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Molecular Virology

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With 164 Figures and 86 Tables

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Preface

Today, knowledge about viruses or distinct aspects of viral infections has become important for our daily life. Continuously, we are confronted with headlines and news concerning, e.g., new influenza viruses, outbreaks of norovirus epidemics, or transmission of otherwise rare zoonotic infections to humans. Due to the major progress in molecular biology and biotechnology, the knowledge on viral infections multiplied during the past decades. Thereby it is possible to characterize and identify new virus types rapidly after their first emergence. Rational development of both antiviral drugs and protective vaccines has reduced the potential danger emanating from viruses for human and animal health.

Indeed, there are hardly any scientific disciplines that have benefited from each other as much as molecular biology and virology. Several important processes in molecular biology and genetics have been initially discovered in viral systems as there is splicing of RNA precursors, nucleosomal structure of double-stranded DNA, or basic immunological defense reactions based on interferon-mediated effects. Additionally, the application of modern methods in molecular biology and biotechnology has led to an advanced knowledge and understanding of the processes involved in the infection caused by human and animal viruses. The *Handbook of Molecular Virology* tries to combine both disciplines to facilitate the understanding between the basic molecular process and the effects that are caused by the respective virus infections and manifested as symptoms or diseases.

First published in Germany in 1996, the present volume represents the translation of the third edition of the text book *Molekulare Virologie*, which appeared in print in 2010. In several places the text has been updated with recently published data. The main focus of all chapters is on virus infections, the knowledge of which is important for human and animal health. The content is divided into two parts: Chapters 1–13 address general aspects of virus replication, structure, and immunological defense. In chapters 14–21, the molecular biology of each virus family is described in detail along with clinical and epidemiological data for the relevant viral infections. Primarily written for readers in Central Europe, the German edition had a special focus on viral infections with relevance for the German-speaking countries. In the English edition, we have tried to adapt the text and deleted details that describe the epidemiology and requirements of notification in Central Europe. For readers that are interested in those data we have included a list of websites of

various national and international public health organizations that provide the respective information.

We thank all the coworkers from Springer, Heidelberg, that have contributed to the English edition as translators and copyeditors (Ariel Quiñones, Dr. Stuart Evans), and project managers (Daniel Quiñones, Dr. Sylvia Blago) who patiently answered all our questions. Furthermore, we are indebted to all our colleagues in virology who we pestered with specific questions in order to obtain the most recent data and results. Dr. Hans Gelderblom supplied almost all the figures that show selected electron microscopic pictures of representative members of each virus family in combination with a chapter describing the basic methods in electron microscopy. Last but not least, we thank all our family members, friends and colleagues, who share our daily lives and who we had to neglect during the writing/translation process.

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Part I

1 Historical Overview

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1.1 Since When Have We Known of Viruses?

"Poisons" were originally considered as the causative agents of illnesses that we know as viral diseases today. At that time, there were no standard methods to detect pathogenic (disease-causing) organisms such as bacteria and protozoa in the supposed "poisonous materials". Only animal experiments performed by Louis Pasteur at the end of the nineteenth century, in which no dilution of the poisonous properties was achieved even after several passages, suggested that the disease-causing agent was able to multiply in the organism. Therefore, there was talk of a reproducible "virus" (Latin for "poison" or "slime") in living organisms, and later also in cells. In St. Petersburg in 1892, Dimitri I. Ivanovski demonstrated that tobacco mosaic disease is caused by an "ultrafilterable" agent, whose size is significantly smaller than that of bacteria: tobacco mosaic virus (bacteria filters have a pore size of

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approximately 0.2 μm, however, most viruses are smaller than 0.1 μm). Soon afterwards, Martinus Willem Beijerinck came to the same conclusion: he developed, for the first time, the notion of a self-replicating, "liquid" agent (*contagium vivum fluidum*). The discovery of foot-and-mouth disease virus by Friedrich Loeffler and Paul Frosch in Greifswald in 1898 was the first evidence of an animal pathogenic virus.

However, it can be retrospectively documented that as long as 3,000 years ago – without knowledge of the nature of the pathogens – practices were implemented which today would be described as vaccinations against viral diseases. In ancient China, India and Egypt, devastating smallpox epidemics must frequently have occurred; Pharaoh Ramses V – as his death mask shows – most likely died of an infection of smallpox virus. As observed at that time, people who had survived the disease were spared from the disease in further epidemics; therefore, they had to have developed some kind of protection caused by the first disease – they were immune. This protective status could also be induced artificially; when dried scabs of smallpox were transmitted to healthy people, they were at least partially protected against smallpox – a measure that we now denominate variolation (the medical term for smallpox is "variola"; ▶ Sect. 19.6). Historical descriptions indicate that smallpox was used as a biological weapon at that time. In the eighteenth century, it was discovered in England and Germany that overcoming milker's nodule disease, which is triggered by a virus related to smallpox, confers protection against genuine smallpox. Edward Jenner must have been aware of these observations in 1796 when he transmitted swinepox and cowpox material, as a sort of vaccine, initially to his first-born son and later to James Phipps, a young cowherd. Both boys remained healthy following exposure to the human pathogenic smallpox virus by inoculation of smallpox pus; in fact, a protective effect was generated by this first deliberate "virological experiment".

Knowledge of this vaccination spread very rapidly from England to the European continent and the USA. The term "vaccination" is derived from the Latin *vacca*, which means "cow". Vaccinations were soon prescribed by law and this led to a gradual reduction of the dreaded disease. In the former German Reich, a first vaccination law was enacted in 1871. However, it still took about 100 years until a human, Ali Maov Maalin, was naturally infected with smallpox (in Somalia) for the last time (in 1977), after the WHO had conducted a worldwide vaccination programme. Today, the disease is considered eradicated.

On a similar basis, i.e., without precise knowledge of the nature of the pathogen, Louis Pasteur developed a vaccine against rabies (▶ Sect. 15.1.5) in Paris in 1885. He transmitted the disease intracerebrally to rabbits in 1882, seeing the causative agent rather in unknown and invisible microbes. As he demonstrated, the pathogen lost its disease-inducing properties by continuous transmission in these animals. In this way, Pasteur achieved the basis for a vaccine virus (*virus fixe*), which, in contrast to the wild-type pathogen (*virus de rue*), was characterized by a constant incubation period. Rubbed and dried spinal cord of rabbits that had been inoculated with the *virus fixe* was no longer infectious, but caused (initially in dogs) protection

against rabies. For the first time, in 1885, Pasteur inoculated a 9-year-old Alsatian boy named Joseph Meister with this material. The boy had been bitten by a rabid dog 2 days before, and finally survived, by virtue of the protective effect induced by the vaccine.

1.2 What Technical Advances Have Contributed to the Development of Modern Virology?

Because of their small size, viruses remained occult to humans for long time. The resolution of the light microscope, which was constructed by Ernst Abbé around 1900, was not sufficiently high to visualize these pathogens; this was only possible with the electron microscope, which was developed by Helmut and Ernst Ruska in 1940. With its aid, the structure of a virus was solved for the first time; that of tobacco mosaic virus. Even obtaining (indirect) evidence of such minute agents, which are not cultivable on artificial media, was impossible before the development of bacteria-proof ultrafilters. They finally allowed the existence of many viruses to be evinced: Walter Reed described yellow fever virus as the first human pathogenic virus in 1900, followed by rabbit myxoma virus and rabies virus in 1903; avian leukaemia virus was discovered by Vilhelm Ellermann and Oluf Bang in 1908, Karl Landsteiner and Emil Popper found poliovirus in 1909 and Peyton Rous discovered Rous sarcoma virus in 1911, which is named after him and represents the first virus associated with the induction of cancer diseases (in this case in the connective tissue of poultry), a notion that had already been suspected by the French bacteriologist Amédée Borrel in 1903.

Even bacteria can be infected by ultrafilterable and transmissible agents, as discovered by Frederik Twort and Felix d'Herelle in 1916 and 1917. They especially noticed the striking ability of these agents to lyse bacteria and, therefore, called them bacteriophages – according to the Greek word *phagein*, which means "to eat". The exploration of the nature of bacteriophages has provided virology with important findings and impulses both in methodological and in conceptual terms. Many of the steps that characterize a viral infection were first discovered in experiments with bacterial viruses: such processes include attachment and penetration, the reproduction-cycle-dependent regulation of gene expression that results in early and late synthesized proteins, and lysogeny, which is associated with the existence of prophages.

1.2.1 Animal Experiments Have Provided Important Insights into the Pathogenesis of Viral Diseases

The study of viruses and their attributes was particularly difficult because they, in contrast to bacteria, could not be propagated in artificial culture media.

However, it could be ascertained that some of the pathogens isolated from diseased people were transmissible to animals, in which they were able to reproduce. For example, human herpes simplex virus was transmitted from human skin blisters to the cornea of rabbits by Wilhelm Grüter in Marburg in 1911. The extraordinary susceptibility of ferrets allowed the isolation of influenza A virus by Christopher Andrews, Wilson Smith and Patrik Laidlaw from pharyngonasal fluid of a sick person for the first time in 1933. Animal experiments also provided many insights into the pathogenesis of viral infections from another point of view. Richard E. Shope discovered rabbit papillomavirus in 1935, and thus the first tumour virus, which – as was later shown – contains a DNA genome. He suspected that such a virus could exist in a latent form as a provirus in the organism. In addition, the discovery that skin cancer can develop from benign skin papillomas is attributed to him. Hence, malignant tumours develop in two or more steps – nowadays a universally accepted notion. Shope further observed that the incidence of cancer differs in different rabbit breeds, and thus genetic traits of the host also influence the development of cancer.

In the framework of animal experiments, Erich Traub made an important observation while studying the virus of lymphocytic choriomeningitis in Princeton in 1935: when pregnant mice were infected with the virus, the virus was transferred to the embryos; mother animals became sick from meningitis, and produced protective antibodies in the further course of the disease. By contrast, the newborn mice remained healthy, but secreted large quantities of the virus for life without developing a specific immune response against the pathogen. This discovery was the first example of an immune tolerance induced by a virus, but the general significance of this phenomenon was not recognized, and the now popular term was not coined (▶ Sect. 16.1.5). Later, lymphocytic choriomeningitis was shown to be an immunologically related disease. The restriction of cytotoxic T lymphocytes by certain genetically determined types of MHC proteins was demonstrated for the first time in this model by Rolf M. Zinkernagel und Peter C. Doherty in 1974. The above-mentioned experiments of Traub evidenced also for the first time the intrauterine transmission of a virus. This raised the question of similar ways of infection in humans. In fact, after a severe rubella epidemic in Australia in 1941, Sir Norman Gregg observed embryopathies when pregnant women were affected by the infection. As demonstrated later, these malformations were the result of intrauterine transmission of rubella virus.

In 1947, coxsackievirus was discovered after transmission of virus-containing stool extracts into newborn mice (Coxsackie is a small town in the US state of New York). Later in New York, Ludwik Gross isolated murine leukaemia virus from blood cells. Besides the importance for tumour virus research, these observations aroused interest in the question concerning the basis of the high susceptibility of newborn animals to viral infections, and suggested investigations on the innate resistance of an organism to infections as well as the time and the causes of its formation.

1.2.2 Cell Culture Systems Are an Indispensable Basis for Virus Research

Laborious and time-consuming experiments were initially the only way to prove the existence of viruses: therefore, there simpler methods were sought. One way involved the observation of so-called inclusion bodies in virus-infected tissues, which were soon judged as an indication for proliferation of the pathogen; as we now know, inclusion bodies are the accumulation of viral proteins and particles in the cytoplasm or the nucleus. The first inclusion bodies were discovered by Dimitri I. Ivanovski; at the same time, Guiseppe Guarnieri discovered similar deposits in cells infected with smallpox virus, then in 1903, Adelchi Negri found inclusion bodies in ganglion cells of rabid animals, which were later named after him. Thus, there were at least simple dye detection methods for some viral diseases. However, virus culture methods became available later.

In the 1930s, it was found that embryonated chicken eggs are appropriate for the propagation of some viral species. Between 1918 and 1920, a pandemic emerging viral disease, Spanish flu, claimed more than 20 million lives, i.e., more than in the First World War. After cultivation of the virus responsible in embryonated chicken eggs in 1933, their haemagglutinating properties were discovered in 1941 (i.e., their ability to agglutinate red blood cells), thereby laying the basis for the development of haemagglutination tests to detect viruses. Another important step in the history of modern virology was the development of the first ultracentrifuges, which became available at about the same time. They made possible the sedimentation and concentration of the minute virus particles. However, the breakthrough in the elucidation of the pathogenetic mechanism of influenza viruses was only possible by the development of molecular biological techniques that allowed the investigation of the genetic material of this pathogen, which exists in the form of single-stranded RNA. Its sequencing revealed the genetic reasons for the previously not understood ability of influenza viruses to change their antigenic properties at periodic intervals (▶ Sect. 16.3.5).

However, it was particularly the donation-funded research of poliomyelitis (▶ Sect. 14.1.5) which provided crucial new insights. Retrospectively, it represents the actual transition to molecular biological research of viral infections. The strong increase in the incidence of polio and the number of deaths – a result of enhanced hygiene standards and the shift of infection rates into later years of life – brought about in the USA the establishment of the National Polio Foundation by Franklin D. Roosevelt, himself a victim of this disease, in the early 1930s. With the funds raised, a major research programme was initiated whose coronation was the discovery of the cytopathic effect by John F. Enders, Thomas H. Weller and Frederik C. Robbins in 1949.

In 1928, Hugh B. and Mary C. Maitland had already introduced the method of tissue culture, in which the cells of small tissue pieces were cultivated in serum-containing liquid media and infected with viruses. Successful viral replication was then demonstrated in animal experiments or by detecting the presence of inclusion bodies. When antibiotics became available in the 1940s, it was then possible to

largely prevent bacterial contaminations in cultures, which led to much simpler handling in this method. Polioviruses were cultivated in embryonic human cells of fixed kidney tissue fragments, and thereby cellular alterations were easily identifiable. This diagnostically valuable cytopathic effect drove the development of virology forwards. It was the basis for the plaque test that was developed by Renato Dulbecco and Marguerite Vogt in 1952, which rendered possible, for the first time, the quantitative determination of the number of infectious particles in cell culture. The capability to cultivate polioviruses under controlled conditions in vitro was the basis for the development of the two polio vaccines: the inactivated vaccine developed by Jonas E. Salk and the live vaccine with attenuated, i.e., weakened polioviruses, developed by Albert B. Sabin. Both vaccines are still in use today. Later, vaccines against measles, rubella and mumps were also produced following the principle applied by Sabin.

By using the method of cell culture, it was possible to cultivate even yellow fever virus, vaccinia virus and rabies virus in vitro. Wallace P. Rowe isolated adenoviruses from cultures of human tonsil tissue after a long cultivation period in 1953. A further development of viral cultivation in vitro provided the method of co-cultivation, which consists in the addition of indicator cells to the tissue cultures, which indicate viral replication by the occurrence of a cytopathic effect. In this manner, the existence of herpes simplex virus was verified in latently infected human dorsal root ganglia in 1971, whereas direct virus detection was not possible at that time. Until then, it had generally been assumed that in the course of viral infections the pathogen would be eliminated completely from the body by the resulting antibodies. The occurrence of herpes blisters as recurrent disease in people with antibodies – known as herpes immunological paradox – refuted that notion (▶ Sect. 19.5). Ernest W. Goodpasture had previously suggested that the trigeminal ganglia should contain a “latent” form of the virus. After such a virus had been detected by co-cultivation, it was recognized that there are a number of infections with latent or persistent viruses, which – independently of the illness symptoms – are excreted either intermittently (e.g., herpes simplex virus) or permanently (such as Epstein–Barr virus). The primary isolation of human immunodeficiency virus (HIV) 1 was also accomplished by co-cultivation of lymph node biopsy material from an AIDS patient with suitable T lymphocytes.

1.2.3 Modern Molecular Biology Is also a Child of Virus Research

Concurrently with the rather practical benefits from developments in viral cultivation, interest in general biological issues became increasingly important. The crystallization of tobacco mosaic virus from liquid media by Wendell Stanley in California in 1935 stimulated discussions as to whether viruses are dead or living matter. The main question concerned, however, the nature and structure of the genetic material, which was found to be nucleic acid by the

seminal experiments of Oswald T. Avery, Colin McLeod and Maclyn McCarty with pneumococci in 1944. In 1952, Alfred D. Hershey and Martha Chase proved that during bacteriophage T4 infections only DNA, but not the protein shell of the virus, penetrates into the bacterial cell. This demonstrated that nucleic acids are the carrier of genetic information. A few years later, in 1955, Gerhard Schramm and Heinz Fraenkel-Conrat showed independently in tobacco mosaic virus that RNA can also be infectious. Schramm and his colleagues had already described in Germany in 1944 that tobacco mosaic virus is composed of RNA and proteins; however, these findings attracted initially only little attention. The base ratios in DNA molecules (A = T and C = G) that were discovered and developed by Edwin Chargaff enabled James D. Watson and Francis H. Crick, in connection with the Rosalind Franklin's X-ray structural analysis, to develop their model of the DNA double helix in 1953. In 1958, Matthew Meselson and Franklin W. Stahl demonstrated that DNA is replicated semiconservatively during cell division. These fundamental insights created the way for the elucidation of basic molecular biological processes which are nowadays generally familiar. The now common molecular genetic, biochemical and immunological methods allow the detection of viruses in the organs and the study of their spread in the organism. The function and effect of viral genes can be explored in isolation and in interaction with other viral or cellular components. Today, many viruses can be cultivated in large quantities in vitro in order to resolve their morphology and particle structure as well as to sequence their genetic information. In the case of non-cultivable viruses, modern molecular biological methods are available which make possible the investigation of the pathogens. Virus–cell interactions can be explored, and provide important insights into the mechanisms of viral replication. On the other hand, many of the molecular processes in eukaryotic cells have been elucidated by using viruses as cell research tools. In this way, the process of RNA splicing was originally described in adenoviruses, in which widely separated gene segments are assembled into single messenger RNA molecules after transcription. The fact that DNA is arranged with histone proteins into nucleosome structures within the nucleus was also first discovered in a virus, simian virus 40. In addition, even enhancers were originally described in viruses, i.e., the specific DNA regions that increase the expression of certain genes in a localization- and orientation-independent manner. Several of these viral regulatory elements have been used for alternative purposes: for example, the most frequently used promoter/enhancer sequences to control the expression of heterologous genes in commercially available vectors are derived from cytomegalovirus. This immediate-early promoter/enhancer region actually regulates the expression of early genes of the virus (see ▶ Sect. 19.5). Furthermore, the transfer of nucleic acid sequences and foreign genes by viral transduction, e.g., using vector systems based on the functions of adenoviruses or retroviruses, is today an indispensable constituent of molecular and cell biology, and has essentially contributed to the development of gene therapy procedures.

1.3 What Is the Importance of the Henle–Koch Postulates?

The study of the epidemiology and pathogenesis of infectious diseases raises the fundamental question of how can it be proved that an illness is caused by infection with a bacterium or a virus. Robert Koch derived four postulates from his work with anthrax bacteria between 1882 and 1890, which his teacher Jacob Henle had previously developed as a hypothesis from the study of so-called miasma and contagions, i.e., the animate or inanimate disease and infection agents:

1. A pathogen must be detected in all cases of a certain disease, but it must be absent in healthy organisms.
2. The pathogen must be cultivable on culture media or in suitable cell cultures in the form of pure cultures.
3. Healthy animals must develop the same disease after inoculation of the pathogen.
4. The causative agent must be reisolated from the infected animals.

Koch noted that the postulates do not comply in all cases. He acknowledged that there are healthy and long-term carriers and that a normal flora of facultative pathogenic bacteria exists. In the realm of virology, not all pathogens comply with these postulates. Positive examples are measles virus (▶ Sect. 15.3.5), human poxviruses (▶ Sect. 19.6.5), canine and feline parvoviruses (▶ Sect. 20.1.6) and classical swine fever virus (▶ Sect. 14.5.7). With regard to viral diseases, Charles River proposed modifications of the postulates in 1937. The exceptions concern preferentially latent or persistent viral infections and the fact that tissue and organ damage or tumour formation cannot always be reproduced as consequences of infection.

Taken from epidemiology, the "Evans postulates" are a worthy supplement (▶ Table 11.1). They show the aetiological importance of a pathogen for a clinical picture if, among others, the pathogen is significantly more frequent in an exposed group and the disease in this population is commoner than in a non-exposed group. Similarly, an immune response should be detectable in the affected collectives. The Evans postulates are valuable particularly for multifactorial infectious diseases, such as canine kennel cough and porcine circovirus infection (▶ Sect. 20.2.6).

The further development of virological and immunochemical detection methods in recent years has allowed the use of additional criteria for the causal relationship between a virus and a disease. These include the detection of a specific humoral or cellular immune response against the pathogen, i.e., IgM or IgG antibodies and specific stimulatable lymphocytes, and the detection of viral proteins, enzyme activities, DNA or RNA by in vitro and in situ methods. The specific detection of viral nucleic acids in tissues is especially important for the aetiological correlation between persistent viral infections and cancer. The fulfilment of Koch's postulates or their modifications is still essential for the development of aetiological relationships between the pathogen and the host.

1.4 What Is the Interrelationship Between Virus Research, Cancer Research, Neurobiology and Immunology?

1.4.1 Viruses are Able to Transform Cells and Cause Cancer

As early as in 1911, it was demonstrated that Rous sarcoma virus can cause cancer. In 1959, Margarete Vogt and Renato Dulbecco observed the transformation from benign to malignant cells after infection with murine polyomavirus in vitro. After animals had been inoculated, these cells generated tumours. Shortly afterwards, it was also discovered that Rous sarcoma virus can transform cells in vitro. Thus, tumour virus research became a driving force of virology. It has enriched the realm of cancer research with decisive impulses both in conceptual and in methodological terms. Experiments with the oncogenic polyomavirus also showed that its dissemination within mouse populations can be followed by serological methods. This aroused the hope that it would be possible to reduce cancer development to viral agents and to study its nature using the classical methods of epidemiology such as virus isolation and antibody detection. In particular, the study of oncogenic retroviruses in animal systems provided seminal insights into the molecular processes that lead to carcinogenesis (▶ Sect. 18.1). By investigating oncogenic retroviruses in 1970, Howard Temin and David Baltimore discovered reverse transcriptase – an enzyme that transcribes the single-stranded RNA of retroviruses into double-stranded DNA. After integration of the viral genetic information into the genome of the host, these viruses lose their individual existence. A few years earlier, Temin had described that an inhibitor of DNA synthesis prevents replication of Rous sarcoma virus, which should not be the case in a typical RNA virus. The integration of a viral genome, which is then present as a provirus, has been associated with tumour development. This event interrupts the continuity of the genome of the cell, as cellular and viral genes can be amplified and destroyed, or their expression can be activated by recombination with viral promoters.

As mentioned above, Shope had already described that carcinomas arise from papillomas by a two-stage or multistage process. The development of cervical carcinoma caused by human papillomaviruses and that of primary liver cancer caused by hepatitis C virus or hepatitis B virus are similar. Even Epstein–Barr virus exerts its tumorigenic effect in a complex way: the viral DNA is detectable in nasopharyngeal carcinoma tumour cells and in various lymphomas (African Burkitt's lymphoma). The cells are infected and immortalized, but do not produce infectious virus particles. Furthermore, chromosomal translocations are found in B-cell lymphomas (▶ Sect. 19.5). However, malignant transformation develops in a multistep process by interaction with other factors, such as malaria, which contributes to a chronic stimulation of cells.

Research on the molecular processes that occur in infections with papillomaviruses, hepatitis B virus and retroviruses has led to the development of vaccines which induce protection against the respective viral infection and are capable of preventing the development of cancer as a long-term consequence.

The vaccination strategy in Southeast Asia that was initiated and promoted by the WHO 25 years ago has led to a significant decrease of primary liver carcinoma, which is caused by hepatitis B virus infections (▶ Sect. 19.1). In veterinary medicine, vaccines against feline leukaemia virus have proved that cats are protected against infections by this exogenous retrovirus and that tumour formation can be prevented (▶ Sect. 18.1). The detailed investigation of the molecular biology and pathogenesis of human papillomavirus infections by the research group of Harald zur Hausen at the German Cancer Research Center (DKFZ) paved the way for the development of appropriate vaccines. These have been available for several years and protect against infections with the highly oncogenic papillomaviruses: they prevent the possible development of cervical carcinoma – one of the commonest cancers in women (▶ Chap. 10, ▶ Sect. 19.3). In September 2008, Harald zur Hausen was awarded the Nobel Prize in Physiology or Medicine for his work concerning the role of papillomaviruses in cervical cancer.

1.4.2 Central Nervous System Disorders Emerge as Late Sequelae of Slow Viral Infections

The term “slow virus infections” was initially coined by Björn Sigurdsson for maedi disease of sheep in Iceland in 1954. Maedi–visna virus causes respiratory symptoms in a slow and progressive disease after very long incubation and latency periods. Maedi–visna virus thus became a model for a range of pathogens that cause diseases with a similar protracted course (▶ Sect. 18.1.6).

The exploration of its pathogenesis revealed that most slow virus infections are caused by pathogens which are usually associated with other diseases. Slow virus infections principally affect the central nervous system and are caused, for example, by measles virus and JC polyomavirus. Subacute sclerosing panencephalitis is probably caused by mutations in measles virus genes, which lead to the emergence of defective virus particles (▶ Sect. 15.3.5). In progressive multifocal leucoencephalopathy, which is triggered by JC polyomavirus, the virus seemingly enters the brain very early and persists there for a long time before the disease breaks out as a result of damage to the immune system (e.g., by infection with HIV; ▶ Sect. 19.2.5). Infections with HIV can also be considered as a slow virus disease. Similarly, prion diseases also progress along the lines of a slow virus infection, but they are caused by non-viral pathogens and have a fundamentally different pathogenesis (▶ Chap. 21).

1.4.3 Interferons Stimulate the Immune Defence Against Viral Infections

Working on yellow fever virus, M. Hoskins, G.M. Findlay and F. MacCallum discovered the phenomenon of interference in 1935: if an avirulent virus was

injected into an experimental animal, the animal was protected from the consequences of infection by a virulent strain when it was applied within the next 24 h, i.e., before the onset of an immune response. In 1957, Alick Isaacs and Jean Lindenmann showed that interferon is responsible for the interference effect. It is species-specific, inducible and belongs to a group of substances that are known as cytokines today. Interferons play an important role in the primary, non-specific defence against viral infections and in stimulating the immune system. The observation that antiviral interferon preparations also have tumour-inhibiting effects was surprising. Generally, cytokines are synthesized when a suitable inducer binds as a ligand to its receptor on the cell membrane, thus triggering specific signal transduction processes in the cell (▶ Chap. 8).

1.5 What Strategies Underlie the Development of Antiviral Chemotherapeutic Agents?

Attempts have been made to develop antiviral chemotherapeutic agents since about 1960. In retrospect, this search can be divided into three stages: the first successful experiments for therapy of a viral infection were performed by Josef Wollensak and Herbert E. Kaufman around 1960 in herpetic keratitis. They used substances that inhibit viral replication in vitro and were known from experimental cancer therapy. However, the selectivity of these substances, i.e., their ability to selectively influence viral and not cellular processes, was only slight because of the high cytotoxicity of the compounds. After the discovery of virus-coded enzymes such as thymidine kinases, DNA polymerases and proteases, it was possible to address the development of specific inhibitors. Antiviral drugs such as amantadine against influenza A virus (▶ Sect. 16.3) and adenine arabinoside and acyclovir (acycloguanosine) against herpes simplex virus (▶ Sect. 19.5) were found by targeted empiricism, i.e., by attempting to find a compound that selectively influences viral reproduction among many compounds with similar effects. The achievement of Gertrude Elion and her staff to use acyclovir as a systemically applicable and selective antiviral drug in herpes encephalitis was an important milestone in chemotherapeutic research. She was awarded the Nobel Prize in Physiology or Medicine in 1988 for her work. After the development of DNA sequencing techniques, the experimental chemotherapy of viral infections entered its third stage. Virus-encoded enzymes were discovered such as the retroviral protease and neuraminidase of influenza viruses, and it was possible to build structural models of enzymes by comparison with proteins of similar functions and known three-dimensional structures. This allows one to identify potential active centres and to develop compounds, also known as "designer" drugs, which accommodate within the active centres and inhibit the viral enzymes. That means deviating from purely empirical research, and is a first step towards a more rational development of antiviral compounds (▶ Chap. 9).

1.6 What Challenges Must Modern Virology Face in the Future?

Molecular virology has achieved significant successes in recent decades: Many infectious diseases can be prevented through the use of modern vaccines or have been completely eradicated (▶ Chap. 10). This ultimately made possible the global elimination of infectious agents such as smallpox virus. Poliovirus, which causes poliomyelitis, is no longer found on some continents, and is currently confined to fewer than ten countries worldwide. In cases in which no preventive vaccination is possible today, e.g., against HIV and some herpesviruses such as cytomegalovirus and herpes simplex virus, a large number of antiviral drugs are available. Although these drugs do not provide a cure, they substantially allow the control of symptoms. These successes might tempt one to assume that virus research has become unnecessary. The assessment of the epidemiological situation by the WHO and the many sensationalistic headlines in newspapers and the media with which we are repeatedly confronted imply the opposite. Because of their frequent and high rates of mutation, viruses are subject to continuous change and development: viruses are permanently compelled to cope with the infected organism and its immune defence systems, always trying to undermine and circumvent them. In particular, viruses that persist in the organism are capable of evading the host immune defence systems by very skilful strategies. The worldwide increase in travel leads not only to contact with new human pathogens, but also to their rapid dissemination. This is demonstrated, for example, by SARS virus infections (▶ Sect. 14.8), the pandemic with the new influenza A virus variant (Mexican flu, “swine flu”) and the threatening potential with regard to humans of new highly pathogenic influenza viruses (▶ Sect. 16.3). New and novel viral diseases which have their origin in the animal kingdom (zoonoses) are also expected owing to increased environmental changes and their serious consequences. Outbreaks of infection with Ebola virus, Nipah virus and Hendra virus are examples. Deforestation of rainforests has led to a change in living conditions for bats, which then infect horses and pigs and, via these intermediate hosts, also humans. Birds carried West Nile virus from Africa to North America, and avian flu virus H5N1 was transported from Asia to Europe by migratory birds. The AIDS pandemic that was induced by human immunodeficiency viruses was originally the result of a zoonotic transmission from monkeys to humans, followed by efficient further dissemination within the human population.

The threat from both new and already well-known viral infections will not decrease because of reduced vaccination, particularly in industrialized countries; therefore, scientists who conduct research in the field of molecular virology will continue to have an ample sphere of activity.

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Viruses: Definition, Structure, Classification 2

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2.1 What is a Virus?

Viruses are infectious units with diameters of about 16 nm (circoviruses) to over 300 nm (poxviruses; Table 2.1). Their small size makes them ultrafilterable, i.e. they are not retained by bacteria-proof filters. Viruses have evolved over longtime period, and have adapted to specific organisms or their cells. The infectious virus particles, or virions, are composed of proteins and are surrounded in some species of viruses by a lipid membrane, which is referred to as an envelope; the particles contain only one kind of nucleic acid, either DNA or RNA. Viruses do not reproduce by division, such as bacteria, yeasts or other cells, but they replicate in the living cells that they infect. In them, they develop their genomic activity and produce the components from which they are made. They encode neither their own protein synthesis machinery (ribosomes) nor energy-generating metabolic pathways. Therefore, viruses are intracellular parasites. They are able to re-route and modify the course of cellular processes for the optimal execution of their own reproduction. Besides the genetic information encoding their structural components, they additionally possess genes that code for several regulatory active proteins (such as transactivators) and enzymes (e.g. proteases and polymerases).

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Table 2.1 Molecular biological characteristics of the different virus families, including some typical prototypes

Virus family	Subfamily/genus	Example	Envelope	Particle size/ shape of the capsid or nucleocapsid	Genome: kind and size
Picornaviridae (▶ Sect. 14.1)	Enterovirus	Poliovirus, coxsackievirus, human enteroviruses, human rhinoviruses	No	28–30 nm/ icosahedron	ssRNA; linear; positive strand; 7,200–8,400 nucleotides
	Cardiovirus	Encephalomyocarditis virus, mengovirus, theilovirus			
	Aphthovirus	Foot-and-mouth disease virus			
	Parechovirus	Human parechovirus			
	Hepatovirus	Hepatitis A virus			
	Erbovirus	Equine rhinitis B virus			
	Kobuvirus	Aichi virus			
	Teschovirus	Porcine teschoviruses			
Astroviridae (▶ Sect. 14.2)	Mamastrovirus	Human, bovine and feline astroviruses	No	27–30 nm/ icosahedron	ssRNA; linear; positive strand; 6,800–7,900 nucleotides
	Avastrovirus	Avian astroviruses			
Caliciviridae (▶ Sect. 14.3)	Norovirus	Norwalk virus	No	27–34 nm/ icosahedron	ssRNA; linear; positive strand; 7,500–8,000 nucleotides
	Sapovirus	Sapporo virus			
	Vesivirus	Feline calicivirus			
	Lagovirus	Rabbit haemorrhagic disease virus			
	Nebovirus	Newbury-1 virus			

Hepeviridae (▶ Sect. 14.4)	Hepevirus	Hepatitis E virus	No	27–34 nm/ icosahedron	ssRNA; linear; positive strand; 7,200 nucleotides
Flaviviridae (▶ Sect. 14.5)	Flavivirus	Yellow fever virus, dengue virus, West Nile virus, tick-borne encephalitis virus	Yes	40–50 nm/ icosahedron	ssRNA; linear; positive strand; 10,000 nucleotides
	Pestivirus	Classical swine fever virus, bovine viral diarrhoea virus			
	Hepacivirus	Hepatitis C virus			
Togaviridae (▶ Sect. 14.6)	Alphavirus	Sindbis virus, Semliki Forest virus, equine encephalitis viruses	Yes	60–70 nm/ icosahedron	ssRNA; linear; positive strand; 12,000 nucleotides
	Rubivirus	Rubella virus			
Arteriviridae (▶ Sect. 14.7)	Arterivirus	Equine arteritis virus, porcine reproductive and respiratory syndrome virus	Yes	40–60 nm/ icosahedron	ssRNA; linear; positive strand; 12,000–16,000 nucleotides
Coronaviridae (▶ Sect. 14.8)	Coronavirinae/ Alphacoronavirus	Human coronaviruses 229E and NL63, feline coronavirus, porcine transmissible gastroenteritis virus	Yes	120–160 nm/helix	ssRNA; linear; positive strand; 25,000–35,000 nucleotides
	Coronavirinae/ Betacoronavirus	SARS-associated coronavirus, mouse hepatitis virus, bat corinaviruses HKU5 and HKU9			
	Coronavirinae/ Gammacoronavirus	Avian infectious bronchitis virus			
	Torovirinae/Torovirus	Bovine and equine toroviruses			

(*continued*)

Table 2.1 (continued)

Virus family	Subfamily/genus	Example	Envelope	Particle size/ shape of the capsid or nucleocapsid	Genome: kind and size
Rhabdoviridae (▶ Sect. 15.1)	Vesiculovirus	Vesicular stomatitis virus	Yes	65–180 nm/helix	ssRNA; linear; negative strand; 12,000 nucleotides
	Lyssavirus	Rabies virus			
	Ephemerovirus	Bovine ephemeral fever virus			
	Novirhabdovirus	Infectious haematopoietic necrosis virus, viral haemorrhagic septicaemia virus			
Bornaviridae (▶ Sect. 15.2)	Bornavirus	Borna disease virus	Yes	90 nm/helix	ssRNA; linear; negative strand; 9,000 nucleotides
Paramyxoviridae (▶ Sect. 15.3)	Respirovirus	Parainfluenza virus	Yes	150–250 nm/helix	ssRNA; linear; negative strand; 16,000–20,000 nucleotides
	Rubulavirus	Mumps virus			
	Avulavirus	Newcastle disease virus			
	Morbillivirus	Measles virus, canine distemper virus, rinderpest virus			
	Henipavirus	Hendra virus, Nipah virus			
	Pneumovirus	Respiratory syncytial virus			
	Metapneumovirus	Human metapneumovirus			

Filoviridae (▶ Sect. 15.4)	Marburgvirus	Marburg marburgvirus	Yes	80–700 nm/helix	ssRNA; linear; negative strand; 19,000 nucleotides
	Ebolavirus	Zaire ebolavirus, Reston ebolavirus			
Arenaviridae (▶ Sect. 16.1)	Arenavirus	Lymphocytic choriomeningitis virus, Lassa virus, Junín virus	Yes	50–300 nm/helix	ssRNA; linear; 2 segments; ambisense strands; 10,000–12,000 nucleotides
Bunyaviridae (▶ Sect. 16.2)	Orthobunyavirus	California encephalitis virus	Yes	100–120 nm/helix	ssRNA; linear; 3 segments; negative strand (ambisense in phleboviruses); 12,000 nucleotides
	Phlebovirus	Rift Valley fever virus, sandfly fever virus			
	Nairovirus	Crimean-Congo fever virus, Nairobi sheep disease virus			
	Hantavirus	Hantaan virus, Puumala virus, Sin Nombre virus			
	Topsovirus	Tomato spotted wilt virus			
Orthomyxoviridae (▶ Sect. 16.3)	Influenza A virus	Influenza A virus	Yes	120 nm/helix	ssRNA; linear; 7 or 8 segments; negative strand; 13,000–14,000 nucleotides
	Influenza B virus	Influenza B virus			
	Influenza C virus	Influenza C virus			
	Thogotovirus	Thogoto virus, Dhori virus			
	Isavirus	Infectious salmon anaemia virus			
Birnaviridae (▶ Sect. 17.1)	Avibirnavirus	Gumboro virus	No	60 nm/ icosahedron	dsRNA; linear; 2 segments; 5,800–6,400 base pairs
	Aquabirnavirus	Infectious pancreatic necrosis virus			
	Entomobirnavirus	Drosophila X virus			

(*continued*)

Table 2.1 (continued)

Virus family	Subfamily/genus	Example	Envelope	Particle size/ shape of the capsid or nucleocapsid	Genome: kind and size
Reoviridae (▶ Sect. 17.2)	Orthoreovirus	Reoviruses	No	70–80 nm/ icosahedron	dsRNA; linear; 10/11/12 segments; 18,000–19,000 base pairs
	Orbivirus	Bluetongue virus, African horse sickness virus			
	Rotavirus	Rotaviruses			
	Coltivirus	Colorado tick fever virus			
	Aquareovirus	Golden shiner virus			
Retroviridae (▶ Sect. 18.1)	Alpharetrovirus	Rous sarcoma virus	Yes	100 nm/ icosahedron or cone	ssRNA; linear; positive strand, transcription into dsDNA; integration; 7,000–12,000 nucleotides
	Betaretrovirus	Mouse mammary tumour virus			
		Jaagsiekte sheep retrovirus (ovine pulmonary adenomatosis virus)			
	Gammaretrovirus	Feline leukaemia virus, murine leukaemia virus			
	Deltaretrovirus	Human T-lymphotropic viruses 1 and 2, bovine leukaemia virus			
	Epsilonretrovirus	Diverse fish retroviruses			
	Lentivirus	Human immunodeficiency viruses			
	Spumavirus	Simian foamy virus			

Hepadnaviridae (▶ Sect. 19.1)	Orthohepadnavirus	Hepatitis B virus	Yes	42 nm	DNA; partially double stranded; circular; 3,000–3,300 base pairs
	Avihepadnavirus	Duck hepatitis B virus			
	Deltavirus (virusoid); infection along with hepatitis B virus as helper virus	Hepatitis D virus	Yes, composition to similar the envelope of hepatitis B viruses		ssRNA; circular; 1,900 nucleotides
Polyomaviridae (▶ Sect. 19.2)	Polyomavirus	BK polyomavirus, JC polyomavirus, simian virus 40	No	45 nm/ icosahedron	dsDNA; circular; 5,000 nucleotides
Papillomaviridae (▶ Sect. 19.3)	Alphapapillomavirus	Human papillomaviruses 6, 10, 16, 18 and 32 (mucosa, oral/genital)	No	55 nm/ icosahedron	dsDNA; circular; 8,000 nucleotides
	Betapapillomavirus	Human papillomaviruses, 5, 9 and 49 (dermal)			
	Gammapapillomavirus	Human papillomaviruses 4, 48 and 50 (dermal)			
	Deltaapillomavirus	Ruminant papillomaviruses (cattle, sheep, deer)			
	Lambdapapillomavirus	Canine and feline papillomaviruses			
Adenoviridae (▶ Sect. 19.4)	Mastadenovirus	Human and canine adenoviruses	No	70–80 nm/ icosahedron	dsDNA; linear; 36,000–38,000 base pairs
	Aviadenovirus	Avian adenoviruses			
	Siadenovirus	Turkey haemorrhagic enteritis virus			
	Atadenovirus	Chicken egg drop syndrome virus			

(*continued*)

Table 2.1 (continued)

Virus family	Subfamily/genus	Example	Envelope	Particle size/shape of the capsid or nucleocapsid	Genome: kind and size
Herpesviridae (▶ Sect. 19.5)	Alphaherpesvirinae	Herpes simplex viruses, varicella-zoster virus, bovine, equine, porcine, canine, feline and gallid herpesviruses	Yes	250–300 nm/ icosahedron	dsDNA; linear; 150,000–250,000 base pairs
	Betaherpesvirinae	Cytomegalovirus, human herpesvirus 6			
	Gammaherpesvirnae	Epstein-Barr virus, human herpesvirus 8, alcelaphine herpesvirus 1 (bovine malignant catarrhal fever virus)			
Poxviridae (▶ Sect. 19.6)	Orthopoxvirus	Variola viruses, vaccinia virus, bovine and simian variola viruses	Yes	350–450 nm/ complex	dsDNA; linear; 130,000–350,000 base pairs
	Parapoxvirus	Orf virus			
	Avipoxvirus	Canarypox virus			
	Molluscipoxvirus	Molluscum contagiosum virus			
	Suipoxvirus	Swinepox virus			
	Yatapoxvirus	Tanapox virus, Yaba monkey tumour virus			

Asfarviridae (▶ Sect. 19.7)	Asfivirus	African swine fever virus	Yes	200 nm/complex	dsDNA; linear; 180,000 base pairs
Parvoviridae (▶ Sect. 20.1)	Parvovirus	Feline panleucopenia virus, canine parvovirus, porcine parvovirus	No	20–25 nm/ icosahedron	ssDNA; linear; 5,000 nucleotides
	Erythrovirus	Parvovirus B19			
	Bocavirus	Human bocavirus, bovine bocavirus, canine minute virus			
	Amdovirus	Aleutian mink disease virus			
	Dependovirus	Adeno-associated viruses			
Circoviridae (▶ Sect. 20.2)	Gyrovirus	Chicken anaemia virus	No	16–24 nm/ icosahedron	ssDNA; circular; 1,700–2,000 nucleotides
	Circovirus	Porcine circovirus, beak and feather disease virus			
Anelloviridae (▶ Sect. 20.2)	Alphatorquevirus	Torque teno virus			
	Betatorquevirus	Torque teno mini virus			
	Gammatorquevirus	Torque teno midi virus			

ssDNA single-stranded DNA, *dsDNA* double-stranded DNA, *ssRNA* single-stranded RNA, *dsRNA* double-stranded RNA

Viruses exist in different conditions. They can actively replicate in cells, and produce a great number of progeny viruses. This is known as a replicationally active state. After infection, some virus types can transition into a state of latency by integrating their genetic information into the genome of the host cell, or maintain it as an episome in an extrachromosomal status within infected cells. Certain viral genes can be transcribed during that time, contributing to the maintenance of latency (herpesviruses). In other cases, the expression of the viral genome is completely repressed over long periods of time (e.g. in some animal pathogenic retroviruses). In both cases, cellular processes or external influences can reactivate the latent genomes, leading to a new generation of infectious viruses. Depending on the virus type, the infection can have different consequences for the host cell:

1. It is destroyed and dies.
2. It survives, but continuously produces small numbers of viruses and is chronically (persistently) infected.
3. It survives and the viral genome remains in a latent state without producing infectious particles.
4. It is immortalized, thus gaining the capability of unlimited cell division, a process that can be associated with malignant transformation into a tumour cell.

2.2 How are Viruses Structured, and what Distinguishes them from Virusoids, Viroids and Prions?

2.2.1 Viruses

Infectious virus particles – also referred to as virions – are constituted of various basic elements (Fig. 2.1): inside, they contain an RNA genome or a DNA genome. Depending on the virus type, the nucleic acid is single-stranded or double-stranded, linear, circular or segmented. Single-stranded RNA and DNA genomes can have different polarity, and in certain cases the RNA genome is similar to messenger RNA, e.g. in picornaviruses and flaviviruses. A single-stranded genome that has the same polarity as the messenger RNA is referred to as a positive or plus strand.

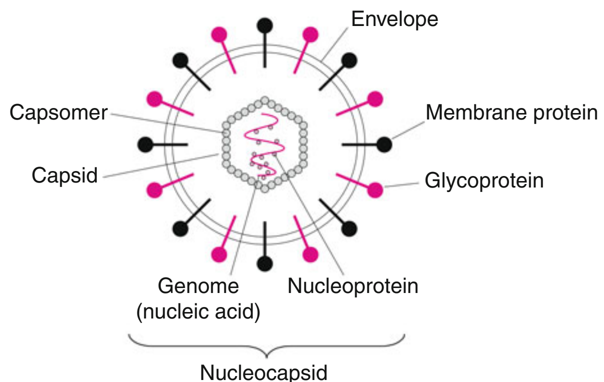
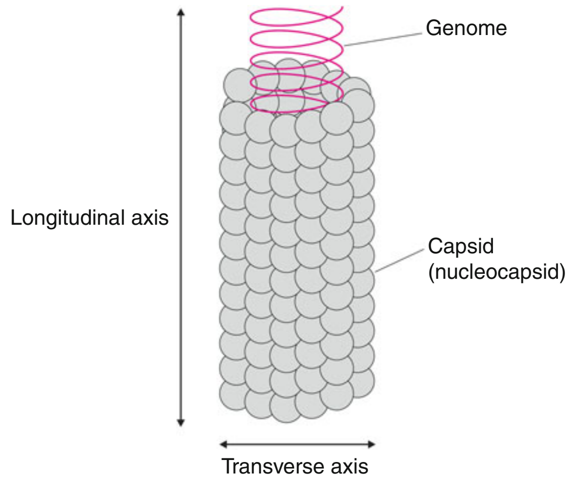


Fig. 2.1 Structure of an enveloped viral capsid



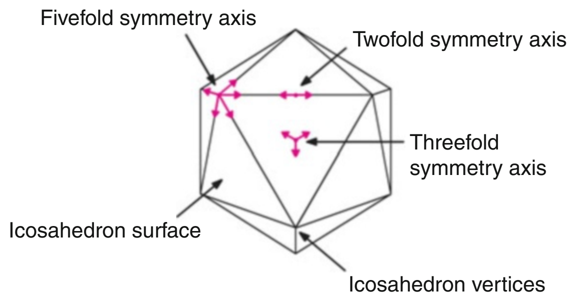


Fig. 2.2 Symmetry forms of viral capsids. (**a**) Helical symmetry; the symmetry planes run parallel to the longitudinal or transverse axis of the particle (e.g. tobacco mosaic virus capsid, nucleocapsid of paraviruses or orthomyxoviruses). (**b**) Cubic-spherical symmetry; icosahedron with rotational symmetry whose centres of the symmetry axes are at the vertices of the icosahedron (fivefold symmetry axis) in the middle of the triangle (threefold symmetry axis) and along the edges (twofold symmetry axis). Picornaviruses, parvoviruses and adenoviruses are examples of viruses with such capsid forms

The genome forms a nucleocapsid complex with cellular histones (polyomaviruses) or viral proteins (e.g. rhabdoviruses, paraviruses, orthomyxoviruses, adenoviruses and herpesviruses). This nucleic acid-protein complex can be surrounded by particular protein structures, the capsids (in polyomaviruses, papillomaviruses, adenoviruses and herpesviruses). In some cases (such as picornaviruses, flaviviruses, togaviruses and parvoviruses), the nucleic acid interacts directly with the capsids. In viruses containing an envelope, the capsid layer can be absent (as in coronaviruses, rhabdoviruses, paramyxoviruses, orthomyxoviruses, bunyaviruses and arenaviruses).

Capsids are rod-shaped or cubic-spherical protein structures. In some virus types, they consist of multimeric units of only one polypeptide, in other cases they are composed of heteromeric complexes. The capsid protein subunits can aggregate into discrete subunits or even into so-called capsomeres, i.e. morphologically distinct structural components. Rod-shaped capsids have a helical symmetry. The two planes of symmetry, i.e. the longitudinal and the transversal axes, differ in length (Fig. 2.2a). By contrast, spherical capsids have an icosahedral structure with

a rotational symmetry; an icosahedron consists of 20 equilateral triangles and 12 vertices (Fig. 2.2b). The symmetry axes have the same length: the fivefold symmetry axis is located at the vertices of the icosahedron; the threefold axis passes through the centre of a triangle, the twofold axis passes along the edges. The number of subunits of an icosahedron can be calculated by the formula $10(n-1)^2+2$, where n indicates the number of morphologically distinguishable structures on the face of a triangle.

The three-dimensional structures of the particles of a number of viruses have been resolved by X-ray structural analyses. Prerequisite is knowledge of the basic composition of the virus, i.e. information on which proteins form the capsid or the virus, as well as the nature of the viral genetic information and the sequence of the structural proteins. In addition, purification of virus particles must be possible and these must be available as a stable highly concentrated virus suspension on the order of several milligrams per millilitre. Finally, the purified virions or, alternatively, viral capsids, which are produced in cell culture or by genetic engineering, must be able to crystallize.

In some virus types, the capsids are surrounded by a lipid bilayer envelope, which is derived from cellular membrane systems. Viral and cellular proteins are embedded in the envelope, and are frequently modified into glycoproteins by sugar groups. Usually, viral surface components are clearly exposed, and they can protrude up to 20 nm from the particle surface. If such a membrane envelope is present, it renders the virus sensitive to inactivation by solvents and detergents. A tegument layer can be situated between the membrane and the capsid (herpesviruses), and contains additional viral protein components.

The exposed proteins and protein domains on the surface of the virus – either in the envelope or in the capsid – are subject to selection pressure by the immune system. Therefore, viruses change by mutation and selection preferentially the amino acid sequences of antibody-binding regions or epitopes, which are responsible for binding neutralizing immunoglobulins. In some species of viruses, this variability of the surface regions leads to the formation new subtypes. In addition to this continuous change of the surface of exposed regions that is determined by mutation and selection, in some virus types another source of variability is possible by genetic recombination, by which even large nucleic acid regions can be exchanged between different viruses. This can lead to substantial changes in the viruses involved and to the generation of new viral species.

2.2.2 Virusoids (Satellite Viruses), Viroids, Mimiviruses and Virophages

Satellite viruses, or virusoids, are small RNA or DNA molecules that code for one or two proteins with which they are associated. Their replication and spread is dependent on the presence of another virus. Virusoids are usually found together

with plant viruses, but also hepatitis D virus, which can only proliferate when the cell is simultaneously infected with hepatitis B virus, is a virusoid (▶ Sect. 19.1.5). Viroids are plant pathogens and consist of a circular RNA (about 200–400 nucleotides) that does not code for proteins and exhibits a complex two-dimensional structure. A central sequence motif is highly conserved and essential for replication of these nucleic acid molecules. Other regions are variable and may be responsible for virulence. These infectious RNA molecules are replicated by cellular polymerases in a rolling circle mechanism (▶ Sect. 3.4), whereby secondary structures are formed at the transitions, which are known as a hammerhead because of their form. They have RNase activity, and autocatalytically cleave the concatemeric RNA strands that result after replication. Ribozymes, small RNA species with sequence-specific RNase activity (▶ Sect. 9.3), are derived from the hammerhead-like RNA structures.

Mimiviruses are a family of very large DNA viruses which were discovered by Didier Raoult in the amoeba *Acanthamoeba polyphaga* only in 2004. These viruses were originally regarded as bacteria because of the extraordinary size of their spherical capsids (400 nm) and protein filaments, which protrude extremely from the surface, conferring the virions with an apparent size of up to 800 nm. Therefore, they were denominated "mimiviruses" as an abbreviation for "mimicking viruses". The DNA genome of mimiviruses comprises 1.2 million base pairs and encompasses more than 1,200 putative genes. Even larger mamaviruses have been discovered in amoebae, which can be infected by parasitic viruses. These significantly smaller viruses (sputnikvirus), also known as virophages, can multiply in amoebae only if they are concurrently infected by mamaviruses. However, sputniks do not use mamaviruses only as a helper virus, but also inhibit their proliferation and morphogenesis, thus making them virtually sick.

2.2.3 Prions

In animals and humans, prions always cause fatal neurodegenerative disorders. They can be transmitted within a species, and – albeit limited – to other organisms beyond species boundaries (▶ Chap. 21). The pathogen responsible (prion, from "proteinaceous infectious particle") does not require a coding nucleic acid in the infectious agent. Prions are composed of the pathological isoform (PrP^{Sc}), which exists especially in β-sheet conformation, and of a non-pathological cellular prion protein (PrP^{C}), which is present predominantly in α-helical conformation. The conversion of the PrP^{C} α-helical conformation into the β-sheet PrP^{Sc} variant is associated with completely different biochemical properties, and is the key pathogenetic basic principle of prion diseases. After its synthesis, the cellular protein PrP^{C} arrives in the cytoplasmic membrane. PrP^{C} is active at the cell surface only for a limited time, and is subsequently degraded in the endosomes. During this process, a small proportion of PrP^{C} proteins are constantly transformed into PrP^{Sc} variants. This process is referred to as prion conversion. PrP^{Sc} proteins cannot be efficiently degraded and accumulate in the cells. The function of PrP^{C} has not been completely resolved.

Experiments with knockout mice containing a deletion of the PrP coding genome sequences revealed that PrP^C appears to be dispensable for development and survival of the mice. However, without PrP^C they cannot develop a prion disease.

Human prion diseases include Creutzfeldt-Jakob disease, kuru and variant Creutzfeldt-Jakob disease. In animals, the most famous representatives are scrapie (sheep), bovine spongiform encephalopathy (cattle) and chronic wasting disease (deer). The peculiarity of prion diseases is that they appear in three manifestations: acquired infectious (exogenous), sporadic (endogenous) and genetic (endogenous). Inasmuch as prions are restricted to the central nervous system, their infectious transmission is generally limited.

2.3 What Criteria Determine the Classification System of Virus Families?

The taxonomic classification of viruses into different families is done by an international commission of virologists and is continuously adapted to current insights. It is based on the following main criteria:

1. The nature of the genome (RNA or DNA) and the form in which it is present, i.e. as a single or a double strand, in positive or negative sense, linear or circular, segmented or continuous; also the arrangement of genes on the nucleic acid is important for the definition of individual families.
2. The symmetry form of the capsids.
3. The presence of an envelope.
4. The size of the virion.
5. The site of viral replication within the cell (cytoplasm or nucleus).

The further subdivision into genera and virus types is largely based on serological criteria and the similarity of genome sequences. The different virus families and their important human and animal pathogenic prototypes are summarized in Table 2.1.

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Viral Proliferation and Replication 3

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3.1 How Is the Infection of a Cell Initiated?

As obligate cellular parasites, viruses do not have their own metabolism; therefore, they must infect cells for reproduction. The virus particles must be able to recognize specific receptor molecules on the cytoplasmic membrane of the host cell and to bind to them. This process is known as attachment. In enveloped viruses, this interaction is mediated by proteins that are embedded within the viral envelope. This is the case in influenza viruses as well as retroviruses and herpesviruses. Binding of viral envelope proteins to cellular surface structures is to some extent very specific: this is the case for the interaction between the surface protein gp120 of human immunodeficiency virus (HIV) and the CD4 receptor, a polypeptide that occurs almost exclusively in the cytoplasmic membrane of T-helper cells and macrophages (▶ Sect. 18.1.5). In other cases, viral proteins bind to cellular structures that are found on various cell types. One example is the binding of haemagglutinin of influenza viruses (▶ Sect. 16.3) to terminal *N*-acetylneuraminic

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acid residues of complex oligosaccharides, which are found as protein and lipid modifications on the membrane surface of various cells.

In non-enveloped viruses, the surface of the capsid proteins contains the structures that are responsible for the more or less specific binding of the particles to particular cells. Picornaviruses, adenoviruses and parvoviruses are such examples. Polioviruses interact with a domain of CD155, a protein of the immunoglobulin superfamily that is localized on the cell surface, by the canyon – a trench-like structure formed by the folding of specific amino acid regions of the capsid protein on the surface of the particle. Most rhinoviruses, which also belong to the picornavirus family, use the cell surface protein intercellular adhesion molecule 1 for specific attachment; other rhinoviruses binds to members of the LDL receptor family (▶ Sect. 14.1.4). Adenoviruses attach to the coxsackievirus and adenovirus receptor, a functionally not characterized cell surface protein, by the knob at the end of the fibre proteins, which are located at the vertices of the icosahedral capsids. The receptor is denominated so because both virus types bond to it (▶ Sects. 14.1.4 and ▶ 19.4.4). The simultaneous interaction of adenoviral penton base proteins, also components of the particle surface, with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins is also necessary for successful attachment (▶ Sect. 19.4.4). This imperative to bind to two different receptor types for successful infection of target cells is also found in other viruses, e.g. in human immunodeficiency viruses and herpesviruses (▶ Sects. 18.1 and ▶ 19.5).

3.2 How Does a Virus Enter a Cell?

After attachment, virus particles that are associated with their respective receptor on the cell surface are translocated into the interior of the cell; this process is referred to as penetration. In non-enveloped viruses, this is usually performed by receptor-mediated endocytosis. This process is generally used by cells to incorporate molecules from the outside into the cytoplasm: the capsid-receptor complexes interact with clathrin-rich membrane sites, where the cytoplasmic membrane invaginates around the bound virus, enclosing it. The resulting vesicle is referred to as an endosome. It invaginates inwards, thus entering the cytoplasm of the cell. Alternative entry routes are through caveolae and caveosomes. For the next steps of the infection cycle, the virus particles must be released relatively quickly from endosomes because these are rich in proteases and other degrading enzymes, which would eventually destroy the virus. Therefore, viruses have evolved mechanisms that allow them to leave the vesicle and to avoid the further endocytosis and degradation process. For such purposes, parvoviruses (▶ Sect. 20.1) possess phospholipase A_2 like enzyme activities, which as part of a capsid protein (VP1) are responsible for release of the virus from the endosome.

Even enveloped viruses have developed ways to escape destruction in the endosomes, as they also penetrate into the cell in part by endocytosis of membrane vesicles. For example, if influenza viruses penetrate a cell (▶ Sect. 16.3), the capsid is surrounded by two membranes in the vesicles, namely the viral envelope and the

vesicle membrane that is derived from the plasma membrane. A fusionally active sequence of the viral haemagglutinin triggers the fusion of the two membranes, leading to the release of the virus particle from the vesicles. This process is found in similar variants in many other enveloped viruses, e.g. in flaviviruses (▶ Sect. 14.5). The fusion of the two membranes is pH-dependent; therefore, acidification of the vesicle interior must occur previously. In contrast to this penetration mechanism, paramyxoviruses (▶ Sect. 15.3) possess in their envelope a special fusion protein that makes possible, in a pH-independent process, the fusion of the viral envelope and cellular membranes during attachment of the particle; in this case, the capsid is released directly into the cytoplasm after merging of the two membranes. The fusion of the viral envelope with the cell membrane is also performed in a similar manner in herpesviruses (▶ Sect. 19.5) and HIV (▶ Sect. 18.1).

3.3 How Is the Viral Genome Released into the Cell?

The release of the viral nucleic acid from the capsid is the result of a still largely unsolved process, which is referred to as uncoating. During this process, the genome of DNA viruses – in herpesviruses also the tegument – is transported by different intracellular transport systems through the nuclear pores into the nucleus. An exception is poxviruses (▶ Sect. 19.6), which replicate as DNA viruses in the cytoplasm of infected cells. After uncoating, the genome of RNA viruses remains as a ribonucleic acid–protein complex in the cytoplasm, where the next steps of the infection cycle occur. This rule is broken only by influenza viruses and Borna disease virus, which replicate as RNA viruses in the nucleus (▶ Sects. 15.2 and ▶ 16.3).

3.4 What Different Strategies Do Viruses Pursue in Gene Expression and Genome Replication?

Viral replication outlines the very complex processes of viral gene expression and genome replication, which are different in all virus types, and finally result in the production of multiple copies of the virus in infected cells. Although viruses possess the genetic information for most of the factors required for their own gene expression and genome replication, specific cellular proteins are often essential for viral gene expression. These proteins usually act as transactivators of viral gene transcription. If they are lacking, the infection cycle cannot continue; the result is that no or only a subset of the viral gene products are synthesized, and the formation of infectious particles remains incomplete. This form of infection, in which the virus is capable of binding to the surface of certain cells and penetrating into them, cannot initiate (or can initiate only partially) the reproductive cycle because of the given intracellular conditions, is referred to as abortive infection. The dependence of replication on the cellular environment is another reason for the cell specificity of viral infections.

Depending on the nature and structure of the viral genome, the replication strategies described in the following sections have been found in the different viruses (the chapters in Part II follow this subdivision).

3.4.1 Positive-Sense RNA Viruses

The RNA genomes of positive-sense RNA viruses (▶ Chap. 14) have the polarity of a messenger RNA (mRNA), and can be directly translated into proteins by using the cellular translation machinery. This results in the synthesis of a large precursor polyprotein in picornaviruses and flaviviruses, whereas two or more different forms of the precursor proteins are found in caliciviruses, togaviruses, arteriviruses and coronaviruses. They are proteolytically cleaved into the viral structural proteins and enzymes. One of the enzymes is very important for genome replication, namely RNA-dependent RNA polymerase. Since this enzyme does not exist in eukaryotic cells, the virus must encode the corresponding genetic information itself. Using the positive-sense RNA genome as a template, this polymerase catalyses the synthesis of a complementary negative-sense RNA, which in turn serves as a template for the production of a large number of new RNA genomes with positive-sense polarity. Important prototypes of positive-sense RNA viruses are picornaviruses, flaviviruses, togaviruses and coronaviruses (▶ Sects. 14.1, ▶ 14.5, ▶ 14.6 and ▶ 14.8).

3.4.2 Negative-Sense RNA Viruses

In contrast to positive-sense RNA viruses, the genome of negative-sense RNA viruses (▶ Chaps. 15 and ▶ 16) does not exhibit the polarity of mRNA; hence, it cannot be directly translated into proteins. This requires transcription of the genome into a complementary RNA molecule, a process that relies on the presence of an RNA-dependent RNA polymerase. Since viral proteins cannot be synthesized directly from the genome owing to its negative-sense polarity, negative-sense RNA viruses must carry this polymerase into the cell as part of the virus particle. The enzyme synthesizes complementary mRNA molecules, which are then translated into viral structural and non-structural proteins. In the following step, the RNA-dependent RNA polymerase is also responsible for the synthesis of a continuous, complementary RNA strand, which serves as a template for the synthesis of negative-sense RNA genomes. Important prototypes of negative-sense RNA viruses are rhabdoviruses and paramyxoviruses (▶ Sects. 15.1 and ▶ 15.3) as well as orthomyxoviruses, which differ from the afore-mentioned viruses by a segmented negative-sense RNA genome (▶ Sect. 16.3). Some virus types that belong to the bunyavirus and arenavirus families (▶ Sects. 16.1 and ▶ 16.2) have segmented genomes as well, and can use parts of them in both positive-sense and negative-sense polarity. Their single-stranded RNA genomes encode proteins in both directions. This highly

efficient use of the coding capacity is referred to as ambisense orientation. It is also found in porcine circovirus, which has a single-stranded, circular DNA genome (▶ Sect. 20.2).

3.4.3 Double-Stranded RNA Viruses

Reoviruses and birnaviruses (▶ Chap. 17) have a double-stranded, segmented RNA genome. In this case, an RNA-dependent RNA polymerase is also found as part of the virus particles, and is carried into the cell during infection. It transcribes the negative-sense genomic fragments into capped, translatable mRNA molecules. These also serve as templates for the synthesis of new double strands. Only reoviruses and birnaviruses follow this principle of conservative replication, in which none of the parent strands are present in the newly synthesized double-stranded RNA molecules.

3.4.4 Retroviruses

These single-stranded RNA viruses (▶ Chap. 18) have a positive-sense genome, but their replication cycle differs completely from that of the previously mentioned virus families. Retroviruses enclose the enzyme reverse transcriptase in their virions, and this is introduced into the cell during infection. The RNA-dependent DNA polymerase activity of reverse transcriptase catalyses the transcription of the RNA genome template into double-stranded DNA, which is subsequently integrated into the cellular genome. This so-called provirus behaves like a normal cellular chromosomal region; it is replicated along with the cellular genome during cell division and is passed on to daughter cells. Transcription and translation occur only from the integrated viral DNA. This produces spliced and unspliced mRNA molecules that are translated into viral structural proteins and enzymes. The unspliced sequence of the provirus spanning the entire mRNA serves as a viral genome, which is packaged into virus particles.

3.4.5 Double-Stranded DNA Viruses

The genome of these viruses (▶ Chap. 19) is transcribed by cellular enzymes after transport into the nucleus. The resulting RNA molecules are then translated into the viral non-structural and structural proteins. Hepadnaviruses (▶ Sect. 19.1) have a partially double-stranded DNA genome, which is completed in the infected cells and is present in the nucleus as a circular molecule. Hepadnaviruses have a reverse transcriptase, which emphasizes, along with some other attributes, their relationship to retroviruses. During the replication cycle, the mRNA that spans the entire genome is transcribed into DNA by this viral enzyme. The smaller DNA viruses such as polyomaviruses (▶ Sect. 19.2) do not encode their own DNA polymerase,

but encode polypeptides that interact with the cellular DNA polymerases, and alter the function of these in such a way that the viral DNA sequences are preferentially replicated. This process begins at the origin of replication and proceeds bidirectionally and semiconservatively; it is very similar to delta or plasmid replication, which is found during replication of circular bacterial chromosomes or episomal DNA molecules.

The more complex DNA viruses such as adenoviruses and herpesviruses (► Sects. 19.4 and ► 19.5) have a tightly regulated gene expression pattern that is divided into early and late phases; these viruses use the cellular transcription and translation machinery as well. Several regulatory and enzymatically active polypeptides are synthesized early, including the viral DNA polymerases and some enzymes that are involved in nucleic acid metabolism, which make possible the replication of double-stranded DNA genomes. The linear genomes of adenoviruses are replicated in a semiconservative mode; this means that each parent strand is used as a template and remains as part of the newly synthesized double-stranded DNA molecules. The replication origins are located at the ends of the double-stranded DNA. The linear DNA genomes of herpesviruses are circularized in the cell. These viruses can have two different replication cycles: during latency, the viral DNA is present as an episome and is replicated by cellular DNA polymerases. In the lytic infection cycle, which leads to the production of progeny viruses, replication occurs according to the principle of sigma replication, which also occurs in some bacteriophages, and is referred to as rolling-circle replication. In this replication mode, a strand of the circular DNA molecule is cleaved at the origin of replication, generating a free 3′-OH end, which is continuously extended by polymerization of further nucleotides by the viral DNA polymerase, whereby the intact DNA strand serves as a template. The 5′ end is continuously displaced from the template strand, just as if it were rolled. In this way, a single strand of DNA is generated that encompasses multiple copies of the herpesvirus genome in concatemeric form, i.e. repeated in series. It is converted to a double DNA strand by discontinuous synthesis of Okazaki fragments, and is cleaved by endonucleases that resolve the concatemers into individual viral genomes. In both adenoviruses and herpesviruses, the synthesis of viral structural proteins is induced only in the late phase of gene expression after DNA replication.

Poxviruses are also double-stranded DNA viruses, but they follow a completely different replication mode. They perform all synthesis reactions in the cytoplasm of infected cells. Therefore, all enzymes that are usually localized in the nucleus cannot be used by poxviruses. These include RNA polymerases, capping enzymes and RNA-modifying enzymes. Therefore, poxviruses possess, in addition to their own DNA polymerase, also the genetic information for these functions. Gene expression and genome replication are also tightly regulated in poxviruses (► Sect. 19.6). The asfarvirus family includes only one animal pathogenic agent: African swine fever virus (► Sect. 19.7). Asfarviruses have a double-stranded DNA genome and are similar to the phytopathogenic iridoviruses in many ways. The replication occurs in the nucleus of infected cells.

3.4.6 Single-Stranded DNA Viruses

The parvovirus, anellovirus and circovirus families encompass viruses with a single-stranded linear or circular DNA genome (▶ Chap. 20). All three families do not encode a viral DNA polymerase; like polyomaviruses, they also use cellular enzymes for genome replication, and these are functionally modified. In this way, complementary double-stranded DNA intermediates are generated that are subsequently transcribed into single-stranded genomes (▶ Sects. 20.1 and ▶ 20.2).

3.5 What Is Morphogenesis?

After replication, both the viral structural proteins and the viral genome are present in multiple copies in the cell. The process of viral morphogenesis describes the orderly assembly of the various components into particulate structures, capsids and ultimately infectious virus particles. Assembly occurs largely without the use of cellular enzymes and other enzymatic activities by interaction between the individual components, and hence is also known as self-assembly. However, there are growing indications that viral morphogenesis cannot proceed entirely without the involvement of cellular functions. Viral proteins, above all cellular chaperones (protein folding catalysts), influence the self-assembly process. The morphogenesis of enveloped viruses is frequently associated with cellular membrane structures. In retroviruses, for example, morphogenesis occurs at the cytoplasmic membrane; however, in herpesviruses, it occurs initially at the inner nuclear membrane, and later on the membranes of the *trans*-Golgi network; in flaviviruses, it occurs at the membrane of the endoplasmic reticulum (▶ Sects. 18.1, ▶ 19.5 and ▶ 14.5).

3.6 How Are Progeny Viruses Released?

One possible way of releasing infectious particles is budding. Here, the preformed capsids aggregate at specific sites in the cytoplasmic membrane, the lipid rafts. In these membrane islands, viral surface proteins accumulate after their transport. The assembled capsids are subsequently enveloped by the protein-containing membrane and are finally released by budding. Depending on the site of the assembly process in the cell, the viral envelope originates from the cytoplasmic membrane, the nuclear membrane, the endoplasmic reticulum membrane or the membrane of the *trans*-Golgi network. If the viral envelope originates from the cytoplasmic membrane, viruses will be directly released into the surrounding region. If viral morphogenesis occurs at the nuclear membrane or at the membrane of the endoplasmic reticulum, release of the virus occurs via transport through the Golgi apparatus to the cell surface and by exocytosis. Some viruses, e.g. HIV, can still perform maturation processes by structural rearrangements in the particles after having been released from the cells. The release of non-enveloped viruses predominantly occurs by lysis of the infected cell. Whether this is an active process induced by the

virus or whether viral replication and the associated interference with the cellular metabolism lead to exhaustion of the cell to the extent that it induces apoptosis resulting in cell death and disintegration is largely unknown and possibly proceeds very differently in the various virus systems.

Replication of viruses, their morphogenesis and their release imply many errors that may lead to the formation of non-infectious, defective virus particles. Defective viruses frequently arise in excess. In many cases, they contain an incomplete replicated genome; in other cases, the loss of infectivity is based on irregular processes during assembly and subsequent viral maturation.

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Pathogenesis

4

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Pathogenesis describes the spread of a virus in the organism and the mutual relationship between the pathogen and its host during infection. These processes can be analysed in several ways by using different histological, virological and immunological methods. Viral infections can be with or without symptoms (also called apparent or inapparent infection courses). In both cases, the host organism responds with immunological defence responses, which usually lead to overcoming the primary disease symptoms and to the elimination of the virus. The immune response may also contribute in the context of immunopathogenesis to specific disease symptoms and either temporary or permanent damage to the host.

The typical course of an acute viral infection ordinarily manifests itself in a stage of disease with nonspecific, flu-like symptoms; in many cases, a direct cure of the disease can be observed afterwards. However, this phase of the disease is frequently followed by a symptom-free interval of only a few days, which is then followed by a second, specific disease stage with typical organ symptoms (Fig. 4.1). In both cases, the immune system can be successful in eliminating the pathogen. Occasionally, viruses can establish persistent infections and remain in the organism for life in spite of the induced immune defence. Some viruses constantly produce progeny viruses that are excreted by the hosts and are transmitted to other uninfected living organisms, e.g. in chronic persistent infections with hepatitis B virus, hepatitis C virus or human immunodeficiency virus (HIV) (► Sects. 14.5, ► 18.1, ► 19.1). In other cases, although the reproduction of the pathogen is controlled, the genetic information remains latent in the cells of certain tissues. The pathogens can be reactivated in certain circumstances to produce offspring

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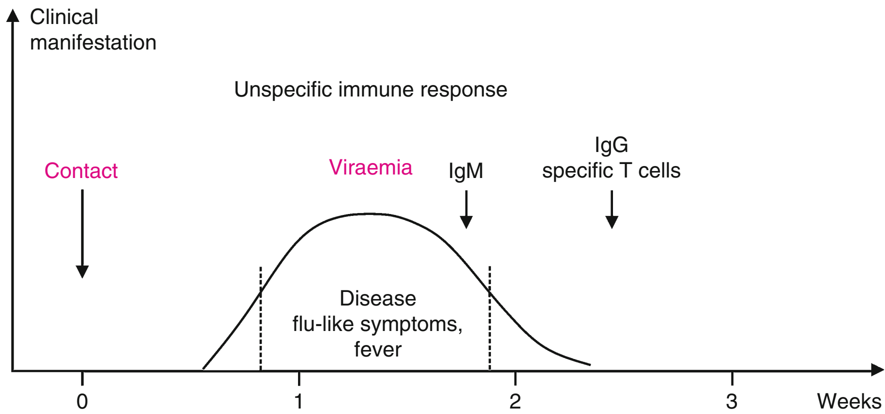

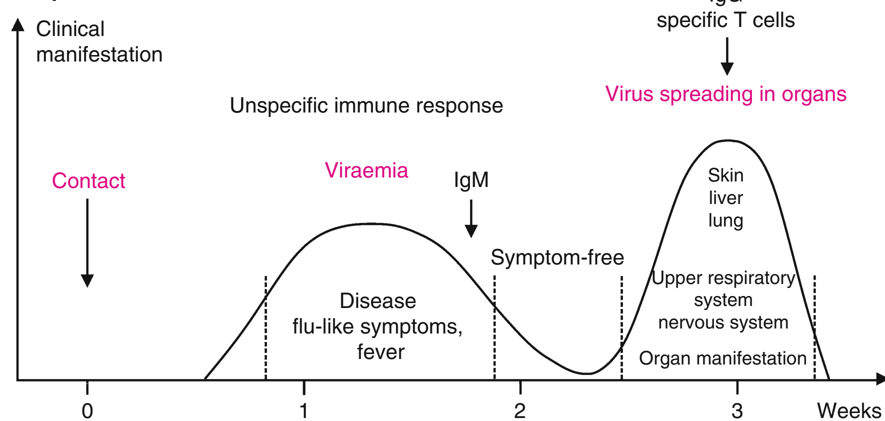

Fig. 4.1 Time course of disease development in acute viral infections. (**a**) Infection with single-phase course of disease with flu-like symptoms and immunological control of the virus. (**b**) Infection with biphasic course of disease; between disease stage I (flu-like symptoms) and disease stage II (organ manifestation) there may be a symptom-free interval of a few days

during the active phase, causing disease symptoms. Well-known examples are varicella–zoster virus and herpes simplex virus, which are members of the herpes-virus family (▶ Sect. 19.5).

The concept of pathogenicity of a virus describes its potential to produce diseases in a given host species. It is based on the activities of viral gene products, which determine in their entirety and in their interaction with each other and with cellular components the disease-causing properties. Frequently, a causative agent is pathogenic only for a sole host species. For example, HIV causes acquired immunodeficiency syndrome (AIDS) solely in humans (▶ Chap. 18), whereas classical swine fever virus, infects only pigs and induces swine fever in them (▶ Sect. 14.5).

The term "virulence" refers to the severity of viral infection. It is the pathogenic potential of a virus and the varying degrees of pathogenic properties within a viral species. Traits embedded in the virulence genes are responsible for the virulence; these can be either attenuated or enhanced by mutations. On the other hand, genetic differences among host species also influence the expression of the pathogenic properties of a virus, which can lead to enhanced or attenuated symptoms and even to resistance to certain infections. Host genes are responsible for these effects, which, for example, prevent attachment of viruses to specific cells or control their spread in the organism. If a specific immune response is already present in an organism because of a previous infection with the same or a related virus (e.g. a vaccine virus), it can also prevent infection or mitigate a disease.

4.1 How Do Viruses Spread in the Organism?

4.1.1 Entry Gates and Initial Replication

Many viruses reach the mucous membranes of the mouth, nose and throat via droplet infection (including coronaviruses, paramyxoviruses, orthomyxoviruses and adenoviruses; ► Sects. 14.8, ► 15.3, ► 16.3 and ► 19.4). In other cases, the genital mucosa is the entry site, for example, of many papillomaviruses or herpes simplex virus type 2 (► Sects. 19.3 and ► 19.5). Many picornaviruses such as poliovirus and hepatitis A virus, but also noroviruses and rotaviruses as members of the calicivirus and reovirus families, gain access to the stomach and intestine via contaminated food, and make contact with the cells of mucous membrane regions (► Sects. 14.1, ► 14.3 and ► 17.2). In the case of flaviviruses and bunyaviruses, the pathogens enter the bloodstream through bites of infected arthropods, and can infect endothelial cells of blood vessels or directly certain blood cells. Similar to salivary secretions of mosquitoes and ticks, viruses also arrive in the bloodstream of an organism parenterally when they are present as contamination in blood transfusions and blood products or in syringe needles or medical surgical instruments which are used for surgical interventions. Needles contaminated with HIV, but also with hepatitis B virus and hepatitis C virus, which are frequently used jointly by several people in intravenous drug abuse are an infection source that enables these infectious agents to penetrate directly into the bloodstream, where they can infect monocytes and $CD4^+$ T cells, in which they multiply (► Sects. 14.5, ► 18.1 and ► 19.1). On the other hand, the rabies virus enters the wound through the bite of infected vertebrates and replicates initially in muscle cells (► Sect. 15.1). Even small skin lesions can provide ideal entry sites for viruses, including papillomaviruses and herpesviruses (► Sects. 19.3 and ► 19.5). Already at the entry points, the viruses encounter cells in which they can multiply locally. However, in the course of evolution, organisms have evolved cells in all tissues which as active components of the innate or acquired immune system counteract the invasion of pathogens and their spread. These include neutrophils, and certain tissues, such as the lymphatic tissues that are associated with mucous membranes, which are also referred

to as gut-associated lymphatic tissue and bronchial-associated lymphatic tissue. The Peyer patches or plaques of the intestinal mucosa have a similar function, and Waldeyer's tonsillar ring of the throat, including the tonsils as lymphoid tissue, has an analogous function as well. The epidermis of the skin contains Langerhans cells, i.e. tissue-specific dendritic cells, which have the function to detect pathogens, to ingest them and to transport them to the nearest lymph nodes, where they trigger further reactions of the immune response. In addition to dendritic cells, macrophages also have important functions in the defence against infections in early infection stages. They migrate into the infected tissue and can phagocytose viruses or their proteins and present the respective peptides on MHC class II antigens on their cell surface (▶ Chap. 7). Macrophages become activated and secrete – similar to virus-infected epithelial or endothelial cells – cytokines and interferons, which in turn stimulate other immunologically active cells. They also contribute to the development of local inflammatory reactions (▶ Chap. 8). If this occurs, for instance, in the mucosal area of the throat, such a reaction can induce the familiar symptoms of cold.

4.1.2 Pathways for Spread of Viruses in the Body

4.1.2.1 Locally Restricted Infections

In some cases, viral reproduction and symptoms remain locally restricted to the entry site. This is true, for example, for human papillomaviruses, which are transmitted from the outside to the skin surface, overcome the outer layers of skin through minor injuries and replicate at the entry site, thereby inducing cell proliferation and leading to the formation of warts (▶ Sect. 19.3). The viruses do not break through the basal lamina of the skin. Spread occurs by the release of infectious papillomavirus particles from the wart, and these can then infect other areas of the skin and can form replication foci. Cytotoxic T cells migrate into these skin areas and recognize infected cells by antigen peptides of viral proteins that are presented on MHC class I antigens. The infection focus remains limited owing to lysis of virus-producing cells. In the case of conjunctivitis caused by adenoviruses, the pathogens arrive from outside the eye, and the infection is confined to the conjunctiva (▶ Sect. 19.4). Immunologically active cells migrate into the eye and provoke an inflammation. Infections with human rhinoviruses affect the upper respiratory tract and remain confined to mucous membranes of this region (▶ Sect. 14.1). Viruses that first infect the mucous membrane of the mouth and throat can spread continuously over the entire mucosa of the respiratory tract after initial replication cycles at the infection site. They can also spread into the middle ear or colonize lower areas of the respiratory tract without dissemination by the bloodstream. This mainly applies to the human pathogenic parainfluenza and influenza viruses (▶ Sects. 15.3 and ▶ 16.3). A similar continuous spread, but in the intestinal mucosa, is found in many enteroviruses and in caliciviruses and rotaviruses (▶ Sects. 14.1, ▶ 14.3 and ▶ 17.2).

Table 4.1 Phagocytosing cells important for dissemination of viruses in the organism

	Cells	Organ
Mononuclear phagocytes	Neutrophils and eosinophils	Blood, connective tissue
	Macrophages, dendritic cells	Connective tissue
	Monocytes	Blood
Reticular endocytic system	Reticular cells	Reticular connective tissue, lymphoreticular tissue in the spleen, lymph nodes, bone marrow, thymus, tonsils
	Sinus wall cells	Spleen, liver, lymph nodes, bone marrow
	Endothelial cells	Blood vessels
	Histiocytes (scavenger cells of different tissues originating from monocytes)	Mesoglia, microglia (central nervous system), Kupffer's star cells (liver), alveolar macrophages (lung), osteoclasts (cartilage, bones), Langerhans cells (skin)

4.1.2.2 Lymphohaematogenic Dissemination

Antigen-presenting cells such as Langerhans cells, dendritic cells and macrophages can identify and ingest viruses or individual viral protein components at the entry site. These cells are loaded with the virus particles and proteins and migrate to the immunologically active centres of the nearest lymph nodes, encountering there other immune cells such as $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes and macrophages, which start to proliferate by contact with the pathogen proteins or with MHC–peptide complexes and by the influence of cytokines secreted by activated immune cells (▶ Chaps. 7 and ▶ 8). This is the reason for lymph node swelling, which is observed in many viral infections. In the lymph nodes there are also cells that can be infected by many viruses. The progeny viruses leave the lymph nodes and are released into the lymphatic fluid and the bloodstream; they generate a primary viraemia, which is difficult to detect, as it is usually transient. Generalized infections are consequences of the dissemination in the body. In other cases, the pathogens are not released. Rather, they remain in a cell-bound state, and are spread by infected cells through the body, e.g. HIV by $CD4^+$ T lymphocytes and macrophages, and cytomegalovirus by granulocytes and monocytes (▶ Sects. 18.1 and ▶ 19.5). The cell-bound or free pathogens reach the reticulohistiocytic system, which consists of different cell types that are able to phagocytose or store substances and particles, and thus also viruses (Table 4.1). They proliferate in them and lead to a commonly pronounced secondary viraemia, which makes possible the efficient dissemination of the virus throughout the organism. The respective viruses only then reach their final replication sites and are able to cause typical symptoms in the affected organs.

4.1.2.3 Neurogenic Dissemination

Some viruses can infect nerve cells during their spread in the organism. In addition to rabies virus, various herpesviruses (such as herpes simplex virus and varicella–zoster virus) have developed this property, which enables them to spread along nerve fibres. During the initial stages of infection, rabies viruses (▶ Sect. 15.1)

migrate from the infected muscle cells into the free nerve endings at the bite site. In this case, viruses are spread neither by the bloodstream nor by the lymph fluid of the body. Rather, they migrate along nerve fibres in the axon of the peripheral nervous system through the spinal cord into the brain. Only in the late phase of infection is there a centrifugal spread back from the brain through the nerves into the periphery. In this way, rabies viruses arrive in the various organs, including the salivary glands, through which they are also excreted. By contrast, herpes simplex virus (► Sect. 19.5), which is preferentially transmitted by direct contact, infects primarily epithelial cells of the skin. The colonization of the peripheral nervous system occurs subsequently, starting from the first proliferation sites in the skin. The viruses infect the free nerve endings and migrate retrogradely via the nerve tracts to the ganglia, where they persist throughout life. In the case of reactivation, the virus migrates back into the skin, where a new relapse occurs. During herpes simplex virus infections of the conjunctiva and the cornea, reactivated pathogens migrate from the ganglia of the nerve fibres into the eye, where they may spread in the epithelium of the cornea and cause inflammations.

4.1.2.4 Organ-Specific Manifestation of Infection

Viruses reach their target organs by lymphohaematogenic dissemination. There, they initially proliferate in the respective endothelial cells that coat the inner surface of all blood and lymph vessels as a single cell layer. They reach the parenchyma, i.e. the specific tissue of an organ, passing through the intercellular spaces between the endothelial cells (free or bound to macrophages, $CD4^+$ T cells or granulocytes). As a result of viral replication, foreign proteins are present in high concentrations. Immunologically active cells are attracted into the infected organ regions and react with secretion of various cytokines and chemokines. This may result in massive inflammatory responses that have immunopathogenetic reasons and are determined by the nature of each infecting virus and the colonization site within the tissue. Details on the pathogenesis and the related manifestation of the disease in various organs will be discussed in the review of the distinct viruses in the corresponding sections. Only some basic mechanisms are described here.

Skin and Mucous Membranes

Viruses that cause colds infect the mucous membranes of the mouth and throat. Frequently, these viruses spread continuously from the mucosa of the respiratory tract, without trespassing into the blood, and thus without haematogenous dissemination. This applies, for example, to rhinoviruses, respiratory syncytial virus and parainfluenza viruses (► Sects. 14.1 and ► 15.3). In other viral infections, concomitant lymphohaematogenic dissemination is found in the organism, during which the mucous membrane of the respiratory tract is secondarily infected once more. This is valid, for example, for measles virus (► Sect. 15.3). However, immunologically active cells such as macrophages and granulocytes, which are present in the mucous membranes, are activated by phagocytosis of virus particles or proteins. They react with the release of various chemokines and cytokines, whereas activated dendritic cells (by intake of viral proteins) migrate

into the nearest lymph nodes and activate B and T lymphocytes. Chemokines and cytokines are usually proteins of low molecular mass, which diffuse through the basement membrane into the vessels, and induce in endothelial cells increased synthesis of adhesion proteins such as intercellular adhesion molecule, vascular cell adhesion molecule and endothelial leucocyte adhesion molecule, to which circulating lymphocytes attach. In this way, the migration of other macrophages, granulocytes and activated lymphocytes is induced, and they move from the vessels towards the infection site. Simultaneously, the activity of cytokines in the infected cells increases the synthesis of MHC class I and class II antigens, which present peptide fragments of viral proteins and induce the formation of specific cytotoxic T cells and T-helper cells. Since the latter, in turn, also secrete cytokines, the induction of cytokines is further reinforced (▶ Chap. 8). All these processes should contribute to inhibit viral replication and to restrain it locally. In some viral diseases, this immunoprotective barrier can be breached, especially in immunocompromised patients. Then, even secondary infections are possible after haematogenous dissemination into the mucosa of the digestive tract. This applies, for example, to cytomegalovirus, which causes epithelial lesions and ulcerations of the intestinal mucosa of immunosuppressed patients.

In viral diseases associated with exanthems such as measles and chickenpox (▶ Sects. 15.3 and ▶ 19.5), the pathogens infect endothelial cells of capillary vessels, and possibly thence skin cells after haematogenous dissemination in the organism. The inflammation, which is caused by viral replication in skin cells and by the subsequent induction of the immune response, manifests itself as a rash. Two exanthem forms can be distinguished:

1. Exanthems in which the pathogens proliferate actively in skin cells, such as herpes simplex virus and varicella–zoster virus (▶ Sect. 19.5), poxviruses (▶ Sect. 19.6), papillomaviruses (▶ Sect. 19.3) and some members of Coxsackie A virus (▶ Sect. 14.1).
2. Exanthems in which the viruses do not replicate actively in skin cells, but are deposited in capillaries as complexes with antibodies. This triggers the immune response, and hence inflammation reactions. Such variants of rash are found in infections with rubella virus and measles virus (▶ Sects. 14.6 and ▶ 15.3), human herpes virus 6 (▶ Sect. 19.5) and parvovirus B19 (▶ Sect. 20.1).

Lung

Lung infections manifest themselves preponderantly as inflammation of the bronchia and bronchioles, or pneumonia; they are caused most frequently by respiratory syncytial virus and by influenza viruses, parainfluenza viruses and adenoviruses, but also by measles virus (▶ Sect. 15.3). By their continuous dissemination, virus particles reach the bronchial mucosa, the finest branches of the bronchial tree, and infect epithelial cells of both bronchi and alveoli. These cells swell, block the alveoli and are eventually shed. Bacterial superinfections of the virus-damaged bronchial epithelium can aggravate the disease and induce secondary bacterial bronchopneumonia and interstitial pneumonia.

Other Organs as Manifestation Sites after Lymphohaematogenous Dissemination

The modes of conjunctivitis that are observed in measles virus infections probably occur after haematogenous dissemination of the virus in the organism, and not by exogenous transmission to the eye, as found with adenoviruses (▶ Sects. 15.3 and ▶ 19.4). During their viraemic, haematogenous dissemination, coxsackieviruses infect particularly the heart muscle and the pericardium, causing inflammations in these organs (▶ Sect. 14.1). Normally, both forms of the disease heal up, but the myocarditis can adopt chronic forms. The liver is infected by different viruses that cause hepatitis after haematogenous dissemination. For example, hepatitis B viruses enter the liver through fissures in the endothelium, in the perivascular spaces, the so-called Disse spaces, and in this way into hepatocytes. There, they bind to specific receptors on the cell surface, penetrate the cells and replicate in them (▶ Sect. 19.1). Apparently, the salivary glands are haematogenously infected by mumps virus (▶ Sect. 15.3). The result is a one- or two-sided parotitis, an inflammation of the parotid glands. In addition to the salivary glands, mumps viruses also reach the testes and the pancreas via haematogenous dissemination; these are organs in which the pathogens proliferate and cause inflammations. Inflammations of the pancreas can also be occasionally caused by coxsackieviruses, which infect the organ parenchyma and islet cells (▶ Sect. 14.1). Both virus types are discussed as triggers of type 1 diabetes mellitus. After haematogenous dissemination in the organism, hantaviruses infect the kidneys and can damage these organs (▶ Sect. 16.2).

The Fetus as a Manifestation Site

The circulatory system of the growing child is separated from that of the mother by the placental barrier. It prevents maternal cells reaching the fetus. However, proteins and low molecular mass substances can pass through this barrier. In pregnant women, haematogenously disseminated viruses such as rubella virus, cytomegalovirus and parvovirus B19 are transported via the bloodstream into the placenta and infect the endothelial cells of this organ. As a result of the placenta being infected, the pathogens can be vertically transmitted to the unborn infant and establish a child infection. As a result, there are lasting embryopathies or fetopathies, which can also lead to the death of the fetus (▶ Sects. 14.6, ▶ 19.5 and ▶ 20.1).

Brain Infections

Between the blood system and the central nervous system, which consists of the brain and spinal cord, there are specific barriers in the organism that separate the central nervous system from the immune system, namely the blood–brain barrier and the blood–cerebrospinal fluid barrier. The blood–brain barrier is particularly pronounced. It consists of a layer of tightly connected endothelial cells and a basement membrane, which coat the capillaries that pervade the brain (tight junctions). Microglia, which are derived from macrophages, and astrocytes sit on the endothelium and surround the vessels with their processes. Interstitial or tissue fluid is secreted by the loops of the villi-rich capillary system, which extends into the subarachnoid space. Cerebrospinal fluid is secreted by the choroid plexus and, in turn, makes contact with the

interstitial fluid. The blood–cerebrospinal fluid barrier is normally impenetrable for proteins, viruses and other insoluble substances. Some viruses, such as rabies virus and Borna disease virus, can circumvent it by neurogenic dissemination along the nerve fibres. They reach the spinal cord and brain and cause meningitis or encephalitis (▶ Sects. 15.1, ▶ 15.2). Other viruses, such poliovirus and tick-borne encephalitis virus, overcome the barriers probably by infection of endothelial cells, as occurs by infecting other organs (▶ Sects. 14.1 and ▶ 14.5). Macrophages are not retained by the blood–brain barrier, and arrive in the brain through the capillaries. If they meet infected cells, virus particles or foreign proteins there, they will secrete cytokines that induce the expression of MHC class I and class II peptide complexes on brain cells, which are normally immunologically non-recognizable, and thus render infected glial cells and neurons vulnerable to cytotoxic T cells. Some viruses, including coxsackievirus, tick-borne encephalitis virus and mumps virus cause inflammation of the pia mater, and this can spread into the cortex (meningoencephalitis; ▶ Sects. 14.1, ▶ 14.5 and ▶ 15.3). Other viruses, such as poliovirus, can also provoke encephalitis or poliomyelitis (inflammation of the grey cells of the anterior horns of the spinal cord) (▶ Sect. 14.1). Demyelination occurs prevalently in infections of the white substance, which is mainly constituted of myelin-containing nerve fibres. They also arise owing to autoimmune processes as a consequence of viral infections, such as the postinfectious encephalitis that is caused by measles virus in humans or by canine distemper virus in dogs (▶ Sect. 15.3). Macrophages elicit immunological defence reactions in the virus-infected brain which are associated with inflammations. If macrophages are infected with viruses, they are another way for the pathogen to overcome the barriers. Therefore, virus-infected macrophages frequently carry the viruses into the brain and release them. Subsequently, the viruses can infect other cells and trigger the symptoms described above. This process is mainly known from brain infections by HIV (▶ Sect. 18.1).

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Cell Damage

5

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The disease symptoms associated with viral infections are based on virus-mediated damage to the infected cells and tissues. A distinction is made between direct cell destruction as a result of viral replication, as found in picornaviruses (▶ Sect. 14.1), and indirect cell destruction, which is generally caused by immunopathological damage. The latter is from damaging effects of the immune response, which was originally developed by the organism for the clearance of pathogens (▶ Chaps. 7 and ▶ 8). For example, in the course of hepatitis B infections, liver cells are attacked and lysed by cytotoxic T cells; therefore, the immune system itself contributes decisively to the emergence of symptoms (▶ Sect. 19.1).

5.1 Cell Death: What Is Necrosis, and how Can Apoptosis Be Recognized?

Necroses are commonly found as a result of a viral infection. Necroses are pathological cell damage based on a combination of direct virus-mediated damage, and indirect immunologically caused injuries. The cells swell, they lose their membrane integrity and—relatively late—the DNA is degraded. Inflammatory reactions occur in the neighbourhood of the necrotic area, and these additionally contribute to the clinical picture.

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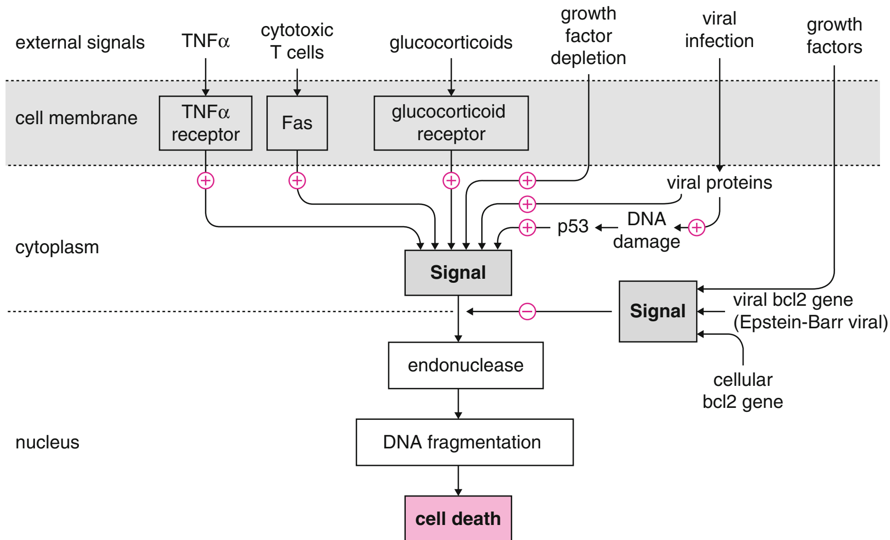

Fig. 5.1 Apoptosis-inducing mechanisms. Various signals and processes that can trigger (+) or inhibit (−) apoptosis (programmed cell death) are illustrated. Different viral gene products are able to promote or hinder apoptosis. The intracellular signalling cascades that lead to cell death are not shown in detail

The apoptotic processes (programmed cell death) which are initiated in virus-infected cells can be clearly distinguished from necrosis. This physiological cell elimination pathway, which proceeds following an established programme, occurs in all multicellular organisms, e.g., in organ differentiation processes, in destruction of non-functional neurons and in removal of a variety of autoreactive T lymphocytes during embryonic development. During apoptosis, only single cells die in an otherwise healthy tissue or organ. Their death can be triggered by different processes (Fig. 5.1) and begins with a contraction of the nucleus, and is followed, relatively late, by disintegration of the plasma membrane and dissolution of the cell. Many apoptotic vesicles are formed in this process. These membrane vesicles include all components of the dying cell, and are rapidly ingested by macrophages, which are located in the region. Inflammatory or immunological reactions are not induced. In contrast to necrosis, the DNA of apoptotic cells is quickly degraded and characteristic fragments are formed, which correspond to nucleosome-associated DNA fragments of 180–200 base pairs.

Biochemically, apoptosis is an inducible, energy-dependent process with increased RNA and protein synthesis, and thus it differs considerably from necrosis. In virus-infected cells, if apoptosis is initiated late during the replication cycle, then progeny viruses are ordinarily released. It is believed that these processes, among others, play a crucial role in the pathogenesis of diseases which are associated with parvoviruses and circoviruses (▶ Sects. 20.1 and ▶ 20.2). On the other hand, the apoptosis of infected cells that is induced early during the replication cycle is a

defence mechanism that strives to prevent efficient viral replication. It can be concluded that apoptosis can exert both positive and negative effects on the life cycle of viruses. Accordingly, viruses have developed a wide variety of molecular mechanisms to evade apoptosis, or to use it cleverly for their own replication.

5.2 What Are the Consequences of Productive Viral Infection for the Cells Affected?

With the first steps of viral replication, i.e. attachment of the virus to the cell surface and penetration, the pathogens begin to influence the cell. Here, decisive factors are the specific features of both the virus and the host organism. The implications of this interaction for the cell are described by the concept of cytopathogenity.

5.2.1 Alterations of Cell Morphology

The most important form of the in vitro recognizable cell damage manifests itself as cell rounding, usually followed by lysis. The change in morphology can be observed ideally in epithelial or fibroblast cultures that grow in cell aggregates as a monolayer and detach from neighbouring cells by the virus-mediated cell rounding. This type of cell damage, which indicates the death of the cell, represents the classic form of the cytopathic effect (Fig. 5.2). It reflects the influence of virus-coded functions on cellular processes that have been adapted to the needs of the pathogen. Thus, the cellular metabolism is selectively switched off during many viral infections. Picornaviruses (▶ Sect. 14.1), for example, are able to translate their messenger RNA genome independently of the presence of the cap-binding complex. This allows them, using a virus-encoded protease, to degrade individual components of this complex, thus preventing the synthesis of cellular polypeptides. Herpes simplex viruses (▶ Sect. 19.5) contain a virus-host shutoff factor as a constituent of the particles, which is introduced into the cell during infection. It inhibits cellular DNA, RNA and protein synthesis. Adenoviruses (▶ Sect. 19.4) regulate the export of messenger RNA from the nucleus to the cytoplasm by the interaction of two viral proteins, the E1B and 34-kDa E4 proteins. During this process, cell-specific transcripts are retained in the nucleus. In this way, solely viral transcripts are exported and translated. This also contributes to the collapse of cellular metabolism. Usually, the composition and integrity of cell components change during viral replication: viral glycoproteins are inserted in the cytoplasmic membrane, MHC antigens present peptides derived from viral proteins, the rate of expression of cellular stress factors (chaperones such as Hsp60, Hsp70 and Hsp90, formerly called heat shock proteins) is increased and differentiation antigens as well as other cell components are produced in increased or decreased amounts in infected cells, thus conferring on them a new appearance. These changes are followed by damage that changes the ion content in cell compartments. Lysosomes predominantly release proteolytic enzymes that enhance cell damage. They are

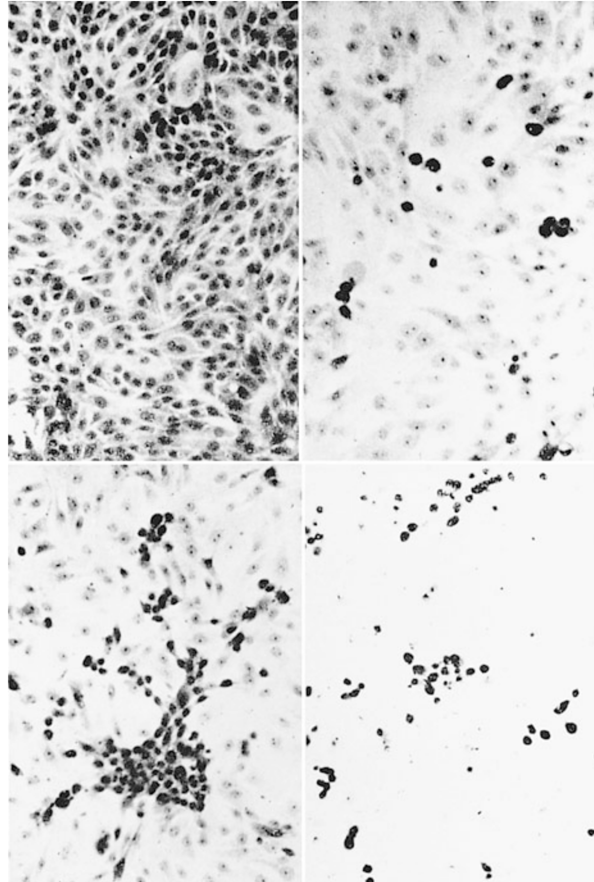

Fig. 5.2 Cytopathic effect with cell rounding represented in a cell culture infected with vaccine polioviruses. *Top left*: uninfected cell culture (HeLa cells). *Top right*: 24 h after infection with vaccine polioviruses, individual cells become rounded and detach from the cell layer, which is attached to the bottom of the culture bottle. *Bottom left*: 48 h after infection, starting from the initial infection focus, vaccine polioviruses infected neighbouring cells, which also begin to exhibit cell rounding. They form a so-called plaque composed of infected rounded cells. At this stage, the surrounding cells are largely undamaged. *Bottom right*: 72 h after infection, all cells are infected in the culture. Almost all cells are detached from the bottom, and only a few adherent cells can be observed on the floor of the culture bottle. The confluent cell layer has disappeared

probably involved in the destruction of microfilaments, microtubules and the cytokeratin skeleton of the cells, leading to their rounded appearance. Furthermore, even the structures of cell junctions that bind the cells to each other and cause them to adhere to their respective surface (in vitro, to the bottom of the culture bottle) are altered, resulting in loss of contacts. This also contributes to the observed cell rounding.

In addition to the occurrence of the cytopathic effect, inclusion bodies were interpreted as evidence that organisms or cultured cells are infected by viruses (Fig. 5.3). These bodies are viral proteins or virus particles which are deposited in the cell and can be detected by staining. Inclusion bodies of RNA viruses are usually localized in the cytoplasm, whereas those of DNA viruses are situated in the nucleus. Exceptions are poxviruses (DNA viruses) with cytoplasmic inclusion bodies as well as influenza viruses and Borna disease virus (RNA viruses) with nuclear inclusion bodies.

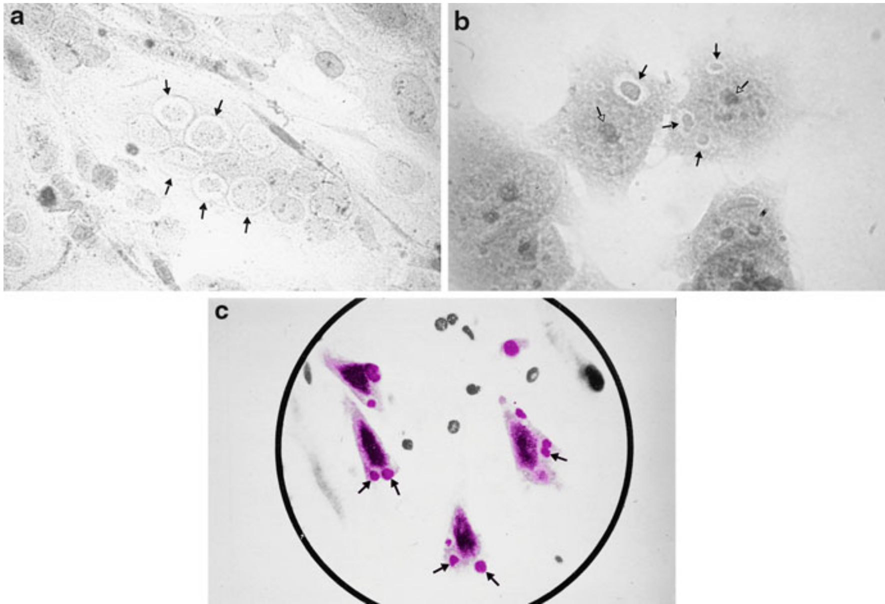

Fig. 5.3 Inclusion bodies. (**a**) Inclusion bodies in HeLa cells infected with herpes simplex virus type 1. *Arrows* indicate the structures that consist of viral proteins, which are deposited and accumulated in the nucleus. They are surrounded by a white halo, by which they can be recognized. (**b**) Cytoplasmic inclusion bodies in HeLa cells infected with vaccinia viruses. Inclusion bodies consist of viral proteins (*black arrows*), and are also surrounded by a halo. Cell nuclei are marked by *open arrowheads*. (**c**) Negri inclusion bodies (*arrows*) in a dot preparation from an animal infected with rabies. Cytoplasmic inclusion bodies composed of viral proteins and lipids can be observed; they can be dyed according to the method of van Gieson, resulting in a purple colour

5.2.2 Syncytia Formation

The formation of giant cells, also referred to as polykaryocytes or syncytia, was observed long ago both in vivo and in vitro (Fig. 5.4). These multinucleated cells were initially found in throat swabs from patients who were infected with measles virus and were denominated Warthin–Finkeldey giant cells. This eponymous property is particularly pronounced in respiratory syncytial virus. Similar syncytia have been detected in smear preparations of skin blisters induced by herpes simplex virus. Syncytia arise by cell fusion, which is induced by viruses in different ways. Upon attachment to the cell surface, paramyxoviruses (▶ Sect. 15.3) have the ability to promote the fusion of their own envelope with the plasma membrane by the activity of the F protein, which is anchored in the viral envelope, a process through which the nucleocapsid penetrates into the cell. This process is independent of the presence of the viral genome; thus, it can also be initiated by the viral envelope alone, which is also known as a virosome. During the course of viral replication, new viral proteins are synthesized and incorporated into the cytoplasmic membrane; they promote

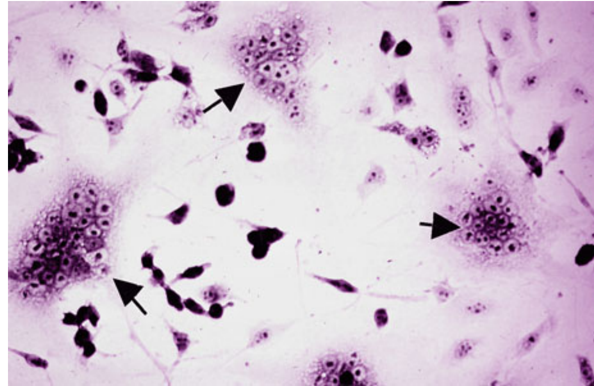

Fig. 5.4 Giant cell formation in a culture infected with respiratory syncytial virus. Several multinucleated polykaryocytes or syncytia (*arrows*) are shown; these are formed by fusion of many cells. *Rounded* cells can be observed in the vicinity; these were induced by the infection

fusion with the membranes of neighbouring cells. Membrane fusion of virus-infected cells with uninfected cells can contribute to the spread of infectious viruses in the organism. Apart from paramyxoviruses and herpesviruses, this kind of fusion is also observed, for example, in infections with human immunodeficiency virus (HIV) and poxviruses (▶ Sects. 18.1 and ▶ 19.6).

5.3 To what Extent Can also Latent Viruses Damage Cells?

Productive viral infections, which are generally associated with the release of large amounts of offspring viruses, can be distinguished from those in which the virus is in a state of equilibrium with the infected cell or organism. In that balanced state, viruses are neither eliminated nor are the cells apparently damaged. Viruses that develop latent or chronic persistent infection forms can cause cell transformation in certain circumstances.

During latency, only the viral genome and a few viral gene products are present in the cell, and the productive cycle is interrupted. Retroviruses integrate their genetic information into the genome of the host cell. Cell damage may occur through the integration of the virus into the genome if functional genes are interrupted (integration mutagenesis). However, the consequences of infection appear predominantly only after activation of the integrated viral genome, and are then caused by the newly synthesized proteins of the virus. The expression of viral genes can be induced by specific factors, which are synthesized, for instance, during differentiation processes of the cell. Thus, expression of the HIV genome is activated only through nuclear factor κB (NFκB), which is present in its active form in immunologically stimulated T cells (▶ Sect. 18.1). In addition to the synthesis of structural proteins and enzymes, the oncogenic alpharetroviruses, betaretroviruses and gammaretroviruses also induce the expression of viral oncogenes, which can transform the cells and promote tumour formation.

In alphaherpesviruses, e.g., in herpes simplex virus, another type of latency is known: the genome is present in an episomal state in nerve cells, i.e. it is present as

a circular DNA molecule within the nucleus. Although there is no synthesis of viruses or proteins, a minimal expression of certain RNA species occurs, and contributes to the maintenance of latency (▶ Sect. 19.5). Cell damage occurs in this case only after reactivation, which can be triggered by various factors. For induction and maintenance of latency, other herpesviruses require the functional activity of viral proteins, which prevent the transition into the productive infection phase and simultaneously also immortalize the cell. Thus, B cells which are latently infected with Epstein–Barr virus are capable of performing infinite cell divisions. Epstein–Barr virus induces the transformation of the cell in connection with additional influences and certain conditions. In papillomaviruses (▶ Sect. 19.3), the transition from the latent infection state to the productive infection mode is dependent on the cell differentiation state in the basal cell layers of the skin: the productive infection mode is associated with the death of the cell and is induced by specific cellular factors that exist only in keratinocytes, i.e. in the uppermost layers of the skin. The highly sensitive methods for detecting nucleic acids using the polymerase chain reaction (▶ Chap. 13) which have been developed in recent years have demonstrated that viral genomes occasionally remain in the organism for long time. In the case of human parvovirus B19, viral genomes can be found in the cells of many tissues even decades after infection (▶ Sect. 20.1). Whether the persistence of the nucleic acid is associated with occasional reactivation of the pathogen or whether it contributes to chronic inflammation by the induction of unspecific immune responses, such as the activation of Toll-like-receptor-mediated defence, is unclear (▶ Chap. 7).

Besides the classic latency, during which no or only minimal expression of the viral genome occurs, persistent or chronic infection modes are also known, in which a continuous, low-level proliferation and release of pathogens occurs. In such cases, an ideal balance between viral reproduction and survival of the host cell is established. These chronic infection forms are found, for example, in hepatitis B virus, Epstein–Barr virus and some adenoviruses; they have evolved mechanisms that allow them to evade elimination by the immune system (▶ Sects. 19.1, ▶ 19.4 and ▶ 19.5). In other species of viruses which commonly replicate lytically, these mechanisms also seem to have great importance. In measles virus, persistent infections may be associated with the emergence of subacute sclerosing panencephalitis (▶ Sect. 15.3). There is increasing evidence that viruses prevent the initiation of apoptosis to avoid even this defence strategy. Hence, for some picornaviruses there are long-lasting infections (▶ Sect. 14.1). Virus mutants often develop in chronic persistent infections. It is unclear to what extent these frequently defective viruses are responsible for cell damage and chronic infections.

5.4 How Do Viruses Alter the Genome of the Host?

Some viral species can be regarded as mutagenic agents in the broadest sense, as they integrate their genomes either completely or partially into the chromosomal DNA of the host cell during replication. As a result of this, cellular genes can be destroyed or silent genes (i.e., non-expressed or low-expressed genes) may be

transcribed by viral promoters, with the consequence that the gene products are then present in high concentrations and become active in the cell. As a consequence of integration processes, both the death of the cell and its transformation to a potential tumour cell are conceivable (▶ Chap. 6). In some viral infections, necrotic or apoptotic processes bring about the destruction or fragmentation of the genome of the host. If the cell recovers, the following repair can lead to translocations, deletions and amplifications of chromosome regions. If these confer a growth advantage to the affected cell, it will be selected in the course of the following cell cycles. Translocations of the short arm of chromosome 14 to chromosomes 2, 8 or 22 occur in Burkitt's lymphoma, which is caused by Epstein–Barr virus, whereby, the cellular *myc* gene is overexpressed. It is uncertain whether this is a result of specific virus-induced translocations or a selection process after general genome damage (▶ Sect. 19.5).

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Transformation and Carcinogenesis

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It was acknowledged long ago that viruses may cause cancer in animals. In 1911, Peyton Rous described viruses as causing sarcomas in poultry. The tumour-inducing virus responsible was later named after him, Rous sarcoma virus. In the following decades, a large number of viruses were discovered that can cause various cancers in poultry and rodents, such as lymphomas, sarcomas and carcinomas. Many of them belong to the family *Retroviridae*, and were classified into the genera *Alpharetrovirus*, *Betaretrovirus* and *Gammaretrovirus*. Most of these pathogens were isolated from inbred strains of the respective species or from cell cultures; under natural conditions, these strains are likely irrelevant as a cause of cancer in the corresponding species. An exception is feline leukaemia virus (▶ Sect. 18.1). The tumorigenic potential of oncogenic retroviruses is based on transformationally active proteins. They are similar to cellular products which are ordinarily involved in the regulation of cell division. In contrast to the cellular products, viral oncogene proteins are altered by mutations in such a way that they are not subject to regulatory control, and are thus constitutively active. In fact, the discovery of viral oncogenes was pioneering and has paved the way for deciphering cellular oncogenes, and thus for understanding the molecular basis of carcinogenesis.

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Evidence for the existence of retroviruses that cause cancer in humans was found only in 1982 when Robert Gallo discovered human T-lymphotropic virus (HTLV; ▶ Sect. 18.1).

Nevertheless, most viruses that are correlated with cancer in humans have a DNA genome. The most important prototypes are papillomaviruses, which cause carcinomas, especially in the genital mucosa, and various malignant skin tumours (▶ Sect. 19.3), hepatitis B virus, which is involved in the development of primary liver cancer in humans (▶ Sect. 19.1), and Epstein–Barr virus as well as human herpes virus 8, which have a close causal relationship to Burkitt's lymphoma and nasopharyngeal carcinomas and to Kaposi's sarcoma, respectively (▶ Sect. 19.5). Hepatitis C virus, a flavivirus (▶ Sect. 14.5) with a single-stranded RNA genome, is associated with liver cancer, like hepatitis B virus. Recently, Merkel cell polyomavirus has been identified as another pathogen that is causally associated with a tumour of humans, namely Merkel cell carcinoma (▶ Sect. 19.2). Aetiologically, it is estimated that approximately 15–20 % of all human cancers are induced by viral infections. Adenoviruses, whose infections in humans could not be clearly associated with malignant diseases, can induce tumours, however, in newborn rodents (▶ Sect. 19.4). Their study has significantly contributed to elucidation of the mechanisms that are involved in cell transformation and tumour development. These insights could be applied to a number of other human cancers. Similarly, this is true for the simian virus 40 (SV40), which infects monkeys under natural conditions, but causes malignant diseases in newborn hamsters and mice (▶ Sect. 19.2). The DNA tumour viruses and hepatitis C virus do not have classic *v-onc* genes such as those found in retroviruses. Today, we know that those viruses principally deactivate the function of cellular tumour suppressors by specific viral regulatory proteins, which induce and maintain the malignant transformation of cells.

6.1 What Characterizes Transformed Cells?

The malignant properties of various viruses are primarily manifested through their ability to generate tumours in vivo. Frequently, this process can also be induced in experimental animal systems, so in many cases proper animal models are available for investigating the underlying molecular mechanisms. However, in vitro systems were and are essential for unveiling the malignant effect; tumour viruses are able to immortalize certain tissue culture cells and transform them in vitro. This makes possible detailed experimental investigations to elucidate the molecular processes that are associated with tumour development. Whereas immortalized cells acquire the capability of infinite cell division by viral activities, transformed cells are additionally characterized by the capability of inducing tumours, when transmitted into appropriate animals. Apart from this fundamental difference, both cell systems share many common characteristics that clearly distinguish them from normal cells.

6.1.1 Morphological Changes

During transformation, the cells change their form: they lose their normal epithelioid or fibroblast-like character and adopt mainly a spherical form. The transformation-associated change of the cytoskeleton is responsible for this effect, and confers on the eukaryotic cells their shape and is involved in cell division, cell motility and cell polarity. The breakdown of intracellular microfilaments is a prevailing process. In non-muscle cells, these microfilaments consist of the globular components β-actin and γ-actin, which are usually organized in long cables with a diameter of 7 nm and are cross-linked by the protein fimbrin. Actin cables form network structures beneath the cytoplasmic membrane, stabilize the shape of the cell and are also involved in the correct positioning of membrane proteins, where they are associated with other proteins such as myosin and tropomyosin, which tense the actin filaments. They are responsible for the short intracellular transport, for example, of vesicles to the plasma membrane. At the sites of the cytoplasmic membrane where the cables end, the cells form contacts with neighbouring cells, or are rooted in the surface of the base (in vitro, on the bottom of the cell culture flask). These regions are referred to as focal contacts. In the cells, there is a dynamic equilibrium of monomeric and polymeric actin. In the presence of Mg^{2+} and K^{+} ions and under ATP binding, globular actin monomers associate to filaments that are stabilized with the polypeptides α-actinin, filamin and fimbrin. During transformation, the cable-like arrangement of filaments is lost, and the actin monomers are diffusely distributed over the entire cell. This facilitates cell motility, which is necessary for tumour formation. Binding of the protein components profilin, gelsolin, vinculin and vilin has a destabilizing effect on the polymerization degree; the actin-bound ATP is hydrolysed during this process. It is unclear what regulates the changes of microfilaments at the molecular level. It is believed that increased phosphorylation of actin and vinculin is involved as it occurs in transformed cells.

Simultaneously with the alteration of actin filaments, a redistribution of transmembrane proteins is observed on the surfaces of transformed cells. They are connected with actin cables at the inner side of the cytoplasmic membrane and lose their position in defined groups, as a result of the collapse of actin cables. This changes, among other things, the concentration of integrins and their distribution. Integrins are membrane-anchored proteins which are connected to the actin cables by their cytoplasmic moiety. Their extracellular domains are associated with fibronectin, which in turn interacts with collagen and laminin. They make up the extracellular matrix, which mediates the interactions between different cells and is responsible for their organization and growth in cell aggregates; it ensures that cells grow as a monolayer in vitro and facilitates adhesion to the base, e.g., the cell culture dish. Owing to their low levels of integrins, transformed cells have significantly lower amounts of fibronectin on their surface and grow in cell clusters (foci). The concentrations of various ions, sugars and amino acid transporting proteins are increased on the surfaces of transformed cells. This is associated with up to tenfold increased metabolic activity in comparison with normal cells. The concentration of

other membrane proteins is also altered: for example, the levels of MHC class I proteins are usually reduced, whereas the levels of proteinases (metalloproteases, cysteine and serine proteases) and collagenases (type IV collagenases, transin/stromelin) are elevated on the cell surface. These proteins are also released into the surrounding region, which augments the potential of transformed cells to invade lymphatic vessels and capillaries and the formation of metastases. As a prerequisite for metastasis, primary tumour cells have to leave the tumour to overcome the basement membrane and to penetrate into the local stroma, e.g., in metastasis of cervical carcinoma that is induced by papillomaviruses (▶ Sect. 19.3). To find access to the circulatory system, to immigrate as a secondary colony into the new target organ and to proliferate there, tumour cells must induce angiogenesis, i.e. the de novo generation of blood vessels that supply the tumour with blood and all the nutrients contained therein.

6.1.2 Cell Growth Changes

In vitro transformed cells differ from normal cells in regard to their growth behaviour. Fibroblasts or epithelial cells which are placed in a cell culture bottle commonly attach to the plastic or glass surface. Subsequently, they divide continuously until a confluent monolayer is formed and the cells have used the entire available area completely. Then, cell division stops. In contrast to normal cells, transformed cells grow in multilayered, three-dimensional aggregates and reach cell concentrations that are five to ten times higher (foci). They are not subject to the so-called contact inhibition. If they are further cultivated in the culture bottle, an equilibrium is established between death of a subset of the cells and continuous division of the remaining subpopulation. Transformed cells grow in culture regardless of contact with plastic or glass surfaces, which is usually necessary for the proliferation of fibroblasts or epithelial cells. This property is an important feature of transformed cells. Therefore, some of these cells may also grow into larger aggregates, so-called cell clones, in a semiliquid medium such as soft agar. The ability to growth in soft agar correlates very well with the ability of transformed cells to form tumours in animal test systems. This behaviour is presumably based on the higher levels of transforming growth factor (TGF)-β, which is synthesized in such cells. TGF-β is a growth factor that stimulates, inter alia, the synthesis of fibronectin and collagen (▶ Chap. 8). The cells are thus provided with a locally limited extracellular matrix, which enables them to adhere together and to grow in grape-like aggregates.

Cells grow normally in vitro only if sufficient amounts of growth factors are available in the culture solution. For this reason, the medium is supplemented with approximately 10–15 % fetal calf serum, which ensures that all essential components are available. Important for cell proliferation are primarily epidermal growth factor, platelet-derived growth factor, various fibroblast growth factors and some hormones. These factors bind to their respective receptors on the cell surface and induce the activation of protein kinases by different signal transduction pathways,

which in a tightly regulated, cascade-like process phosphorylate various cellular proteins, including transactivators. Transactivators bind to the serum response elements in the promoter region of growth-factor-dependent genes and induce their expression. The newly formed gene products initiate cell division. By contrast, transformed cells divide independently of the presence of growth factors in the culture medium. They are able to grow in media containing no or very little serum. Transformed cells produce many of the necessary factors themselves, thus stimulating their autocrine proliferation. Moreover, they often secrete tumour growth factors (TGF-α and TGF-β), which also stimulate autocrine cell growth (▶ Chap. 8). In some transformed cells, the growth factor receptors are altered in such a way that they stimulate an active state even in the absence of the respective factor, incessantly transmitting signals into the cell.

6.1.3 Autocrine Cell Growth Stimulation by Viruses

Autocrine stimulation mechanisms are probably involved in the origin of HTLV-mediated T-cell leukaemia in humans. HTLV encodes a Tax protein that indirectly affects transactivation by interacting with nuclear factor κB (NFκB) and factors of the cyclic AMP response element binding (CREB) protein family, whereby they are activated, bind sequence-specifically to Tax response elements in the viral long terminal repeat promoter and induce transcription of the integrated viral genome (▶ Sect. 18.1). Furthermore, cellular genes are also expressed whose promoters contain CREB- or NFκB-dependent DNA regulatory elements. These include the genes encoding granulocyte–macrophage colony-stimulating factor, interleukin-2 and the α chain of interleukin-2 receptor (▶ Chap. 8). The increased expression of these cytokines and their corresponding receptors induces cell proliferation in an autocrine stimulation loop. It is the first stage in the development of HTLV-mediated T-cell leukaemia.

Even Epstein–Barr virus, which latently infects and immortalizes B lymphocytes, can stimulate autocrine proliferation of these cells (▶ Sect. 19.5). Latent Epstein-Barr virus nuclear antigen 2 (EBNA2) transactivates the promoters, which control the expression of latent viral genes. In addition, EBNA2 induces the synthesis of various cellular proteins, including that of CD23. Latent membrane protein 1 (LMP1), another viral polypeptide that is synthesized during latency, is also able to do so. The CD23 protein is found in two versions: the membrane-bound CD23 is a low-affinity IgE receptor; on the other hand, the secreted form of the protein serves as B-cell growth factor and promotes proliferation of infected B lymphocytes. The Epstein–Barr-virus-specific LMP1 gene product has also a number of other functions that are essential for transformation of latently infected cells. There is evidence that this envelope protein acts similarly to some of the classic oncogenes of retroviruses, and is a constitutively active growth factor receptor, which continuously triggers and maintains a signalling cascade. Apparently, it is similar to members of the nerve growth factor receptor family, which includes the TNF receptor. They constantly

transmit signals into the infected cells, resulting in induction of the expression of NFκB-dependent genes, among other things. As a result, apoptosis is inhibited, and increased concentrations of adhesion molecules, transferrin receptor, and CD23 proteins are induced.

6.2 What Is the Effect of Inactivation of Tumour-Suppressor Proteins?

Tumour suppressors, also called antioncogenes, are regulatory proteins that control cell division. They regulate the transition of proliferating cells from the G_1 phase, or in resting cells from the G_0 phase, to the S phase, in which the genome is replicated, and many other synthesis processes are performed (Fig. 6.1). The timing of the cell cycle is determined by synthesis and degradation of cyclins, whose concentration increases in a phase of the cell cycle, and then decreases during the following stages. Cyclins regulate, in turn, the activity of cyclin-dependent protein kinases (CDKs), which influence the phosphorylation status and thus the activity of various cell-cycle-specific transcription factors.

Many viruses can replicate only in dividing cells, where they perform productive infections that are associated with the generation of progeny viruses. Whereas some viruses – such as the autonomous parvoviruses (► Sect. 20.1) – have developed a strong tropism for dividing cells, others are able to accelerate the cell cycle in infected cells by inducing the S phase. They encode proteins which inhibit cellular factors that regulate, prevent or impede the entry of the cell into the S phase. All viruses that induce cancer in humans have such qualities and express the corresponding genes early during the infection cycle. They inactivate cell division regulators, and induce the transition from the G_1 or G_0 phase to the S phase. In DNA tumour viruses, viral replication is generally associated with cell death, which is induced by apoptosis mechanisms during the late phase of the infection cycle. If the lytic infection is interrupted, the viral gene expression is arrested at an early stage, and viral replication does not occur. Generally, the cellular milieu is responsible for these abortive infections. Thus, the lytic infection cycle of papillomaviruses (► Sect. 19.3) occurs only in skin keratinocytes, but not in the poorly differentiated cells of the basal layers. Cellular proteins, which are produced only in a specific stage of differentiation, facilitate the entry into the late viral replication phase. In other viruses, such as the oncogenic adenoviruses and SV40, an abortive infection occurs when the pathogen infect cells of non-natural hosts, in this case rodents (► Sects. 19.2 and ► 19.4). In addition, the viral genome is occasionally integrated completely or partially into the host-cell DNA, as may occur in hepatitis B virus and papillomavirus infections. This process disrupts the continuity of individual viral genes; as a result, there is often uncontrolled overexpression of viral genes, whose products interact with cellular tumour suppressors and induce continuous cell division, since the lytic replication cycle is blocked at the same time. This can eventually lead to carcinogenesis. Even if a growing number of tumour-suppressor

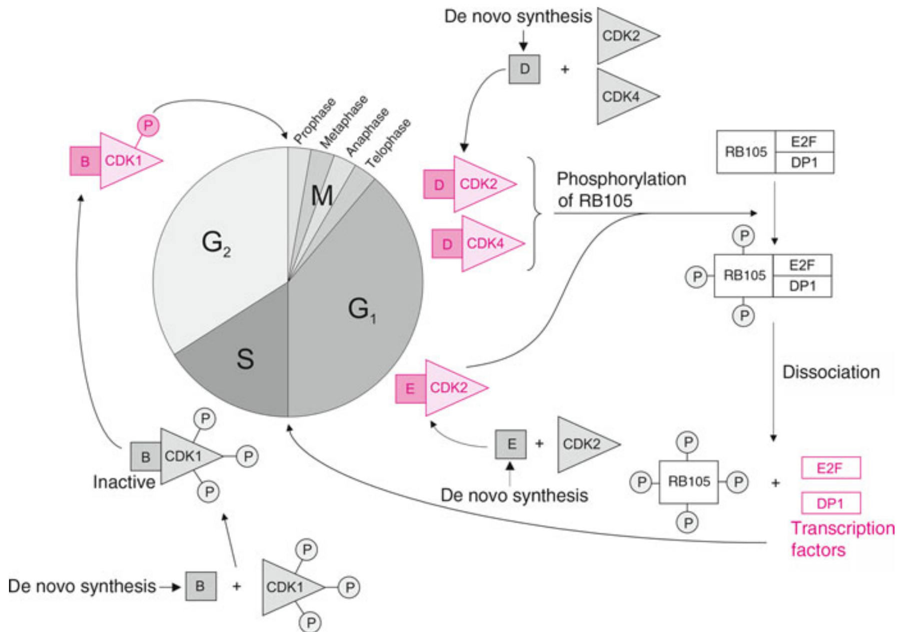

Fig. 6.1 Cell cycle phases (*M* mitosis, G_1 presynthesis phase, *S* synthesis phase, G_2 postsynthesis phase) and the factors involved in regulating the transitions. Cyclin D is synthesized in the early G_1 phase, binds to cyclin-dependent kinase 2 (*CDK2*) and cyclin-dependent kinase 4 (*CDK4*) and activates them. These kinases phosphorylate, among other proteins, retinoblastoma protein RB105, which is present in a complex with transcription factors E2F and DP1. In the later G_1 phase, cyclin E is synthesized, and interacts with CDK2, which performs the complete phosphorylation of RB105. E2F and DP1 are released from the complex, and act as transactivators of various cellular genes. These genes encode proteins which in turn regulate the transition from the G_1 to the S phase of the cell cycle. At the beginning of the S phase, cyclin B is synthesized, and interacts with the phosphorylated form of cyclin-dependent kinase 1 (*CDK1*). During the S and G_2 phases, CDK1 is dephosphorylated and regulates, in its monophosphorylated form, in complex with cyclin B, the entry of the cells into mitosis. *Squares* cyclins, *triangles* different cyclin kinases, *red* active components, *grey* inactive components

genes are known, viral gene products influence the activity principally of two groups of these protein families. On the one hand, tumour suppressor p53; on the other hand, the family of retinoblastoma proteins (RB105/RB107). The following sections provide a brief overview of the functions of these two protein classes.

6.2.1 Protein p53

The p53 gene is highly conserved among mammalian species. In humans, the p53 gene is located on the short arm of chromosome 17. It encodes a protein of about 393 amino acids residues. Normally, p53 has a short half-life of 6–15 min, it is

phosphorylated and is localized in the nucleus, where it assembles into tetrameric complexes. Increased concentrations of p53 are found in cells which were exposed to UV radiation or γ-radiation or were incubated with radioactive substances, chemotherapeutic drugs or DNA-damaging agents. All these compounds can cause mutations, i.e. changes in the DNA sequence.

The protein p53 can be functionally divided into four principal domains: the transcriptional transactivation domain resides in the amino-terminal region, which predominantly contains acidic amino acids. It is followed by a proline-rich region. The central DNA-binding core domain consists of a variety of structural motifs that are evolutionarily conserved; this important region is responsible for the sequence-specific DNA-binding activity and the correct folding of p53, thus essentially contributing to its tumour-suppressor activity, as demonstrated by the fact that it is the target of more than 90 % of p53 mutations found in human cancers. The carboxy-terminal region contains several functional motifs, including the tetramerization domain, three nuclear localization signals, which flank the oligomerization domain, a single putative nuclear export signal located within the tetramerization domain and a basic negative regulatory domain.

The protein p53 interacts with specific promoters containing p53 consensus sequences or with transcription factors that bind to p53-responsive promoters, and transactivates them. This induces the synthesis of proteins, which arrest the cell in the G_1 phase and delay the transition to the S phase. For example, the protein p21 is strongly induced, and interacts with the CDK2 and CDK4, and inhibits them. CDK2 and CDK4 have the function to phosphorylate various proteins, such as RB105. If RB105 is not phosphorylated, it remains associated with the E2F and DP1 transcription factors, which thus cannot exert their transactivation functions. This prevents the cell from entering the S phase of the cell cycle and provides more time to repair DNA damage (Fig. 6.1). If DNA repair is not successful, p53 induces apoptosis in eukaryotic cells (programmed cell death), and thus also prevents tumour formation. To a certain degree, the function of p53 is comparable to the SOS response, which is induced under similar DNA-damaging conditions in bacteria. This regulatory mechanism delays replication of the bacterial chromosome, so more time is available for the repair systems to correct the DNA damage before it can be manifested as mutations in DNA daughter molecules after subsequent replication rounds.

Mutations in the p53 gene in tumour cells lead to the synthesis of p53 variants which are not properly folded, no longer associate to form tetramers, and have thus lost the ability to function as sequence-specific DNA-binding transcription factors. Mutations in only one allele of the p53 gene are usually sufficient since wild-type and mutant proteins form inactive heterotetramers. In cells carrying p53 gene mutations, further mutations occur in the entire genome because of the impaired activity of p53, and these additionally contribute to malignant transformation in a multistep process. Viruses have developed diverse mechanisms to inactivate the cell-cycle-regulating effect of p53, and to create conditions that are necessary for their own reproduction. The T antigen of SV40 (▶ Sect. 19.2), the 55-kDa E1B protein of adenoviruses (▶ Sect. 19.4) and the X protein of hepatitis B virus (▶ Sect. 19.1) bind to p53 and prevent the formation of functionally

active tetramers. In papillomaviruses (▶ Sect. 19.3), the viral E6 protein induces complex formation between cellular E3-ubiquitin ligases and p53 and initiates its ubiquitin-dependent proteolytic degradation, thereby decreasing the intracellular concentration of p53.

6.2.2 Retinoblastoma Proteins

The retinoblastoma gene was first described in children with eye cancer. In these patients, both alleles of a gene are defective; the gene is located on the long arm of chromosome 13 and encodes a protein with a molecular mass of about 105 kDa (RB105). Later, mutated forms of this protein were also detected in osteosarcomas, soft tissue sarcomas and in breast, lung and bladder cancers. Phosphorylation of RB105 and the similar protein RB107 depends on the phase of the cell cycle. The aforementioned CDK2 and CDK4 are responsible for the phosphorylation of these proteins (Fig. 6.1). They are active, i.e. proliferation-inhibiting, in their dephosphorylated state. In this form, they bind to transcription factors E2F and DP1, which as free proteins interact with DNA elements in the promoters of specific genes in a sequence-specific fashion; such genes include the genes encoding the cellular enzymes thymidine kinase, dihydrofolate reductase, DNA polymerase-α and CDK2. All these proteins are required during the S phase of the cell cycle. The transactivation functions of transcription factors E2F and DP1 are inhibited in association with RB105. Therefore, the entry of cells into the S phase is blocked. If retinoblastoma proteins are phosphorylated, the complex with the transcription factors is dissolved. The latter can bind to the corresponding promoters and induce the expression of the genes that they regulate. Viruses that exclusively replicate in proliferating cells possess factors which influence the functions of retinoblastoma proteins. The papillomavirus E7 protein, the T antigen of SV40, the hepatitis C virus NS5B protein, and the E1A proteins of adenoviruses bind to RB105/RB107. These interactions abolish the complex formation with transcription factors E2F and DP1, which in turn become activated, and exert their transactivation activities (▶ Sects. 14.5 and ▶ 19.1–19.4).

6.2.3 Alternative Pathways to Induce Proliferation

Epstein–Barr virus has probably developed alternative ways to stimulate proliferation in latently infected cells. Epstein–Barr virus nuclear antigen leader protein is a gene product that is synthesized in B cells during the latent infection cycle. Although it can interact with the tumour-suppressor proteins p53 and RB105, it does not apparently influence their regulatory functions (▶ Sect. 19.5). However, Epstein–Barr virus nuclear antigen leader protein activates along with EBNA2 the expression of the cyclin D2 gene. The cooperative effect of the two latent viral proteins together with the antiapoptotic activity of Epstein–Barr-virus-encoded RNA induces the transition from G_0 into G_1 in resting B cells.

6.3 How Can Tumour Cells Evade the Immune Response?

Tumours can develop in vivo only if transformed cells are unrecognizable to the immune defence systems, and if they are able to evade the immune response. This applies also for tumours which are induced by viruses. Cells infected with adenoviruses or Epstein–Barr virus can elude the antiviral effect of interferon by synthesis of adenovirus-encoded virus-associated RNA molecules or Epstein–Barr-virus-encoded RNA molecules, respectively. Both virus types also reduce the concentration of MHC class I proteins on the cell surface. This prevents infected cells from being recognized and destroyed by cytotoxic T lymphocytes (▶ Chap. 7, ▶ Sects. 19.4 and ▶ 19.5). Epstein–Barr virus nuclear antigen 1 (EBNA1) also has an important function in this process: this protein is expressed in all latently infected cells; it acts as a transactivator and also increases its own expression. Concomitantly, it binds to the viral origin of replication oriP, at which the episomal replication of the viral genome is initiated during the latent state. Hence, EBNA1 is responsible for maintaining the immortalized state. Despite the long-lasting production of this viral protein, the cells are not recognized as foreign: a domain within EBNA1 which consists of repeated glycine–alanine residues prevents degradation by the proteasome, i.e. it inhibits the process that is essential for the formation of MHC antigen–peptide complexes (▶ Sect. 19.5).

Other human tumour viruses can also avoid immune defence systems. Papillomaviruses (▶ Sect. 19.3) evade most immune responses by infecting preferably the outer skin layers, and thus occupying an ecological niche, which is not accessible to many immunologically active components. Hepatitis B virus produces and secretes large amounts of the surface protein HBsAg, which intercepts virus-specific neutralizing immunoglobulins, which thus cannot exert their antiviral activity.

6.4 Are Viruses Capable of Suppressing Apoptosis?

Apoptotic processes are probably associated with the occasionally observed phenomenon of spontaneous tumour regression. Although little is known about these processes, there is a growing body of evidence that transformation and tumour formation can solely be successful if viruses induce not only cell proliferation and have mechanisms to evade the immune system, but also prevent the induction of apoptosis. These processes are best studied in Epstein–Barr virus, which has complex mechanisms for immortalization and transformation. The latent protein LMP1 induces the expression of the cellular proto-oncogene c-bcl-2 (Fig. 6.1), which in turn prevents the cell triggering apoptosis by inducing the Fas signalling pathway. The same effect is exerted by the adenovirus 19-kDa E1B protein and a protein of Epstein–Barr virus which is encoded in the BHRF1 open reading frame ORF. They have sequence and functional homology to the protein Bcl2 and also counteract the induction of apoptosis (▶ Sects. 19.4 and ▶ 19.5). Besides tumour viruses, several other viruses that can establish persistent infections have developed

mechanisms to suppress apoptosis; these include the poxviruses (▶ Sect. 19.6). On the other hand, viruses that induce acute infections also seem to have functions that suppress apoptosis in a tissue-specific manner, such as SARS virus (▶ Sect. 14.8).

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Immunology 7

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The immune defence mechanisms by which an organism combats viral infections can be divided into two systems. On the one hand, there are the unspecific, non-adaptive immune reactions, which recognize and eliminate invading foreign pathogens. This so-called natural or innate immune system becomes primarily active after a virus has overcome the external physical protection barriers of the body (skin, mucous membranes). It consists of dendritic cells, granulocytes, monocytes, macrophages and natural killer cells (NK cells). They have proteins that serve as receptors, e.g. Toll-like receptors (TLRs) and complement receptors, for specific structures of pathogens and for the soluble products of the innate immune system (acute-phase proteins, factors of the complement system, cytokines, chemokines and interferons). The effects and functions of cytokines, chemokines and interferons will be discussed separately in ▶ Chap. 8. The specific, adaptive immune response is the second line of defence, and is developed only during or after the establishment of an infection. It includes antibody-producing B cells – the humoral

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immune system – as well as T-helper (T_H) cells and cytotoxic T lymphocytes, which collectively constitute the cellular defence system. The adaptive immune reactions can selectively recognize certain pathogen types or subtypes, and in the case of a reinfection, they are able to recognize the pathogens again and eliminate them. They are long-lasting and a subset of stimulated lymphocytes transform into memory cells during their development, which confers on the organism an efficient protective immunity against infections with the same pathogen. The systems of the specific and non-specific immune responses are in close contact with each other, particularly via cytokines, chemokines and interferons. An immune response is generally triggered by antigens. These may be the infectious pathogens, individual protein components or sugar structures. The immune system recognizes these as foreign, and thus can distinguish between endogenous and exogenous components. However, the antigens must be of a certain size to trigger different immune responses. Molecules with a molecular mass of less than 3–4 kDa are usually incapable of doing that.

7.1 What Are the Cellular and Molecular Components of the Immune System that Constitute the "First Front" Against Invading Pathogens?

7.1.1 Dendritic Cells

Dendritic cells are white blood cells which are characterized by highly ramified outgrowths or projections of the cell membrane (Latin *dendriticus* meaning "ramified" or "branched"). Plasmacytoid (lymphoid) dendritic cells and myeloid dendritic cells originate from haematopoietic progenitor cells in the bone marrow; myeloid dendritic cells probably differentiate alternatively into macrophages direct from monocytes. As immature dendritic cells, they migrate from the blood into almost all tissues of the body, where they establish a dense network of sentinel cells, which ingest extracellular components by phagocytosis and endocytosis, similarly to neutrophils and macrophages. The Langerhans cells of the skin and mucous membranes, the Kupffer cells of the liver, the interdigitating cells of the spleen and lymph nodes, interstitial dendritic cells and the M cells of mucosa-associated lymphoid tissue are tissue dendritic cells. They constantly check their environment for invasion and the presence of pathogens (viruses, bacteria, fungi), which they recognize by interacting with receptors of the pattern recognition receptor family. These include TLRs that recognize pathogen-specific structures and bind to them (Sect. 7.1.5). In dendritic cells these contacts induce the synthesis and secretion of large amounts of interferon-α (IFN-α) and interferon-β (INF-β) as well as proinflammatory cytokines; this gives rise to the activation of granulocytes and macrophages, which move to the infection site. In the course of this process, dendritic cells mature and phagocytose the pathogens. Like macrophages, they can present peptides which result from the degradation of pathogen proteins in complex with MHC class II proteins on their cell surface, thus inducing the defence

responses of the specific immune system. Unlike macrophages, dendritic cells are able to leave the tissues to which they had migrated, and reach in activated form, via streaming lymph fluid, the spleen and lymph nodes, the local organizing centres of the immune defence. There, they interact as antigen-presenting cells with B and T lymphocytes, and thus are decisively involved in the induction of the specific immune response: they activate T_H lymphocytes and macrophages, regulate the cytokine pattern of the emerging T_H lymphocytes into interferon-γ (IFN-γ)-producing T_H1 cells or IL-4 producing T_H2 cells, initiate the formation of cytotoxic T lymphocytes and induce, using the assistance of T cells, the differentiation of plasma cells into antibody-producing B lymphocytes. Therefore, dendritic cells are highly specialized to stimulate specific immune responses and are an important link between the unspecific and the specific immune defence systems.

Although follicular dendritic cells morphologically resemble the plasmacytoid and myeloid dendritic cells, they have completely different functions. They are resident and non-motile cells in the germinal centres of lymph nodes and other secondary lymphoid organs (e.g. spleen, tonsils, Peyer patches). They probably do not originate from bone marrow precursor cells and cannot phagocytose and degrade pathogens or protein components of pathogens. By contrast, on their surface they have Fc immunoglobulin receptors and C3 complement receptors through which they bind to antigen–antibody complexes. They present these complexes to B lymphocytes and induce their proliferation and maturation. In the course of this, for instance, the immunoglobulin class switch occurs (Sect. 7.2.2).

7.1.2 Granulocytes

Approximately 60–70 % of all circulating white blood cells are granulocytes, which have a polymorphic nucleus divided into three to four segments and possess a large number of lysosomes in the cytoplasm. The latter are referred to as granules owing to their appearance in microscopic images. These cells are produced in the bone marrow, from which they migrate into the blood. They have a relatively short half-life of 2–3 days. Because of the different dyeability of their granules, which primarily contain proteases and other degrading enzymes, they can be divided into neutrophil, eosinophil and basophil subgroups.

In particular, neutrophils, which represent the largest subpopulation (about 90 %), are involved in the first defence mechanisms against viral infections; humans produce about 10^{11} neutrophils per day. During the first few hours after their formation they still do not have a segmented nucleus; thus they are called unsegmented. To reach the infected tissue, neutrophils leave the bloodstream and the vascular system in a multistage process that is mediated by adhesion molecules and chemokines (chemotactic, soluble, attracting proteins which are released by activated dendritic cells). They attach to endothelial cells by adhesion molecules on their cell surfaces, such as L-selectin, vascular cell adhesion molecule 1 and intercellular adhesion molecule 1, which in turn – also chemokine-mediated– produce the corresponding ligands (E-selectin and P selectin, integrins).

This enables granulocytes to pass through the intercellular spaces of the endothelium which line the blood capillaries. In the course of this, they migrate to the inflammation or infection sites along a concentration gradient of chemokines, to which they bind by the corresponding chemokine receptors on their surface, and are the first immune cells to arrive there. Upon contact with the pathogens, neutrophils release the content of their granules into the surrounding region, or they phagocytose the pathogens. These are then surrounded by membrane vesicles, the phagosomes, which fuse with intracellular granules to form phagolysosomes. In this way, the agents are confronted with a plethora of degrading enzymes (including proteases, lysozymes, myeloperoxidases, hydrolases and muraminidases) and are killed. Granulocytes are stimulated by interaction with pathogens. As a result, granulocytes produce a variety of inflammatory factors such as IL-8, IL-1, IL-6 and TNF-α as well as leukotrienes and prostaglandins. In particular, IL-8 has a chemotactic effect and lures more granulocytes and T lymphocytes to the infection site. The substances released act not only in an immunoregulatory manner. Some of them, e.g. IL-1, are also involved in the development of fever and the increase of algesthesia. Phagocytosis is more effective when the surfaces of the pathogens are complexed with antibodies, which are generated at a later stage of infection. It is part of the antibody-dependent, cell-mediated cytotoxicity (ADCC). If neutrophils are not activated during the first 6 h after their emergence, i.e. if they not were confronted with infectious and/or inflammatory reactions, apoptosis will naturally be induced in them; dead granulocytes are then degraded in the liver by the macrophages that reside in this organ.

Eosinophils and basophils represent only about 2–5 and 0.2 % of white blood cells, respectively. Mast cells are tissue cells of mucous membranes and connective tissues. Their function is very similar to that of basophils. The main function of eosinophils is defence against large extracellular parasites, which cannot be phagocytosed owing to their size, such as worms (helminths). Eosinophils are attracted by chemotactic substances, attach to the parasites and pour out the content of their granules to the surrounding region, i.e. enzymes, oxygen radicals and cytotoxic proteins such as major basic protein (MBP); MBP is a group of small, arginine-rich proteins that are toxic to helminths, but also to mammalian cells, especially the cells of the bronchial epithelium. Eosinophils are also capable of phagocytosing small pathogens or IgE-containing immune complexes. Basophils and mast cells are involved in the development of allergic immune reactions, as they have IgE receptors on their surface and release histamine, heparin, proteases and leukotrienes upon attachment of IgE-containing antigen–antibody complexes. Histamines are also released upon contact of mast cells with MBP of eosinophils, which respond in turn with the release of histaminase and arylsulphatase, thus counteracting the histamine release. Disturbances in these processes can lead to allergic reactions.

7.1.3 Monocytes and Macrophages

Monocytes and macrophages belong, along with granulocytes and dendritic cells, to the mononuclear phagocytes, which are the most important cells of the innate

immune system. Approximately 2–8 % of blood cells are monocytes. They are large, contain many lysosomes in their cytoplasm and have a well-pronounced Golgi apparatus. Both MHC class I and MHC class II proteins are anchored in their cell membrane. Monocytes originate from myeloid stem cells in the bone marrow, which initially develop to monoblasts under the influence of growth factors such as granulocyte–macrophage colony-stimulating factor and monocyte colony-stimulating factor. Monoblasts further differentiate into monocytes, which leave the bone marrow and circulate for 20–30 h in the blood before they migrate as macrophages into different tissue and organ systems. Similar to neutrophils, macrophages also follow the paths that are mediated by adhesion molecules and chemokines, leave the bloodstream and arrive early at the infection site. They can ingest and digest foreign material by phagocytosis, i.e. pathogens or protein components derived from them. During this process, peptides are formed from digested proteins. These peptides interact with MHC class II proteins, and are finally presented on the cell surface of monocytes and macrophages. In this way, they become antigen-presenting cells that activate the specific immune system, namely the T_H lymphocytes (Sect. 7.2.1). Furthermore, activated macrophages produce specific surface proteins such as CD14, CD16 and CD86 (CD stands for cluster of differentiation), and TLR2 and TLR4. Like TLR2 and TLR4, CD14 interacts with lipopolysaccharide (endotoxin)-negative bacteria. If these receptors come in contact with lipopolysaccharides, they stimulate macrophages to phagocytose foreign material and to release inflammatory mediators such as TNF-α, IL-1β and IL-6.

7.1.4 Natural Killer Cells

Natural killer cells (NK cells) originate from precursor cells in the bone marrow. Unlike T lymphocytes, NK cells remain in the peripheral lymphoid tissues, where they develop into large, granular lymphocytes, which possess characteristic surface markers. One of their principal functions is the clearance of virus-infected cells and tumour cells. NK cells induce apoptosis preferentially in cells that exhibit very low levels of MHC molecules on their surface. This mode of recognition and elimination of virus-infected and transformed cells is of crucial importance, as they often reduce the number of MHC proteins on their surfaces in order to escape the MHC-dependent specific immune responses. Fundamentally, NK cells are programmed to destroy all nucleated cells. However, MHC class I molecules on the cell surface inhibit this activity; hence, NK cells selectively induce apoptosis in those cells that do not exhibit MHC class I expression. Therefore, NK cells have killer cell immunoglobulin-like receptors (KIR) on their surface, which recognize and bind to MHC molecules. The KIR–MHC interaction leads to an inhibitory signal that suppresses the killer activity of NK cells. If this interaction is absent, then killing activatory receptors, which are also anchored in the cell membrane of NK cells, release toxic messengers which induce apoptosis in cells with poor expression of MHC molecules. Unlike granulocytes and macrophages, NK cells are always

functionally active; thus, they do not need to be converted into an activated state by binding of specific cytokines. However, their activity can be enhanced by IL-12 or IFN-α and IFN-β, which are secreted, for example, by activated dendritic cells, monocytes and macrophages. NK cells produce large amounts of IFN-γ and other cytokines (IL-1, TNF-α), which leads to further immunological activation steps (▶ Chap. 8). Therefore, NK cells have, in addition to their cytotoxic effect, also an immunoregulatory importance.

7.1.5 Toll-Like Receptors

Toll-like receptors (TLRs) are members of a family of proteins that belong to the non-specific, innate immune system. TLRs are found in all vertebrates, including fish and reptiles. Their name is derived from the *Toll* gene, which was discovered in *Drosophila melanogaster*. It displays homology to the gene that encodes the IL-1 receptor. This suggests that the TLRs are members of an evolutionarily very old system. More than ten different TLRs have been discovered in most animal species, including humans, some of which exist only in certain species, such as in mice, but not in humans. TLRs are produced especially in dendritic cells, monocytes and macrophages, and belong to the group of pattern-recognition receptors. They are anchored in the cytoplasmic membrane (TLR1, TLR2, TLR4, TLR5, TLR6, TLR11) or in the endosomal membrane (TLR3, TLR7, TLR8, TLR9) and serve to detect pathogen-associated molecular patterns. These are structures that exist only on or in pathogens (viruses, bacteria, fungi). Therefore, TLRs endow the innate immune system with the ability to distinguish between "self" and "non-self". TLR1 interacts specifically with peptidoglycan components of Gram-positive bacteria, whereas TLR4 binds to lipopolysaccharides of gram-negative bacteria and TLR5 recognizes flagellin, a protein of bacterial flagella (Table 7.1). The specific recognition of viral pathogen structures is performed mainly by TLR3, TLR7, TLR8 and TLR9, which are anchored in the endosomal membrane: TLR3 binds double-stranded RNA, TLR7 and TLR8 interact with single-stranded RNA and TLR9 binds to unmethylated CpG motifs in single-stranded and double-stranded DNA. Double-stranded RNAs are characteristic molecules that occur as genome components or intermediates of genome replication of RNA viruses; they do not exist in uninfected cells. The same applies to single-stranded DNA or unmethylated CpG motifs. These virus-specific components make contact with TLRs after they have been phagocytosed by dendritic cells, and are present in endosomes (Fig. 7.1). Intracellular signalling cascades are induced after interaction of various TLRs with different pathogen-specific ligand structures by means of their leucine-rich domains, which are located extracellularly or in the endosomal lumen. Some TLRs can also form heterodimeric complexes, the ligand-binding properties of which are then modified or can overlap. As a result of ligand binding, Toll/IL-1 receptor (TIR) domain proteins, such as myeloid differentiation primary response gene 88 (MyD88) and similar proteins that act as adapters, can attach to the conserved TIR domains of different TLRs, which are oriented to the cytoplasm.

Table 7.1 Human toll-like receptors (*TLR*), their ligands and the pathogens that activate them

	Localization	Ligands	Pathogen
TLR1/2	Cytoplasmic membrane	Triacylated lipopeptides	Bacteria, mycoplasma
TLR2	Cytoplasmic membrane	Peptidoglycan	Gram-positive bacteria
		Lipoarabinomannan	Mycobacteria
		Modulin	*Staphylococcus aureus*
		Zymosan	Fungi
		Glycosylphosphatidylinositol	*Trypanosoma cruzi*
		Haemagglutinin	Measles virus
TLR2/6	Cytoplasmic membrane	Diacylated lipopeptides	Mycoplasma
TLR3	Endosomal membrane	dsRNA	Viruses
TLR4	Cytoplasmic membrane	Lipopolysaccharides	Gram-negative bacteria
		Viral membrane proteins	Respiratory syncytial virus
			Mouse mammary tumour virus
TLR5	Cytoplasmic membrane	Flagellin	Bacteria
TLR7	Endosomal membrane	ssRNA	Viruses
		Guanosine analogues	
TLR8	Endosomal membrane	ssRNA	Viruses
TLR9	Endosomal membrane	Unmethylated CpG motifs in DNA	Bacteria, viruses
TLR11	Cytoplasmic membrane	Profilin-like structures	*Toxoplasma gondii*
		Protein components	Uropathogenic *Escherichia coli*

ds double stranded, *ss* single stranded

This multistage process that involves IL-1 receptor associated kinases leads to the activation of members of the Iκβ kinase (IKK) family, which phosphorylate Iκβ, the inhibitor of nuclear factor κB (NFκB). As a consequence, the transcriptional factor NFκB can enter the nucleus and induce the expression of various proinflammatory cytokines and adhesion proteins. Some members of the IKK family phosphorylate interferon regulatory factors 3 and 7, which thus exert their activity as transactive proteins inducing the expression of IFN-α and IFN-β. Alternatively, the signal translation can also be MyD88-independent by addition of (TIR-domain-containing adapter-inducing IFN-ß (TRIF) to the TIR domains of TLRs involving the participation of phosphatidylinositol 3-kinase. Nevertheless, all the different pathways converge in the activation of the synthesis of inflammatory cytokines and adhesion factors as well as class I interferons.

Hence, TLRs are at the front line of the immune defence. After entry of a virus or other infectious agents, they become active first, and initiate the next steps of both the non-specific and the specific immune responses.

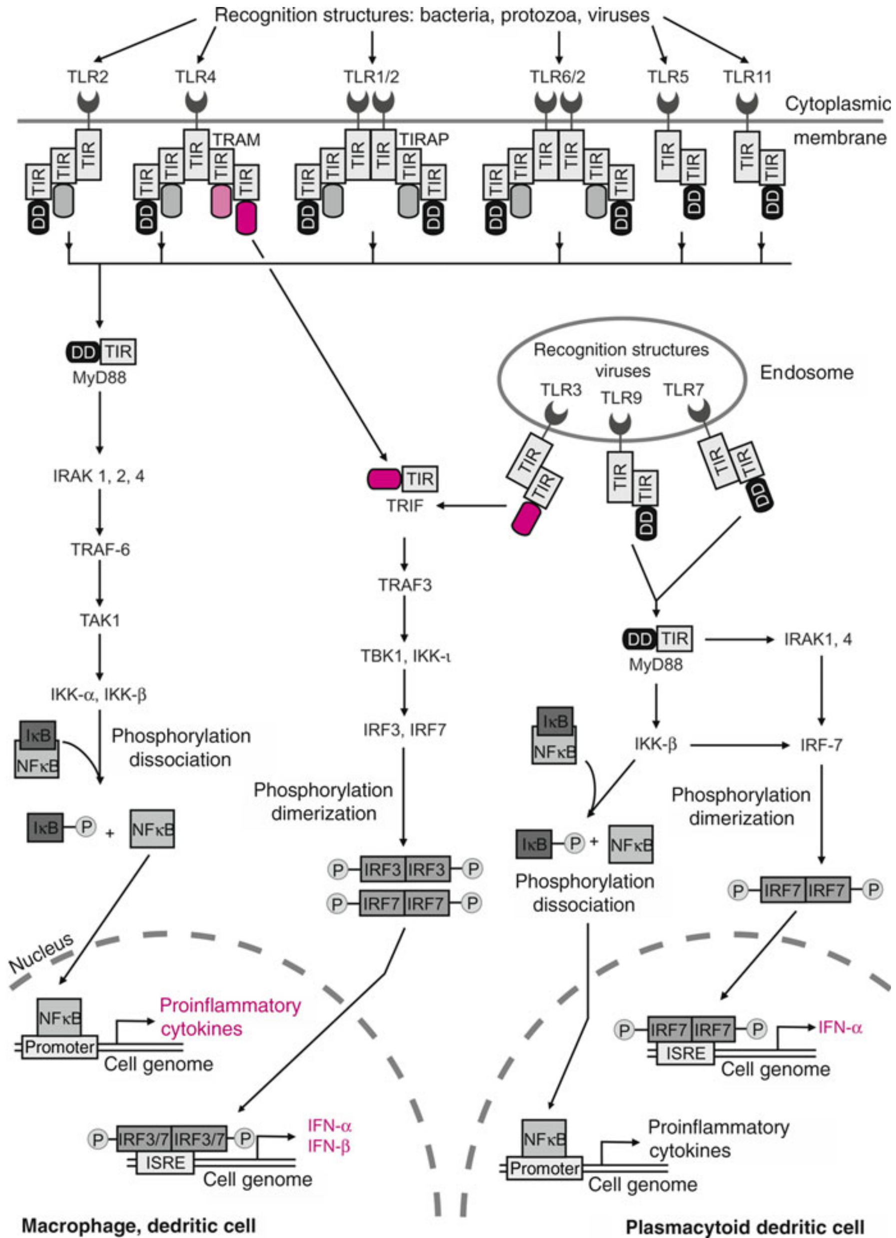

Fig. 7.1 Toll-like receptor (*TLR*)-mediated activation pathways (simplified illustration). TLRs are anchored in the cytoplasmic membrane (TLR1/2, TLR2, TLR4, TLR6/2, TLR5, TLR11) or endosomal membrane (TLR3, TLR7, TLR9) of dendritic cells or of macrophages/monocytes. The various TLRs interact with pathogen-specific molecular patterns. This process induces structural changes in the cytoplasmic Toll/IL-1 receptor (*TIR*) domains of the TLRs, which is followed by the interaction of TIR domain proteins, e.g. myeloid differentiation primary response gene 88

7.1.6 Acute-Phase Proteins

This large group of different proteins is part of the systemic reaction of the body to infection, inflammation or tissue injury. They are produced in hepatocytes, and their synthesis increases substantially by IL-1 and/or IL-6 stimulation. Both interleukins are secreted by activated granulocytes and macrophages, and are transported to the liver via the bloodstream. C-reactive protein (CRP) belongs to the acute-phase proteins, and is a non-specific marker of inflammation; it received its name because it reacts, together with Ca^{2+} ions, with the C polysaccharide of pneumococci. After an immunological stimulus, CRP is detectable in the blood within a few hours. It binds to phosphocholine, and thus recognizes phospholipid components in membrane components of viruses, bacteria and the body's own cells that have been destroyed. CRP complexed with phosphocholine activates the complement system. It exerts an opsonizing effect, and is phagocytosed by neutrophils and macrophages. In this way, it activates other immune defence reactions.

Other members of acute-phase proteins include factors controlling blood coagulation such as fibrinogen, which is the precursor of fibrin, α_1-glycoprotein and proteinase inhibitors such as α_2-haptoglobin, α_2-macroglobulin, α_2-antichymotrypsin, α_1-antitrypsin and C1-esterase inhibitor, which regulate coagulation and kinin cascades. In addition, various components of the complement system, such as the C3 component, are constituents of acute-phase proteins.

7.1.7 The Complement System

Complement is one of the most important mediators of inflammatory reactions. Inflammation is a consequence of infection, and is causally connected with the processes that are induced by the non-specific immune response. Complement is activated by two different pathways: the so-called classical pathway and the alternative activation pathway. However, both lead to the same mechanism, the lysis of virus-infected cells, bacteria, parasites or tumour cells. The classical pathway is induced by antigen–antibody complexes. By contrast, the alternative pathway is activated independently of the presence of immunoglobulins. The complement system connects the specific and the non-specific part of the immune system, but it is also a link between the cellular and the humoral branch

Fig. 7.1 (continued) (*MyD88*) and TIR-domain-containing adapter-inducing interferon-ß (*TRIF*), characterized by death domains (*DD*). This interaction may induce various kinases, e.g. IL-1 receptor associated kinase (*IRAK*), Iκβ kinase (*IKK*), TNF-associated factor (*TRAF*) and TRAF-family-associated nuclear factor κB (*NFκB*) activator binding kinase (*TBK*), and signal cascades, resulting in the activation of NFκB or interferon regulatory factor (*IRF*) 3/7. These factors are functionally active and induce the transcription of genes encoding proinflammatory cytokines and class I interferons (I*FN*). *ISRE* interferon-stimulated response element, *TIRAP* Toll/IL-1 receptor domain containing adaptor protein, *TRAM* Toll/IL-1-receptor-domain-containing adapter-inducing interferon-ß-related adaptor molecule

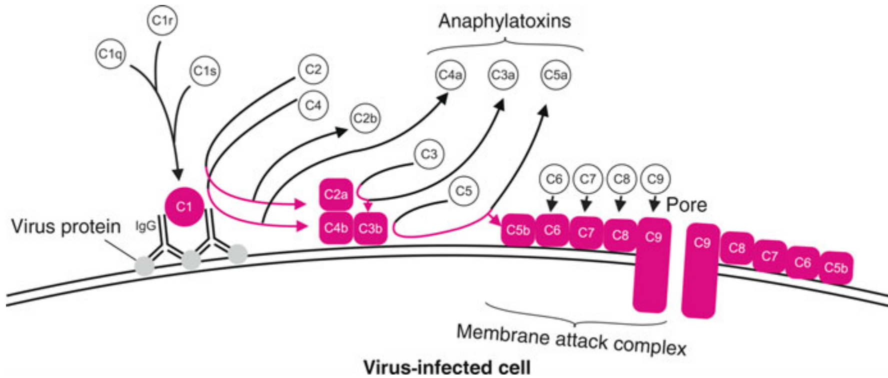

Fig. 7.2 Complement activation via the classical pathway by a virus-infected cell containing viral proteins on its cell surface. Antibodies can specifically bind to these proteins. Two adjacent IgG antibodies or one bound IgM antibody can induce attachment of the complement components C1q, C1r and C1s to C1. C1 is a protease that cleaves component C4 into C4a and C4b as well as component C2 into C2a and C2b. C4b and C2a form a complex which attaches to the cytoplasmic membrane and, in turn, cleaves component C3. C3b is covalently bound to the structures of the cell surface. The complex of C4b, C2a and C3b processes component C5 into C5a and C5b. The latter, in turn, interact with the cell surface, causing the attachment of the membrane attack complex composed of C6, C7, C8 and C9, which induces the destruction of infected cells by pore formation. The C4a, C3a and C5a proteins, which are cleaved during this process, act as anaphylatoxins. The active components of the complement cascade are shown in *red* and their inactive precursors are shown in *black*

of the immune response. Both complement activation pathways are based on several factors, which activate each other in a cascade-like process. The central reaction in which the two pathways converge is the proteolytic cleavage of complement component C3 into C3a and C3b by C3 convertase. In the alternative pathway, this transformation is induced spontaneously by certain sugar structures on the surface of bacteria, viruses, fungi and protozoa as well as tumour and virus-infected cells. In the classical pathway, it is indirectly triggered by antibodies bound to the surfaces of the respective pathogens or cells. The three subcomponents C1q, C1r and C1s attach to the Fc region of immunoglobulins to form the active unit (Fig. 7.2). This acts as a protease and processes component C4 and then component C2, whereupon the cleavage products C4b and C2a bind to the membrane surface of the target structure, i.e. the infected or transformed cell, the virus or bacterium, the fungus or protozoan. They form as a complex the above-mentioned C3 convertase of the classical pathway. During cleavage of C3, the conformation of the C3b subunit is modified in such a way that a reactive thioester bond is exposed. It reacts with functional side groups of amino acids, which are accessible on the surface of pathogens, cells or antigen–antibody complexes. As a result, the C3b subunit is covalently bound. The C3b subunit marks the structures as foreign, thus giving rise to a signal that induces phagocytosis by granulocytes, macrophages and monocytes.

Binding of the C3b component to target structures on membranes concomitantly activates the lytic pathway of the complement system. At its inception, C5 convertase

is formed, and comprises the C4b, C2a and C3b complex in the classical pathway. In the alternative pathway, the cleavage of the C5 component into C5a and C5b occurs independently of C4b and C2a. C5b binds to the surface-associated C3b and brings about the accumulation of factors, C6, C7, C8 and C9, which together make up the membrane attack complex. This complex is formed in the cell membranes, and intersperses them with pores; the pathogens or the cells are destroyed by lysis.

The small components C4a, C3a and C5a, which are cleaved as soluble products of C4, C3 and C5 during complement activation, make up the anaphylatoxins. These proteins play an important immunoregulatory role, since they induce the release of the histamine-rich content of granules of basophilis and mast cells into the surrounding region. They increase the permeability of vascular walls and induce contraction of smooth muscles. C5a exerts a chemotactic effect on macrophages and neutrophils, which subsequently migrate to the infection site and induce the further mechanisms of the immune defence.

7.2 What “Weapons” Are Available for the Specific Immune Response?

7.2.1 T Lymphocytes

T lymphocytes have a pivotal role in regulating the immune response, and in detecting and eliminating virus-infected or tumour cells from the organism. The specific recognition of altered cells occurs via T-cell receptor, a protein complex that is anchored in the plasma membrane of T lymphocytes. It is a heterodimer composed of an α and a β chain in about 95 % of T lymphocytes. Cells with γδ T-cell receptors are rarely found; these consist of heterodimers of a γ chain and a δ chain on the cell surface. A large proportion of them carry neither CD4 nor CD8 receptors. The function of γδ T cells has not been definitively resolved; they probably have regulatory functions and are involved in the suppression of the cellular immune response after an infection. They can also bind soluble proteins, such as phosphate derivatives and MHC class I like molecules, and are probably involved in eliminating epithelial infections. All protein chains of T-cell receptors are anchored in the cytoplasmic membrane of T cells through a carboxy-terminal, hydrophobic amino acid sequence. Their surface-exposed part possesses both a constant domain and a variable domain, which are stabilized by disulphide bonds. The variable domains are responsible for the specificity of the different T lymphocytes. Using them, they recognize foreign structures on the surface of their target cells. The diversity of T-cell receptors is achieved by a sophisticated process in which the genetic information of 50–100 different V gene segments is combined with that of a few D, J and C gene segments by somatic recombination and alternative splicing during differentiation of immature thymocytes into T lymphocytes, a process that occurs in the thymus during embryogenesis. Similar processes also occur during the generation of the variable regions of immunoglobulins (Sect. 7.2.2).

The CD3 protein complex is bound to the T-cell receptor. This complex is a heterotrimer composed of membrane-anchored γ, δ and ε chains, which transmit a signal to the ξ subunits, and thus into the cell, upon binding of T-cell receptor to foreign structures (Fig. 7.3). Other proteins that are associated with T-cell receptor are either CD4 or CD8 receptors, except in γδ T cells. Their occurrence leads to the subdivision of T lymphocytes into the subgroups of $CD4^+$ T_H cells and $CD8^+$ cytotoxic T cells. They mediate the interaction of T cells with either MHC class II or MHC class I antigens during specific recognition of exogenous structures.

In addition to the interaction of the T-cell receptor with an MHC–peptide complex on the side of antigen-presenting cells, a co-stimulatory signal is required for activation of T cells. This is usually mediated by the interaction of B7 proteins (B7.1 and B7.2) or CD40 proteins on antigen-presenting cells with their ligands, CD28 (cytotoxic T lymphocyte antigen 4) or CD40 on T cells. This co-stimulatory interaction is a prerequisite for IL-2-mediated proliferation of T lymphocytes. If these co-stimulatory signals fail to occur or are repressed by certain virus-specific factors, T cells can become anergic, i.e. functionally inoperative or unresponsive, or eventually die by induction of apoptosis. $CD4^+$ T cells with high CD25 expression have been identified as important regulatory T cells, which decisively determine the immune response to self-antigens, and thus to self-tolerance and autoimmunity.

7.2.1.1 Cytotoxic T Cells

$CD8^+$ cytotoxic T lymphocytes recognize virus-infected cells, lyse them and thus contribute decisively to restricting the infection in the body. By the T-cell receptor complex and the CD8 receptor, these cells bind to MHC class I proteins, which present peptide fragments of viral proteins as foreign components. MHC class I proteins or antigens are present on all cells of an organism, with the exception of brain cells (▶ Sect. 4.1.2). These proteins are heterodimers composed of a membrane-anchored α chain and a β_2-microglobulin subunit (Fig. 7.3a). The surface-exposed moiety of the α chain is divided into three domains, the α_1 and α_2 domains are folded in such a way that an antiparallel β-sheet floor is formed, on which two α-helices are supported. The structure resembles a groove or cavity in which the ß-sheet forms the floor, and the α-helices shape the brims that line the trench (Fig. 7.4). Peptides with a length of approximately nine amino acids can be accommodated in this cavity. They are bound by a combination of hydrophobic and ionic interactions. These peptides are fragments of viral proteins that are recognized as foreign by the T-cell receptor of cytotoxic T cells, which then binds to them. If the cell is not infected, the hole is occupied by peptides originating from the cell itself. These are not recognized as foreign because cytotoxic T cells with such specificities are retained in the thymus and eliminated during T-cell maturation. Loading of MHC class I proteins with peptides occurs in the endoplasmic reticulum (ER). This requires an active synthesis of viral proteins, a situation that exists only in infected cells, in which the viral genes are expressed and translated into proteins during viral replication (Fig. 7.5a). After synthesis, a small proportion of them are delivered to the proteasome complex in the cytosol and are degraded. The resulting peptides are transported into the ER lumen by a peptide transporter

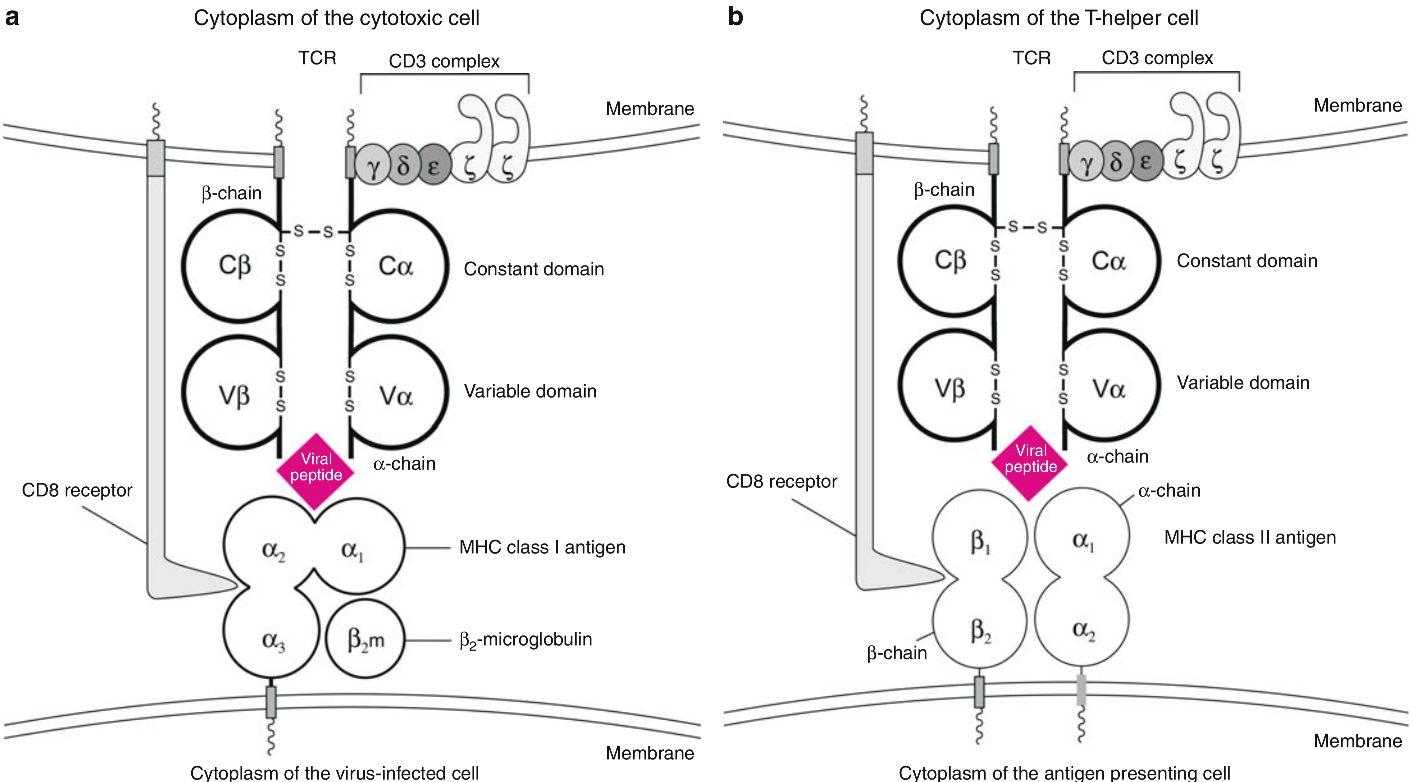

Fig. 7.3 The most important components in the interaction of T lymphocytes with antigen-presenting cells. (**a**) Recognition of virus-infected cells by cytotoxic T lymphocytes. The complex of MHC class I antigen and β_2-microglobulin contains a peptide (*red*) in its antigen-binding cavity; this is derived from

(transport-associated protein) that is integrated in the ER membrane. There, the peptides are accommodated into the cavity of MHC class I molecules, which are synthesized in the ER as membrane-anchored proteins. During this process, the moiety that protrudes into the lumen is later exposed at the cell surface; before the formation of stable complexes with β_2-microglobulin and the peptide, the MHC α chain is bound to calnexin, an ER-membrane-associated protein that acts as a chaperone preventing the MHC amino acid chain from prematurely adopting its final folded state. The finally formed complex of peptide, α chain and β_2-microglobulin is transported through the Golgi vesicles and the *trans*-Golgi network to the cell surface. It is anchored in the membrane, where it can be recognized by the T-cell receptors of $CD8^+$ T lymphocytes. Then, T lymphocytes release cytotoxic factors (granzymes), radicals and perforins. The latter oligomerize under the influence of Ca^{2+} ions, and are embedded in the membrane of the cell that was recognized as foreign, they intersperse it with pores, leading to cell lysis. A prerequisite for this process is that the corresponding T lymphocytes have been stimulated by cytokines such as IL-2 and IFN-γ, which are secreted by T_H cells. Additionally, cytokines such as IL-1, TNF and IFN-α, which are released from the cells of the innate immune system, e.g. by activated macrophages, increase the activity of cytotoxic T lymphocytes. Therefore, they represent a close connection between the unspecific immune responses and the specific cytotoxic T-cell response. Cytotoxic T cells are also capable of triggering a suicide programme (apoptosis; ▶ Fig. 5.1) by their Fas ligands via contact with Fas receptors on the surface of target cells. In addition to these cytotoxic functions aimed at killing antigen-presenting cells, $CD8^+$ T lymphocytes can also bring about a non-cytotoxic antiviral response by releasing INF-γ.

Every person possesses the genetic information encoding up to six different α chains of MHC class I antigens, which, in humans, are known as human leucocyte antigens (HLA), whereby two molecules are of type HLA-A, HLA-B and HLA-C, respectively. These genes reside in human chromosome 6 and are inherited

Fig. 7.3 (continued) the degradation of viral proteins that are synthesized in the infected cell (productive viral infection, internal). This complex is specifically recognized by the T-cell receptor (*TCR*), which interacts with it by the variable domains of its α and β chains. Irrespective of this, the CD8 receptor protein (*shaded in grey*) of the cytotoxic T cell binds to a conserved domain in the MHC class I antigen. The protein interactions induce overall structural rearrangements in the T-cell receptor, and these are transmitted into the cell by the CD3 complex. The T lymphocyte releases cytotoxic proteins and perforins, which lyse and kill the infected cell. (**b**) Recognition of antigen-presenting cells by T-helper cells. The MHC class II antigen, which consists of an α and a β chain, contains in its antigen-binding cavity a peptide (*red*) that is derived from the degradation of viral proteins of other cells (external) and was actively ingested by a cell by endocytosis, e.g. a macrophage. This complex is specifically recognized by the T-cell receptor (*TCR*), which interacts with it by the variable domains of its α and β chains. Independently of this, the CD4 receptor protein (*shaded in grey*) of the T-helper cell binds to a conserved domain in the MHC class II antigen. The protein interactions induce structural rearrangements in the T-cell receptor, and these are transmitted into the cell by the CD3 complex. The T-helper cell reacts with the release of cytokines

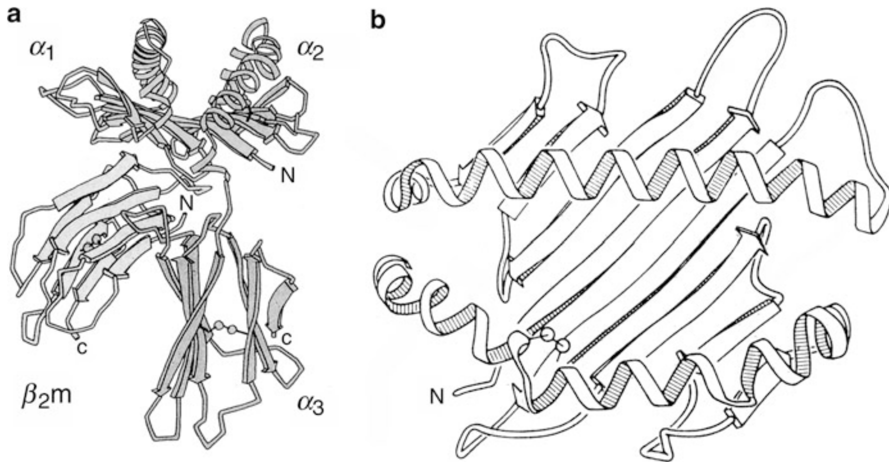

Fig. 7.4 Structure of the surface-exposed part of the MHC class I antigen (HLA-A2). (**a**) The complex of HLA-A2 and β_2-microglobulin, based on data from X-ray structure analysis. Folding of the amino acid chain of HLA-A2 into the three domains, α_1, α_2 and α_3, leads to the formation of the antigen-binding groove or cavity by the two α-helices of the α_1 and α_2 domains, which are represented by *spirals*. In the α_3 domain, *c* indicates the carboxy terminus. This site of the amino acid chain represents the transition into the transmembrane region. This region was removed by proteolytic cleavage to generate the crystal structure. (**b**) The antigen-binding groove of HLA-A2 (*top view*). The floor is constituted of six antiparallel β-sheets (shown by *arrows*), which are lined by two α-helices lying on each side of the floor (*spirals*) (From Bjorkman 1987)

according to Mendel's laws. HLA molecules can be classified into different haplotypes, which differ in the amino acid sequences of α chains. Today, we know more than 30 different haplotypes of the HLA-A type, more than 60 of the HLA-B type and more than 15 of the HLA-C type, which can usually be divided into further subtypes. This high diversity in combination with the genetic inheritance rules determines that each person has his or her own set of HLA haplotypes. Thus, every human cell has a characteristic "make up", except nerve cells and brain cells as well as certain cells of the eye. The different amino acid composition is primarily manifested in the residues lining the antigen-binding groove. Therefore, the different HLA haplotypes can bind only specific peptide segments. Today, it is known that this is the reason for the different genetic ability of individuals to react immunologically to infectious diseases. For example, if an individual has an HLA subtype that can poorly bind peptides of a specific viral protein, the respective infected cells will be neither recognized nor eliminated.

This mechanism also explains the genetically conditioned increased susceptibility of cheetahs to infections with feline corona virus and the relative high frequency of the associated feline infectious peritonitis (▶ Sect. 14.8). Cheetahs have a very narrow genetic base and are not able to present important protective epitopes of the viral envelope protein on their MHC class I molecules. The strongly reduced cellular immunity against this virus explains the frequent incidence of feline infectious peritonitis in cheetahs, a disease that occurs in domestic cats only sporadically.

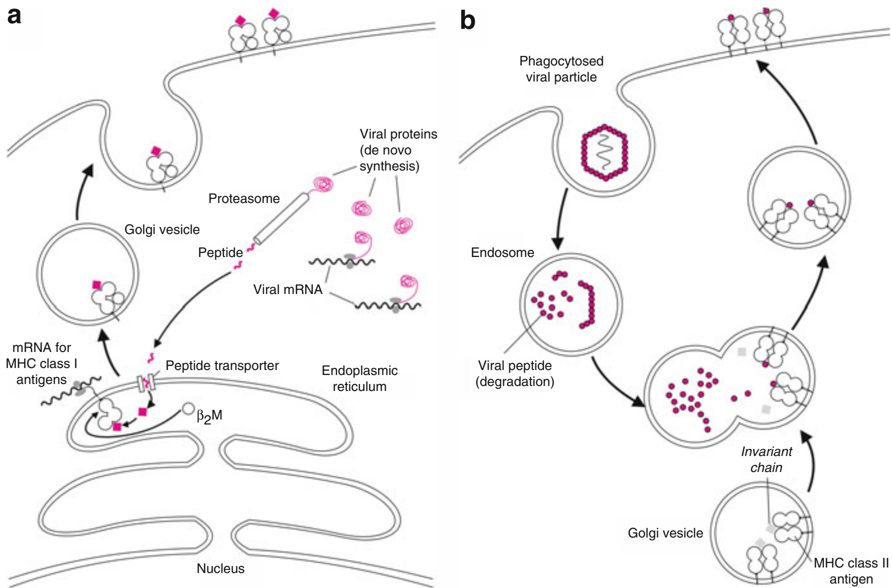

Fig. 7.5 Mechanisms of antigen processing, and loading of MHC antigens. (**a**) Loading of MHC class I antigens. Viral proteins (*red*) are synthesized in virus-infected cells during infection. A subset of them are degraded by the proteasome in the cytosol. A transporter protein, which is located in the membrane of the endoplasmic reticulum, delivers the resulting peptides into the lumen of the endoplasmic reticulum. They attach to the antigen-binding groove of MHC class I molecules, which are embedded in the membrane of the endoplasmic reticulum, and project into its lumen. There, they associate with β_2-microglobulin ($\beta_2 M$). The MHC class I–peptide complexes are transported via the Golgi apparatus and the *trans*-Golgi network to the cell surface. (**b**) Loading of MHC class II antigens. A viral component originating from another cell is ingested by a macrophage and degraded by enzymes in the endosome. This process produces peptides (*red*). MHC class II molecules are synthesized in the endoplasmic reticulum and their external domains are translocated into the lumen. These form a complex with a small protein, the invariant chain (*shaded in grey*), which binds to the antigen-binding cavity. At the *trans*-Golgi stage, vesicles fuse with endosomes, which contain the ingested and degraded viral components. The invariant chain is then degraded and replaced by viral peptides, which bind to the antigen-binding cleft. The MHC class II–peptide complexes are further transported to the cell surface, where they are anchored. *mRNA* messenger RNA

7.2.1.2 T_H Cells

The receptors of T_H cells bind in combination with CD4 to MHC class II antigens, which similarly to the class I antigens described earlier, contain peptides of viral origin. MHC class II antigens are present only on potentially antigen-presenting cells, such as monocytes, macrophages, dendritic cells and B and T lymphocytes. They are heterodimers composed of an α chain and a β chain, which are anchored in the membrane (Fig. 7.3b). The amino-terminal α_1 and β_1 domains are folded into an antigen-binding cavity, which can accommodate peptides with a length of up to 20 amino acids. In this case, binding seems not to be dependent on the specific

amino acid sequence of the peptides, as known for MHC class I antigens. MHC class II antigens also possess a high genetic diversity: every person has one DP, DQ and DR allele, which are also subdivided into many different haplotypes. They are also coded on human chromosome six, and are also inherited according to Mendel's laws. Theoretically, every person has six different HLA class II genes, of which there are many haplotypes and subtypes.

The process of loading HLA class II molecules with peptides is different from for HLA class I proteins: the proteins, from which the peptides are derived, are not synthesized in the antigen-presenting cells. Instead, HLA class II molecules bind fragments of proteins of other cells, e.g. virus-infected cells, which were phagocytosed by a cell carrying HLA class II molecules that has arrived in the endosomes, where they are proteolytically degraded (Fig. 7.5b). After their translation at the ER membrane, HLA class II molecules are also located in this cell compartment. After their synthesis and during transport, they are complexed with a third, small protein, the invariant chain. This is accommodated within the antigen-binding groove of the HLA class II heterodimer. The invariant chain is cleaved by proteolysis in the acidic pH environment only when the complex reaches the endosomes via the Golgi apparatus. This prevents the incorporation of cellular "self" peptides during transport. In the endosome, external, internalized foreign peptides encounter endogenous HLA class II proteins. They form complexes and are transported to the cell surface, where they become anchored and presented to $CD4^+$ T_H cells.

The interaction with antigen-presenting cells causes T_H lymphocytes to release many different cytokines, thus stimulating the activity of other immunologically active cells. During their first contact with an antigen, naive T_H cells (T_H0 cells) secrete the entire range of possible factors. This quality is lost when T_H0 cells differentiate into T_H1 or T_H2 cells. By secreting IL-2 and IFN-γ, T_H1 cells promote especially the activation of other T_H cells, as well as that of cytotoxic T cells and macrophages. In contrast, T_H2 cells release preferentially IL-4, IL-5, IL-6 and IL-10, and stimulate the proliferation and differentiation of pre-B cells into antibody-producing plasma cells. Therefore, T_H cells that are activated by antigen recognition regulate the immune response with the help of cytokines (▶ Chap. 8).

7.2.1.3 Regulatory T Cells

$CD4^+$ and $CD25^+$ T lymphocytes, which are able to control autoreactive cells, were discovered only in 1995. Today, some phenotypes of these T cells are known to exert a regulatory effect not only on the innate, but also on the adaptive immune system. Regulatory T cells ensure that activated immune cells do not inflict excessive damage to the tissue owing to their inflammatory and cytotoxic properties. If there are not enough regulatory cells, immune cells can be activated without impediment. If they are self-reactive T cells, then they will lead to autoimmune disease and massive tissue damage. If there are too many regulatory T cells, the immune system is strongly suppressed. In such a case, infections cannot be adequately controlled and transformed cells are not eliminated. The risk of cancer increases.

Regulatory T cells also seem to play a role in viral infections. For example, different disease courses have been detected in infections with herpes simplex virus

or hepatitis C virus. The viruses can be eliminated by the immune system if regulatory T cells do not restrict the immune response. However, this also leads to strong tissue damage, which is lethal if it is permanent.

7.2.2 B Lymphocytes and Antibodies

7.2.2.1 Antibody Molecules and their Functions

Antibodies, or immunoglobulins, are bifunctional molecules. On the one hand, they have highly variable domains in the Fab (fragment antigen binding) region, which allow them to interact specifically with virtually every imaginable antigen (Fig. 7.6). This interaction allows in certain cases the direct neutralization of viruses when, for example, an infection of cells is prevented. On the other hand, in all molecules (subclasses) antibodies have an identical Fc (fragment constant or crystalline) region. Its functions include binding to Fc receptors that are located on the cell surface of macrophages, monocytes and neutrophils; this binding induces phagocytosis of the antigen–antibody complex. Furthermore, antigen–antibody complexes activate the classical pathway of the complement cascade, which, in turn, facilitates phagocytosis of complexes, leading to lysis of infected cells. Furthermore, the ADCC response of neutrophils is also one of the effects induced by immunoglobulins. These cells bind by their Fc receptors to antibodies that are associated with viral surface proteins on infected cells, damaging them by releasing the content of their granules.

General Structure

Antibodies are glycoproteins that are released in large amounts by plasma cells into the bloodstream. They consist of two light chains and two heavy chains, which are arranged in a Y-shaped basic structure (Fig. 7.6). Light chains are composed of a variable amino-terminal domain and a constant carboxy-terminal moiety. Within the variable domain there are regions with a highly increased variability in the amino acid sequence. These complementarity determining regions interact with the respective antigen and determine the specificity and affinity of binding. The individual domains are stabilized by intramolecular disulphide bonds. In humans, there are α and κ variants of light chains, which are characterized by differences in the constant region. Immunoglobulins can be classified into the classes IgM, IgG, IgA, IgD and IgE on the basis of their heavy chains, which differ in terms of type and size. Heavy chains also have a variable domain in the amino-terminal regions, which is followed by a differing number of constant domains: there are three constant domains in the heavy γ, α and δ chains of IgG, IgA and IgD, whereas four constant domains are present in the μ chain of IgM and in the ε chain of IgE. Light and heavy chains are combined together in such a way in the antibody molecule that the variable amino-terminal region and the following constant domains of the corresponding light and heavy chains interact with each other. They form the two arms of the Y-shaped structure, which are also known as Fab regions. One intermolecular disulphide bond covalently links the light and heavy chains. Heavy chains dimerize beyond the

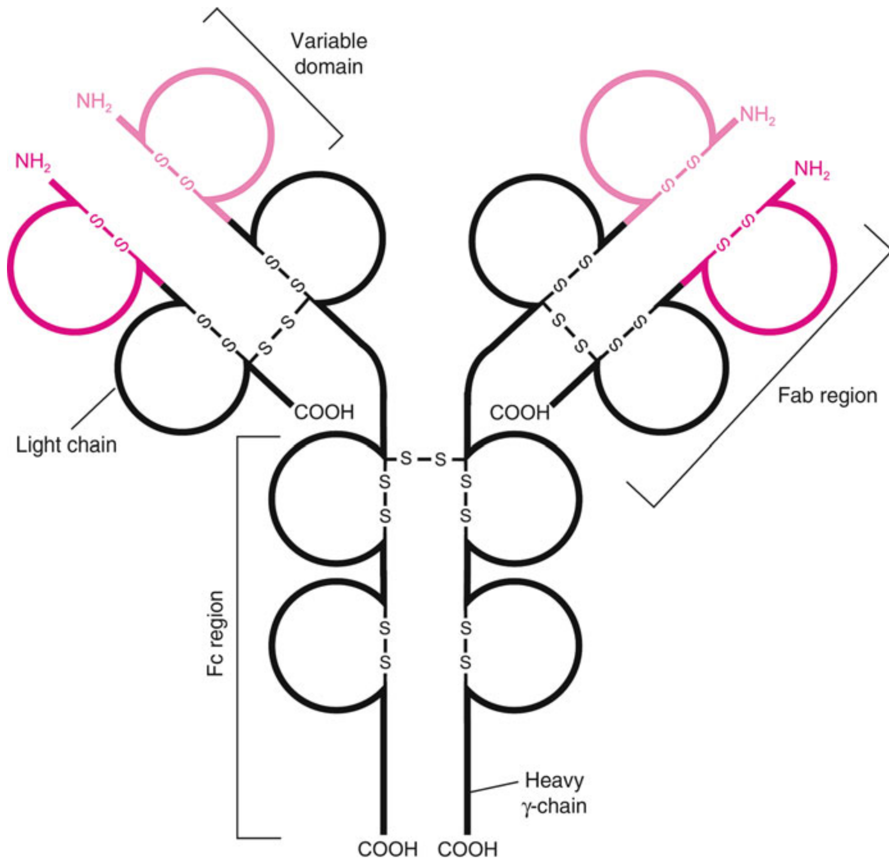

Fig. 7.6 Structure of a typical antibody molecule represented by IgG. The folding of each domain and its stabilization by disulphide bonds is indicated schematically. Variable domains are depicted in *red* and constant domains are illustrated in *black*

second constant domain and form the stem of the Y-shaped structure, also known as the Fc region. The two heavy chains are also interconnected by a disulphide bridge.

IgM Antibodies

A variant of these immunoglobulins is present in the cytoplasmic membrane of pre-B cells, and functions as an antigen receptor; the B cell and in consequence also the plasma cell remains specific for the corresponding antigen that binds to them. Another IgM variant is secreted by the cells upon antigen stimulation; it is important for the early activation of the complement system. The IgM antibodies released are present as complexes that are constituted of five units, whose Fc regions are linked by short peptides, the J chains. IgM molecules are the first antibodies against a particular pathogen produced during an infection. Their proportion to total immunoglobulin in serum is about 10 %. IgM antibodies bind antigens with a relatively low affinity.

IgD Antibodies

Similar to IgM, IgD is produced in small quantities in the early phase of infection, and is also present as a membrane-associated molecule on B cells. it accounts for less than 1 % of total immunoglobulin. Its function has not been conclusively resolved. It is believed that IgD also acts an as antigen receptor and is necessary for the antigen-induced differentiation of pre-B cells into plasma cells.

IgG Antibodies

IgG constitutes 75 % of total immunoglobulin, and is the most abundant antibody population in serum. It is the most important antibody and confers a protective immune response in cases of repeated contact with the same pathogens. In contrast to IgM, IgG has a very high affinity; thus, it binds to antigens with high specificity. There are four different IgG subclasses, which have different functions, and are produced depending on the pathogen and the antigen type. The IgG1 and IgG3 subclasses predominate in the early phase of viral infections, and are the only IgG subclass that can activate the complement system. In the case of infections that occurred a long time ago, only IgG3 antibodies are still found. IgG2 antibodies are induced principally by bacterial polysaccharide structures. IgG-specific Fc receptors are located on macrophages, monocytes and neutrophils, which are stimulated to phagocytose by binding of the antigen–antibody complex. Furthermore, IgG antibodies induce the mechanisms of the ADCC response.

IgA Antibodies

The proportion of IgA in the total serum immunoglobulin is only 15 %. However, it is the predominant antibody in the mucous membranes and body secretions such as saliva, bronchial fluid and urogenital secretions. It is produced in the plasma cells of the submucosa, and is secreted into the mucous membranes by an active mechanism mainly through epithelial cells. For this purpose, the monomeric IgA molecules are linked to dimers by a J chain. IgA dimers bind to an IgA receptor on the “back” of epithelial cells, they are internalized together with the receptor and are transported to the “front” of the epithelium. The receptor protein remains associated with the IgA dimer as a secretory piece, and stabilizes the complex in the mucous membrane. IgA play an important role in the local defence mechanism against infections in mucosal regions, and in the prevention of recurrent infections with the same pathogen type.

IgE Antibodies

IgE antibodies are produced particularly against parasites, and induce the release of histamines by binding to the corresponding receptors on mast cells and basophils by their Fc region. IgE is found only in trace amounts in the serum of healthy people. However, its level is significantly increased in allergic individuals. Here, in the case of recurrences of the same antigen, it is jointly responsible for the allergic reactions and the resulting histamine and prostaglandin release, for example, in the bronchial tree, and for the anaphylactic shock reaction.

7.2.2.2 Generation of Antibody Diversity and Antibody Subclasses

B cells produce antibodies, and are thus required, along with T lymphocytes, for the establishment of a specific immune response. B cells originate from bone marrow pluripotent stem cells, which are the progenitors of all haematopoietic cells, and develop into precursor B cells by the influence of cytokines (IL-3). IL-4, IL-5 und IL-6 are necessary for further differentiation into pre-B cells. During this process, somatic recombinations occur at the DNA level, and lead to rearrangements of the variable regions of immunoglobulin genes. Initially, the D and J segments of heavy chains are mutually combined. Subsequently, one of more than 100 V segments is randomly added in front, so that a defined arrangement of VDJ regions is present in the pre-B cell. These are joined to the constant regions of the μ chain by splicing. Subsequently, the V and J segments of the light α and κ chains are rearranged and linked with the C regions by splicing during transcription. The corresponding protein chains are synthesized, and are transported to the cell surface. In this phase, pre-B cells have membrane-bound IgM molecules on their surfaces and secrete small amounts of IgM molecules with the corresponding specificities. The many millions of possible combinations of the VDJ regions of heavy chains, or the VJ segments of light chains, give rise to the huge antibody diversity, which ensures that virtually for every potential antigen, there is a specific IgM molecule. They are continuously present in low concentrations in the relevant cells as well as in the peripheral blood, and can neutralize pathogens. The soluble IgM antibodies form complexes with antigens and act as inducers of the complement cascade. On the other hand, the membrane-associated IgM molecules act as antigen receptors and induce the incorporation of the resulting membrane-bound antigen–antibody complex. The proteins are degraded in the endosomes, where foreign antigen peptides can bind to HLA class II proteins. From there they return to the cell surface as an MHC complex. In this phase, pre-B cells become antigen-presenting cells, which are bound by T_H cells with the corresponding specificities of T-cell receptors, and then produce a large number of different cytokines (▶ Chap. 8). Thereby, B cells are stimulated to proliferate, and differentiate into plasma cells. The immunoglobulin class switch also occurs in this phase, i.e. the variable domains of heavy chains are combined by alternative splicing with the corresponding segments of γ chains or – depending on the cytokine signals received – the other heavy chains. Mutations occur in the DNA sequences encoding the highly variable complementarity determining regions in the V domains. Antibodies obtain their final, high-affinity specificity by this maturation step via somatic hypermutation.

7.2.2.3 When Does a Specific Immune Response Occur in an Organism?

The unspecific, non-adaptive immune responses are innate, and are already present in newborn organisms. In contrast, the specific cellular and humoral defence mechanisms develop postnatally. To be able to develop the high-affinity binding of T-cell receptors of T_H and cytotoxic T cells as well as that of the variable regions of antibodies, the immune system and the organism must have contact with the respective viruses and other infectious agents. These processes occur after birth, except for transplacentally transmitted viruses. If the embryo is infected during

gestation, it is able to develop its own IgM and IgG antibodies only from the 22nd week of pregnancy. In humans, newborns receive a first specific protection by maternal antibodies (maternal immunity), which reach the bloodstream of the child during pregnancy as well as via breastfeeding, and assume explicit protective functions (maternal passive immunity) for about 6 months; thereafter, they are gradually degraded. The basis for this is the human placenta type (haemochorial placenta), which is based on an extensive dissolution of structural parts of the maternal placenta. In this case, the maternal blood directly irrigates the fetal capillaries that are surrounded by the chorion membrane, which is permeable to IgG antibodies.

The situation is different in animals: in horses and swine, there is a complete separation of the fetal and maternal side of the placenta (epitheliochorial placenta), whereas in ruminants and carnivores (canines and felines) the endothelium of the uterus, and to differing degrees, also the submucosa of the uterus are disintegrated (syndesmochorial placenta in ruminants, and endotheliochorial placenta in carnivores). The different types of placentas have a direct influence on the transfer of maternal antibodies: the more completely the maternal and fetal sides are separated from each other, the more impermeable is the placenta to immunoglobulins. In contrast to humans, immunoglobulins are transmitted in ruminants and swine exclusively and in dogs and cats predominantly via colostrum, the protein-rich first milk, which contains vitamins, antibodies and leucocytes and is produced until a few days after birth. The resorption of antibodies by young animals is effective only during the first few hours of life.

7.3 How Does the Antiviral Defence Elicit Autoimmune Diseases?

The immune system enables organisms to develop antibodies and T-cell receptors with specificities for many millions of antigens. At the same time, it must be ensured that they recognize only foreign antigens without attacking the structures of the organism itself. When the immune system no longer distinguishes between "self" and "foreign" and reacts against the body's own structures and cells, autoimmune reactions can develop. In the other case, a tolerance is established.

Normally, during embryogenesis and later also in the thymus, T cells with receptors are selected and retained, and these recognize the body's own structures. They do not reach the peripheral blood, but they perish through apoptosis, i.e. by induction of programmed cell death. This clonal selection ensures that no T_H cells and cytotoxic T lymphocytes with self-specificities are present in the organism. Inasmuch as the antibody production is dependent on the help of T cells, immunoglobulins with corresponding self-recognition should not be present. Viral infections occasionally trigger autoimmune reactions. Some viruses encode proteins that resemble cellular polypeptides, but are not identical with them. This is the case, among others, in measles virus, which codes for a protein with similarity to the basic myelin of the brain, in human immunodeficiency virus (HIV), which possesses

several protein segments with homology to various cell components, and in Epstein–Barr virus (▶ Sects. 15.3, ▶ 18.1, and ▶ 19.5). Because of these similarities, these viruses induce immunological cross-reactions with the respective cellular proteins. The imitation of cellular proteins is referred to as molecular mimicry. After a relevant infection, cytotoxic T lymphocytes may be present, and these attack and destroy the body's own cells. Cross-reactive T_H cells can induce the production of immunoglobulins that are directed against cellular proteins. The resulting antigen–antibody complexes can trigger all kinds of defence responses, ranging from the attack of neutrophils with the release of inflammatory factors to the activation of the complement cascade with its cell-damaging effects. If these complexes accumulate in the synovial space, then the induced immune responses can cause severe arthritis. Such processes are a possible cause of postinfectious reactive arthritis that, for example, is associated with rubella virus or parvovirus infections (▶ Sects. 14.6 and ▶ 20.1).

However, there are also other mechanisms. During the primary infection, Epstein–Barr virus induces infectious mononucleosis, a polyclonal activation of T cells that react with an increased cytokine release, and thus also stimulate B cells polyclonally (▶ Sect. 19.5). Many B and T lymphocytes are stimulated regardless of the antigen specificity. In many patients with an acute Epstein–Barr virus infection, immune responses against various endogenous structures are found; the increased activation rate is manifested as lymph node swelling and lymphadenopathy. The organism reacts to viral infections with the production of IFN-α, IFN-β and IFN-γ. These interferons induce, among other things, an increased expression of MHC class I and MHC class II proteins on the surface of uninfected cells. This MHC overexpression can lead to immunological side effects, and the corresponding cells can be attacked by T lymphocytes. In the case of Epstein–Barr virus infections, this leads to mononucleosis. In this case, the non-specific proliferation can lead to monoclonal or polyclonal malignant cells and Burkitt's lymphoma by additional, long-lasting stimulatory factors (such as malaria, chromosomal translocations). Even the effect of superantigens leads to immunological attack on the body's own cells. On the one hand, such protein molecules bind to specific V_β chains of T-cell receptors, and on the other hand, they bind to MHC class II proteins on the surface of antigen-presenting cells. In this way, both cell types are brought into contact with each other in an antigen-independent manner. This non-specific stimulation leads to oligoclonal expansion of T cells with certain β chains in the receptors, which are also directed against the body's own cellular structures. In addition, the release of corresponding cytokines can be observed. There is evidence that rabies virus, mouse mammary tumour virus and several other retroviruses – possibly also HIV – have superantigens (▶ Sects. 15.1 and ▶ 18.1).

7.4 How Can Viruses Evade the Immune System?

Several viruses have evolved mechanisms which enable them to evade the immune response of their hosts. This ability can often be attributed to inaccurately functioning viral enzymes such as RNA polymerases, which usually replicate the viral

genomes. Consequently, especially RNA viruses (e.g. hepatitis C virus, HIV and influenza viruses) exhibit very high mutation rates, and continuously change the sequence and structures of their surface-exposed protein regions under the selective pressure of antibodies, and thus escape the neutralizing effect of immunoglobulins (quasi-species; ▶ Sects. 14.5, ▶ 16.3 and ▶ 18.1). Furthermore, HIV alters the cytokine and chemokine patterns that are produced by infected cells. As a result, the immune system and in particular the cytotoxic T lymphocytes become ineffective (▶ Sect. 18.1).

Other viruses have also found simple but ingenious mechanisms to circumvent the immune response of the host organism and to establish persistent infections. Papillomaviruses (▶ Sect. 19.3) infect, for example, the cells of the outermost layers of the skin, an ecological niche that is not easily accessible to the immune system. Herpes simplex viruses do not produce viral proteins in nerve cells during latency; hence, the immune system cannot recognize them as infected cells. By contrast, hepatitis B viruses (▶ Sect. 19.1) produce large amounts of hepatitis B surface antigen (HBsAg) during infection, which is then present in high concentrations in the blood and is captured by HBsAg-specific neutralizing antibodies.

In addition to these rather untargeted mechanisms to evade the immune response, many viruses, especially DNA viruses, have developed special approaches by which they can specifically suppress defence responses. This enables them to establish persistent infections. The commonest strategy is the reduction of MHC antigens on the surface of infected cells. This impedes recognition by the cellular immune response; this alternative is used by adenoviruses, herpesviruses (cytomegalovirus, human herpesvirus 8 and Epstein–Barr virus) and also by human immunodeficiency viruses. The infected cells can no longer present viral peptide antigens, cytotoxic T lymphocytes do not recognize infected cells and the virus escapes elimination (▶ Sects. 18.1, ▶ 19.4 and ▶ 19.5). The molecular mechanisms which viruses have developed for this purpose are varied: by the action of specific viral proteins, adenoviruses and cytomegaloviruses retain newly synthesized MHC class I proteins in the ER, and hinder their transport to the cell surface. On the other hand, human immunodeficiency viruses has protein functions which destabilize the surface-exposed MHC proteins in order to induce their endocytosis and subsequent proteolytic degradation. To prevent MHC-depleted cells being attacked by NK cells, cytomegaloviruses encode an additional MHC class I protein homologue that interacts with KIR receptors, thus preventing the development of the cytotoxic activity of NK cells (▶ Sect. 19.5). De facto, in almost all immune attack strategies against viruses, there are examples of specific escape or infiltration strategies in the context of viral immune evasion.

Interferons have a strong antiviral activity (▶ Chap. 8). To avoid this non-specific immune response, several viruses interfere with the signalling pathways that activate the expression of interferon genes. They prevent the phosphorylation of signal transducer and activator of transcription (STAT) proteins and induce their degradation (paramyxoviruses; ▶ Sect. 15.3) and have protein components that inhibit the activity of protein kinase R or 2′-5′-oligoadenylate synthetase, impede its binding

to double-stranded RNA, or degrade it (reoviruses, human immunodeficiency viruses, adenoviruses, Epstein–Barr virus, poxviruses; ▶ Sects. 17.2, ▶ 18.1 and ▶ 19.4–19.6). Alternatively, many viruses have developed mechanisms that inhibit cytokine synthesis or restrict their effects. Asfarviruses code for a protein with homology to the factor Iκβ, which prevents activation of NFκB, and thus inhibits the synthesis of many immunologically important gene products by interfering with the signalling pathway (▶ Sect. 19.7). Especially, pox viruses, but also some herpesviruses, produce proteins that are homologous to different cytokines or chemokine receptors, which are secreted by infected cells and intercept the soluble mediators of the innate immune response in the blood (▶ Sects. 19.5 and ▶ 19.6). However, particularly herpesviruses use the synthesis of chemokine analogues to block the corresponding receptors, which represents a frequently used viral strategy to suppress the immune response.

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8 Cytokines, Chemokines and Interferons

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In 1935, M. Hoskins, G.M. Findlay and F. MacCallum discovered the phenomenon of interference: animals that were inoculated with avirulent yellow fever viruses were found to be protected against infections with the wild-type virus in the following 24 h. The cause of interference remained unclear for a long time until Alick Isaacs and Jean Lindenmann discovered the substance interferon in infected embryonated chicken eggs in 1957. Initially, it was thought that the defence mechanism mediated by interferon was directed against “foreign nucleic acids”, since interferon could effectively be induced by the double-stranded RNA of reoviruses. However, this effect was not very selective. It soon became apparent that even uninfected cells are affected by interferon and that the administration of interferon to animals can provoke serious adverse side effects. In addition, it also became evident that there is a basal interferon concentration in the organism which is physiologically determined and is sustained by frequent viral infections. Interferons exert many functions in the cell, and fulfil important functions in regulating cell physiological processes.

Interferon was only the first of a large number of inducible cytokines that were subsequently discovered. As early as in 1866, it was reported that tumours can regress after bacterial infections. In 1975, Elizabeth Carshwell and colleagues showed that macrophages of mice which had been infected with the tuberculosis

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BCG vaccine (named after *Bacillus* Calmette–Guérin) and treated with *Escherichia coli* lipopolysaccharides produced a cytotoxic, tumour-destroying factor, which was designated as tumour necrosis factor (TNF). The emergence of fever as a symptom of central medical importance also attracted early interest. It was possible to distinguish between exogenous and endogenous fever-inducing substances (pyrogens). Purified influenza viruses were used as an exogenous pyrogen, and the endogenous activity that was induced by that "external stimulus" was detectable in the serum, and was denominated interleukin-1 (IL-1) in 1971. This substance is produced by, for example, stimulated macrophages and granulocytes, and along with lectins (e.g. phytohaemagglutinin) can activate lymphocytes. In recent years, it has been found that Toll-like receptors (TLRs), through their ability to recognize pathogen-associated molecular patterns and to interact with them, induce the expression of interferons and various cytokines by triggering a signalling cascade; this is essential for the early immune defence steps (▶ Sect. 7.1). These findings have decisively contributed to a better understanding of the fundamental processes involved in the induction of the antiviral immune response.

8.1 Which Classes of Cytokines Are Distinguished, and what Are their Functions within the Immunological Effector Systems?

Cytokines constitute a part of the unspecific, non-adaptive immune defence, and are thus essential for the control of pathogens during primary contacts. They are active at the time of infection, when specific antibodies or cytotoxic T cells are not yet available. They regulate and coordinate the interaction of immune effector systems, i.e. the cellular and humoral immune response, which alone are not able to control the spread of a pathogen in an organism and to cause its elimination. Most cytokines are produced and secreted in immunologically active cells. They exert their biological activity by binding to specific receptors in the cytoplasmic membrane of certain cells. In this way, they induce a signalling transduction pathway that culminates with a specific effect. In addition to interferons, the following factors belong to the cytokine group today: interleukins, TNFs, colony-stimulating factors (CSFs), chemokines and transforming growth factors (TGFs).

8.1.1 Interferons

Fundamentally, interferons are classified into type I and type II interferons; furthermore, there are interferon-like cytokines, which are known as type III interferons (Table 8.1).

8.1.1.1 Type I Interferons

The acid-stable type I interferons can be divided into eight subgroups: In humans, IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω have been described; they are all encoded

Table 8.1 Currently known interferons (*IFN*) and their properties

Subgroup	Name	Species	Receptor	Triggered signal transduction pathways and proteins involved
Type I IFNs	IFN-α	Humans	IFNαR1/IFNαR2	Jak1, Tyk2, STAT1, STAT2, STAT3, STAT4, STAT5, PI3K, Akt, NFκB, MAPK, p53, PRMT1
		Vertebrates		
	IFN-β	Humans	IFNαR1/IFNαR2	
		Vertebrates		
	IFN-δ	Ruminants	IFNαR1/IFNαR2	
	IFN-ε	Humans	IFNαR1/IFNαR2	
		Mice		
	IFN-κ	Humans	IFNαR1/IFNαR2	
	IFN-τ	Swine	IFNαR1/IFNαR2	
	IFN-ω	Humans	IFNαR1/IFNαR2	
		Vertebrates		
	IFN-ζ	Mice	IFNαR1/IFNαR2	
Type II IFNs	IFN-γ	Humans	IFNγR1/IFNγR2	Jak1, Jak2, STAT1, STAT3, STAT5, PI3K, Akt, NFκB, MAPK
		Vertebrates		
Type III IFNs	IL-28A/IFN-λ2	Humans	IFNλR1/IL-10R2	Jak1, Tyk2, STAT1, STAT2, STAT3, STAT5
	IL-28B/IFN-λ3	Vertebrates		
	IL-29/IFN-λ1			

MAPK mitogen-activated protein kinase, *NFκB* nuclear factor κB, *PI3K* phosphatidylinositol 3-kinase, *PRMT1* protein arginine *N*-methyltransferase 1, *STAT* signal transducer and activator of transcription

in the short arm of chromosome 9. To date, IFN-δ, IFN-τ and IFN-ζ (limitin) have only been detected in animals. With the exception of IFN-ε, IFN-δ and IFN-τ, type I interferons are antiviral cytokines. They are synthesized in virus-infected cells, are released into the environment and generally function species-specifically by binding to receptors in the plasma membrane of other cells, in which they exert a protective effect against the infection. They inhibit viral replication, but also cell division, and thus also have tumour-inhibiting properties. All type I interferons are secreted by cells, and exert their effects by binding to IFN-α receptor (IFNαR), a heterodimer composed of an IFNαR1 and an IFNαR2 chain, which is expressed on almost all cell types (Table 8.1).

Molecular Properties

IFN-α and IFN-β are the longest known and best studied interferons. They have a similar serological behaviour, which is based on a sequence homology of about 50 %; they are 166 amino acids in length, and have a molecular mass of 20 kDa. Human IFN-α, originally also called leucocyte interferon, has 12 similar subtypes, which are encoded by a multigene family and exhibit a homology of 85–90 % at the amino acid level. IFN-β (fibroblast interferon) has only one subtype. Little is known about the function of IFN-ε. It has been detected in humans and mice; in the latter,

it is constitutively expressed in the tissues of the placenta and the ovaries and presumably has functions in reproduction; it is uncertain whether it also exerts an antiviral effect. IFN-κ is produced in keratinocytes and has – albeit low – antiviral activity. IFN-ω has been found in all animal species examined, except mice. Only one subtype of IFN-ω has been found in humans. Like all type I interferons, it has a molecular mass of about 20 kDa and has an antiviral effect. IFN-ζ has been detected only in mice. It comprises 161 amino acids and has an antiviral effect like IFN-α; however, its antiproliferative effect on lymphocytic and myeloid progenitor cells is significantly weaker. IFN-δ and IFN-τ have been detected in cattle and swine, and have an important function in maintaining early pregnancy. They are synthesized in trophoblasts, inhibit prostaglandin synthesis, and thus prevent the prostaglandin-induced regression of the corpus luteum and the abortion of pregnancy. Whether they also have a physiological antiviral effect has not been studied (Table 8.1).

Synthesis

The expression of IFN-α and IFN-β genes is induced most notably in dendritic cells, monocytes and macrophages that have phagocytosed pathogens, and in all types of infected cells containing pathogen-specific components of viruses (pathogen-associated molecular patterns), which interact with the various TLRs. In the case of viral infections, TLR3, TLR7, TLR8 and TLR9 are primarily important. These TLRs are predominantly anchored in the endosomal membrane; they come into contact with viral nucleic acids in this cell compartment, where the viruses are present after uptake by phagocytosis. These interactions trigger multistep signalling cascades that culminate in the activation of the transcription factor nuclear factor κB (NFκB) and/or interferon regulatory factors (IFRs), particularly IRF3 and IRF7. These enter the nucleus and interact with the control elements in the promoter regions of various cytokine genes, including the IFN-α and INF-β genes, which are subsequently upregulated (▸ Sect. 7.1).

In addition, there are also other ways to induce the synthesis of IFN-α and INF-β. If unnaturally large amounts of double-stranded RNA or triphosphorylated RNAs that are not capped at the 5′ terminus are present in the cytoplasm of a virus-infected cell (two types of molecules that hardly exist in uninfected cells), this will also induce the expression of IFN-α and IFN-β. This process is initiated by a subfamily of pattern-recognition receptors: the retinoic acid inducible gene I (RIG-I)-like helicases (RLH). The RIG-I protein is a cytoplasmic RNA detector. It consists of two amino-terminal caspase activation and recruitment domains (CARDs), which are followed by an RNA helicase moiety, which is referred to as DExD/H box owing to it having a conserved amino acid motif. Similar RNA helicase domains have been found in melanoma-differentiation-associated protein 5 (MDA-5) and LGP2 protein – together with RIG-I they constitute the RLH family – and in Dicer enzymes. The latter cleave double-stranded RNA into about 20–25 nucleotide fragments, which are involved in the regulatory process of RNA interference as small interfering RNAs. The RNA helicases RIG-I and MDA-5 bind to double-stranded RNA; RIG-I also binds to 5′-triphosphorylated single-stranded RNA.

This binding activates their ATP-dependent helicase activities, and causes the CARDs to bind to the protein IFN-ß promoter stimulator 1 (IPS-1), which also has an amino-terminal CARD, and is associated with mitochondrial membranes. IPS-1 is an adaptor protein that mediates the interaction with TNF-receptor-associated factor (TRAF) 3, whereby the inducible IκB kinase and the TRAF-family-associated NFκB activator (TANK)-binding kinase 1 become activated. Both kinases perform the phosphorylation of IRF3 and IRF7, which form homodimers or heterodimers, enter the nucleus, bind to the interferon-stimulated response element (ISRE) promoters of IFN-α and IFN-β genes and a number of other interferon-inducible genes, and induce their expression (Fig. 8.1). Alternatively, the Fas-associated death-domain-containing protein (FADD)-dependent activation of the NFκB, which binds to NFκB-dependent promoters in the nucleus, can also be achieved by binding of IPS-1 to the CARDs of RIG-I or MDA-5. In this way, the expression of a number of genes encoding proinflammatory cytokines is activated. These processes can be also induced experimentally by double-stranded RNA, or some synthetic substances such as polyinositol cytidine acid.

Effect

All type I interferons bind to IFN-α receptor. This interaction triggers cellular signal transduction pathways, which entail a variety of responses: establishment of an antiviral state, inhibition of cell division, increase of the concentration of MHC class I antigens and other surface markers (tumour markers) on the cell surface, induction of expression of proapoptotic proteins (e.g. caspases) or repression of antiapoptotic factors (such as Bcl-2) and release of other cytokines (Table 8.2).

The first step of the cascade is the conformational change of IFNαR. This conformational change activates the kinases Tyk2 und Jak1, which are associated with the intracellular domains of IFNαR1 and IFNαR2 (Fig. 8.2). These receptors are phosphorylated and give rise to the binding of STAT2 and STAT1 to the IFNaR2 chain ("STAT" is an acronym for "signal transducer and activator of transcription"), and thereby they are phosphorylated and activated as well. In this state, STAT1 and STAT2 detach from the receptor complex on the inner side of the cytoplasmic membrane and interact with the protein p48. The STAT1/p48 and STAT2/p48 complexes enter the nucleus and bind – usually together with other cell-specific transactivators – to ISRE promoters, which regulate the expression of interferon-stimulated genes. Depending on the type of cell, its differentiation and other factors, additional proteins such as STAT3 and STAT5 can also be integrated into the signalling pathway. Sometimes, other signalling cascades are also used, such as the phosphatidylinositol 3-kinase pathway or the mitogen-activated protein kinase pathway. In addition, IκB, the inhibitor of NFκB, is also phosphorylated. As a consequence, the inhibitor becomes inactivated, leading to induction of all cellular genes that are regulated by NFκB. These genes and the ISRE are the real targets of the interferon effect. Their activation induces the synthesis of many different proteins (Table 8.2). This explains the diversity of the above-mentioned interferon-induced mechanisms.

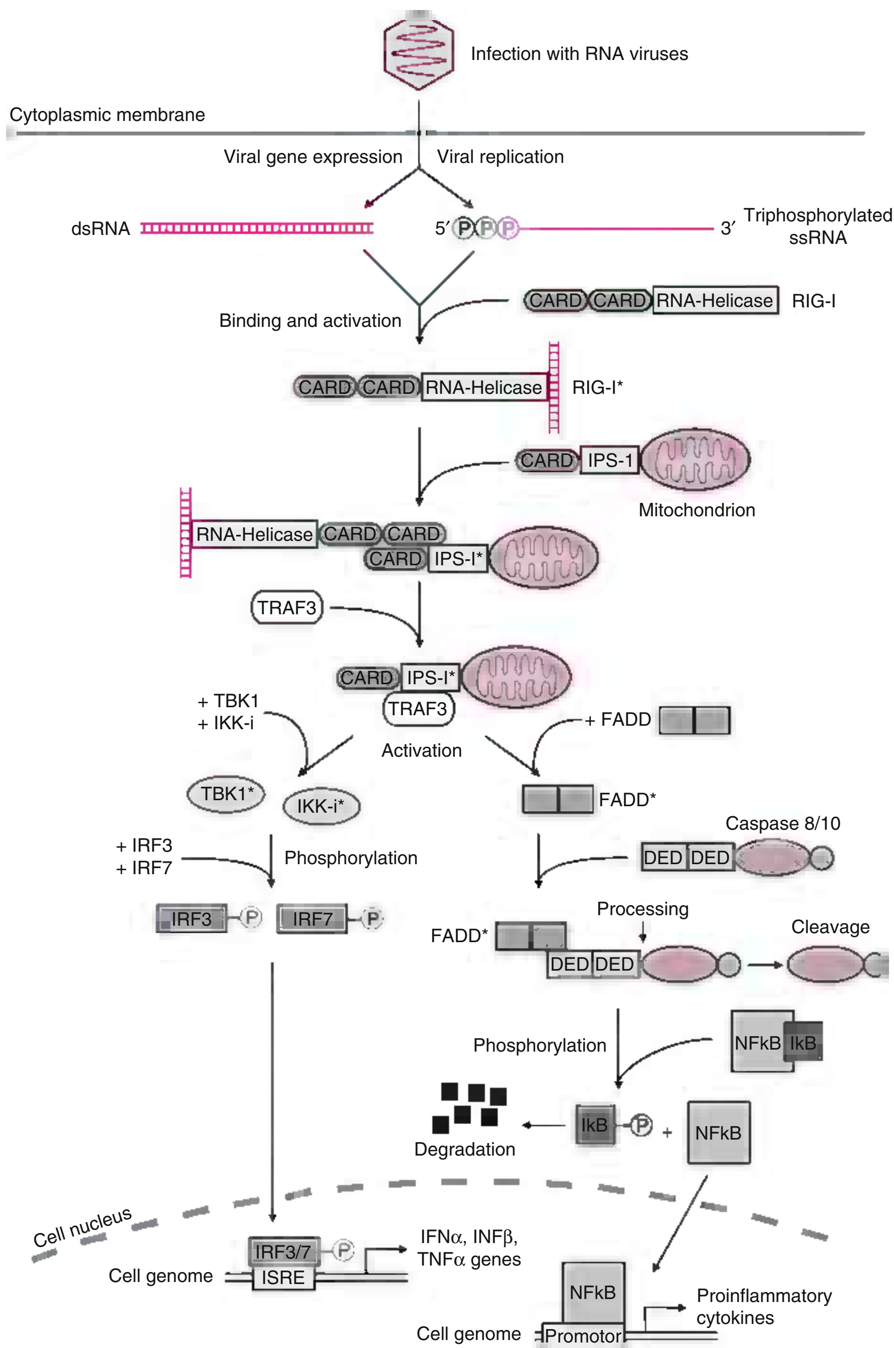

Fig. 8.1 Induction of interferon (*IFN*) synthesis mediated by activation of retinoic acid inducible gene I (*RIG-I*) helicases by viral RNA (simplified representation). *Asterisks* indicate activated

Table 8.2 Principal effects of the most important interferons (*IFN*) on the expression of cellular proteins

	IFN-α	IFN-β	IFN-ω	IFN-γ
Protein kinase R	+++	+++	+++	+
2′-5′-Oligoadenylate synthetase	+++	+++	+++	+
Mx proteins	+++	+++	+++	–
MHC class I	+++	+++	+++	+++
MHC class II	+	+	+	++
Proapoptotic factors (e.g. caspases, Bak, Bax)	+++	+++	+++	–
Antiapoptotic factors (e.g. Bcl-2)	–[a]	–[a]	–[a]	–[a]

[a]Inhibition

Generally, proteins that are produced by the effect of type I interferons influence viral replication: they lead to inhibition of protein synthesis. One pathway leads through the interferon-mediated induction of 2′-5′-oligoadenylate synthetase. If double-stranded RNA is present in the cell as part of the viral genome or as an intermediate of viral replication, 2′-5′-oligoadenylate synthetase catalyses the esterification of up to five ATP residues to oligoadenylates. They bind to an RNase and activate it. The RNase degrades the single-stranded RNA of cellular and viral messenger RNA molecules, thereby destroying the genetic information of single-stranded RNA viruses. This inhibits both the viral and the cellular protein synthesis. Furthermore, interferon induces the expression of the protein kinase R, which also requires the presence of double-stranded RNA for its activity. It phosphorylates and inactivates the translation elongation factor eIF2. Consequently, protein synthesis is terminated. Moreover, interferons induce the synthesis of a number of other cellular proteins. Noteworthy is primarily the increased expression of MHC class I antigens. This augments the ability of cells to present viral protein fragments on their surfaces, which in turn facilitates the recognition and lysis of infected cells by cytotoxic T cells. In addition, IFN-α and IFN-β induce the expression of MHC class II proteins, albeit to a lesser extent. Mx proteins

Fig. 8.1 (continued) variants of the respective enzymes or factors. RIG-I helicase is present in the cytoplasm of the cell and interacts with viral RNA species [doubled-stranded RNA (*dsRNA*); uncapped, 5′-triphosphorylated single-stranded (*ssRNA*)] and becomes activated (*RIG-I**). The caspase activation and recruitment domain (*CARD*) of RIG-I* interacts with the CARD of IFN-ß promoter stimulator 1 (*IPS-1*), which in its activated form (*IPS-1**) interacts with TNF-receptor-associated factor 3 (*TRAF3*). TRAF-family-associated nuclear factor κB (*NFκB*) activator (TANK)-binding kinase 1 (*TBK1*) and inducible IκB kinase (*IKK*-í) phosphorylate the transactivators interferon regulatory factor 3 (*IRF3*) and interferon regulatory factor 7 (*IRF7*) (*left*), which are transported into the nucleus, bind to the IFN-stimulated regulator element (*IRSE*) promoters and induce the gene expression of IFN-α, IFN-β and TNF-α. IPS-1* can activate Fas-associated death-domain-containing protein (*FADD*) by an alternative stimulation process, which in further steps induces the phosphorylation of IκB (*right*). This leads to the release of NFκB as a transactivator, which in turn can induce the synthesis of proinflammatory cytokines in the nucleus. *DED* death effector domain

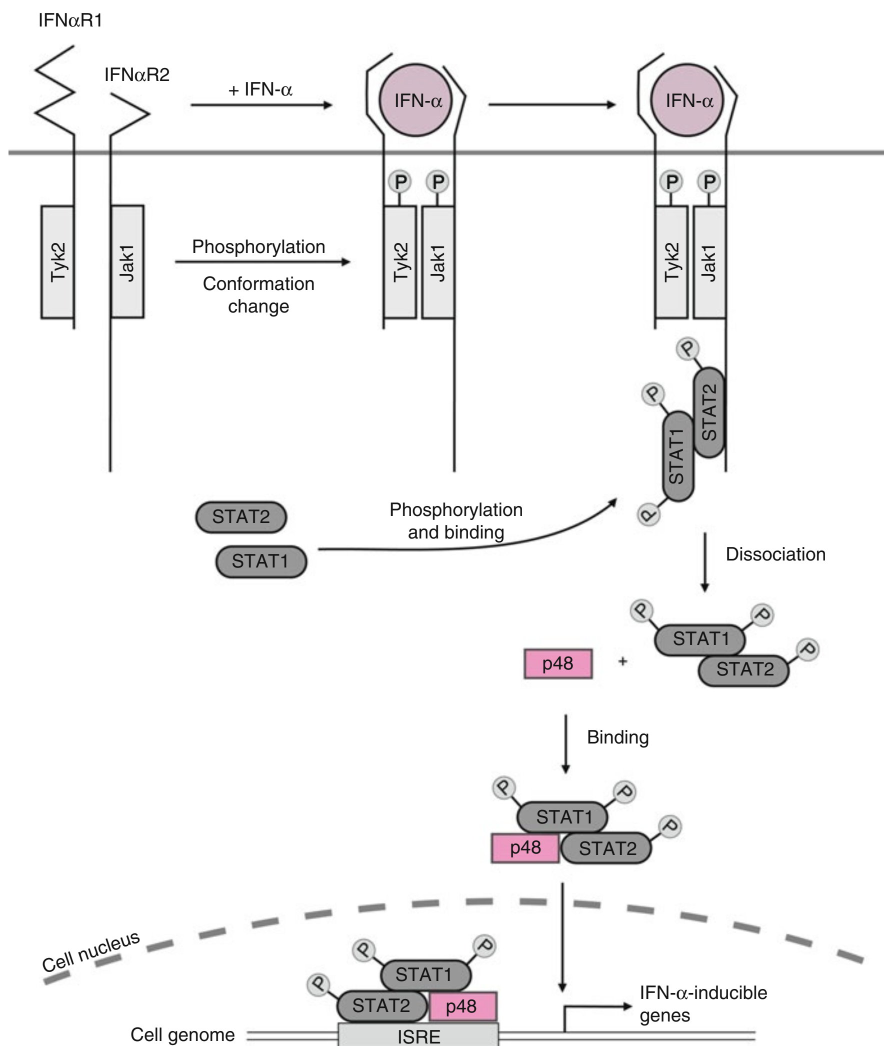

Fig. 8.2 Signalling cascade induced by interaction of IFN-α with IFN-α receptor (*IFNαR*) (simplified representation). IFNαR is anchored in the cytoplasmic membrane. It is a heterodimeric protein. The intracellular domains of the subunits IFNαR1 and IFNαR2 are associated with the kinases Tyk2 and Jak1. Upon binding of IFN-α to the receptor complex, IFNαR changes its conformation and the kinases are phosphorylated. This causes binding of STAT1 and STAT2 proteins and their phosphorylation. Phosphorylated STAT1 and STAT2 proteins detach from the complex, interact with additional cellular proteins (p48) and enter the nucleus. They bind to ISRE promoters and activate the expression of IFN-α-inducible genes

constitute a special group of IFN-α and IFN-β inducible polypeptides. They belong to the protein superfamily of dynamin-like enzymes and possess GTPase activity. Two type I interferon inducible Mx genes have been discovered in most mammals; in humans, they are the MxA and MxB genes. MxA proteins accumulate in the

cytoplasm and inhibit the proliferation of influenza viruses as well as other viruses, such as measles virus, parainfluenza viruses, hantaviruses, vesicular stomatitis virus and bunyaviruses (▶ Sects. 15.1, ▶ 15.3 and ▶ 16.2). Their mode of action is not completely understood. There is evidence that they influence the intracellular transport of viral proteins, and also interfere with replication and morphogenesis. No antiviral activity has been found for the MxB proteins so far.

8.1.1.2 Type II Interferon

IFN-γ is an acid-labile type II interferon which is found in all mammals, and has only one subtype. It is a homodimer of two glycosylated protein subunits, which are 146 amino acids long and have a molecular mass of 22 kDa, and have homology neither with IFN-α and IFN-β nor with other type I interferons. In humans, the gene encoding IFN-γ is located on chromosome 12. IFN-γ was originally denominated immune interferon. It has been found in all animal species studied so far. IFN-γ is principally produced by subtype 1 T-helper cells, which are stimulated by antigen contact. This stimulation can be caused by activated dendritic cells, macrophages, natural killer cells and to a lesser extent also by cytotoxic T cells. IFN-γ induces its effects by binding to IFN-γ receptor (IFNγR), a homodimeric protein composed of two IFNγR1 chains. Binding of IFN-γ to IFNγR1 causes complex formation with two IFNγR2 chains and induces, like type I interferons, a signalling cascade, in which the kinases Jak1 and Jak2, which are associated with the IFNγR1 and IFNγR2 chains, initially phosphorylate each other and subsequently the tyrosine residue at position 457 of IFNγR1 chains (Fig. 8.3). This induces binding of STAT1 proteins and their phosphorylation. The phosphorylated and activated STAT1 dimers are transported into the nucleus and bind to the corresponding ISRE motifs of IFN-γ-regulated genes. Similar to type I interferons, other signalling pathways using alternative kinases and other transcription factors can also be involved in activation (Table 8.1). The effect of IFN-γ gives rise to MHC class II protein expression and release of further cytokines, as well as to an increase of the effector function of cytotoxic T cells, natural killer cells and mononuclear phagocytes; therefore, IFN-γ is regarded as one of the pivotal cytokines involved in the induction of the specific immune response. IFN-α and INF-β as well as IFN-γ reinforce each other mutually, and influence the expression of the cytokine network.

8.1.1.3 Type III Interferons

The family of type III interferons has only recently been created. It comprises three members: IFN-λ1 (also known as IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B; Table 8.1) Type III interferons are encoded on chromosome 19 in humans. They resemble type I interferons, have molecular masses of about 20 kDa and are monomers; only IFN-λ1 is glycosylated. Similar to type I interferons, the synthesis of IFN-λ is induced in plasmacytoid dendritic cells through activation of TLR3, TLR7, TLR8 and TLR9, which interact with virus-specific pathogen structures, mainly viral RNAs (see ▶ Sect. 7.1). This leads to phosphorylation and activation of IRF3, which acts as a transactivator and binds to specific

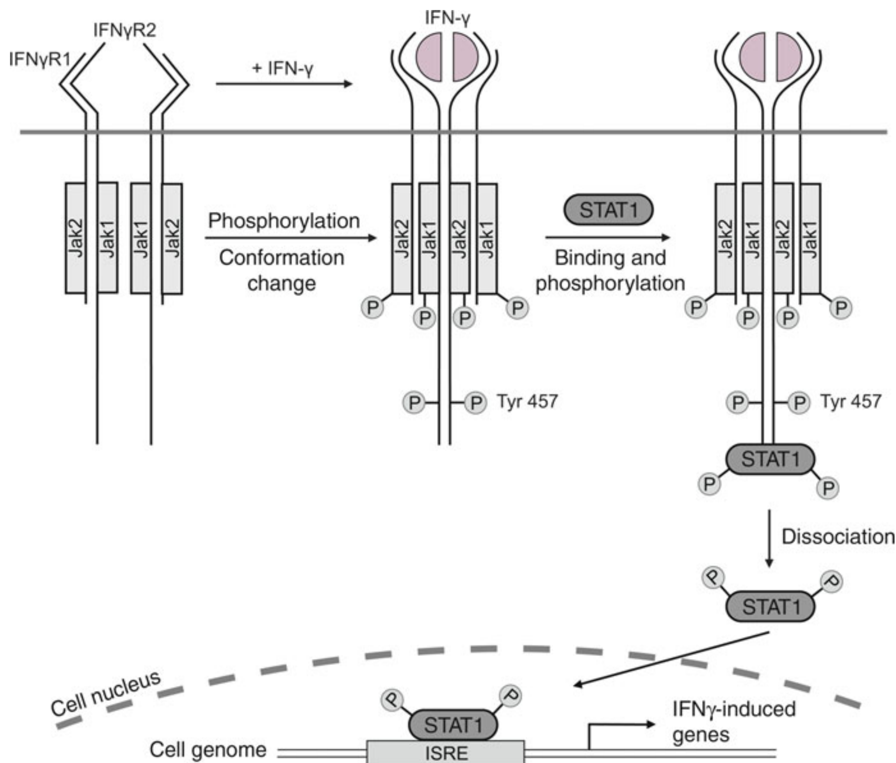

Fig. 8.3 Signalling cascade induced by interaction of IFN-γ with the IFN-γ receptor 1 (*IFNγR1*)/IFN-γ receptor 2 (*IFNγR2*) complex (simplified representation). IFNγR1 chains are anchored in the cytoplasmic membrane. By interacting with the homodimeric IFN-γ, they associate both with each other and with two chains of IFNγR2. The intracellular domains of IFNγR1 and IFNγR2 are associated with the kinases Jak1 and Jak2. The IFNγR1/IFNγR2 complex changes its conformation upon binding of IFN-γ and becomes phosphorylated along with Jak1 and Jak2. This induces binding of STAT1 and STAT2 proteins and their phosphorylation. The phosphorylated STAT1 proteins dissociate from the complex to form dimers, which are transported into the nucleus, where they bind to ISRE promoters and activate the expression of the IFN-γ-inducible genes

sequence elements in the promoter of IFN-λ genes. The various subtypes of IFN-λ bind to a heterodimeric receptor, composed of the β subunit of IL-10 receptor (IL10R2) and the IFNλR1 protein (also known as IL28Rα). The induced Jak/STAT signal transduction pathway, which is triggered by binding to the receptor complex, largely resembles that induced by type I interferons (Table 8.1, Fig. 8.2); however, there are still few data concerning details of the mode of action of IFN-λ. It has been found that IFN-λ induces the production of Mx proteins. Type III interferons exhibit a very broad antiviral effect, which is preferentially manifested in epithelial cells.

8.1.2 Interleukins

Interleukins are proteins with molecular masses between 8 and 70 kDa. Most of them are glycosylated, some act as monomers (IL-1), others act as homodimers (IL-17) or as heterodimers (IL-12). Many have a number of subtypes that are combined in interleukin families (IL-1, IL-17). Interleukins are produced and released by various cells of the immune system mainly after stimulation by interferons, cytokines, microorganisms, antigens, lectins or lipopolysaccharides, and act by binding to specific receptors on the surface of immunologically active cells. Cells which do not belong to the immune system also have such receptors and are influenced by interleukin binding. However, the term "interleukin" is derived from their first described activity, namely to "mediate between leucocytes". To date, more than 35 interleukins are known, and are numbered according to the order of their discovery. They exert regulatory effects on the various activities of the immune system, which they interconnect and regulate. Because of the increasing number and variety of interleukins, a complete tabular listing is not included. Table 8.3 summarizes the properties of the best studied interleukins. The molecular characteristics of all known interleukins, their functions and their mode of action, as well as the cell types that are responsible for their synthesis can be found at http://www.copewithcytokines.org.

8.1.3 Tumour Necrosis Factors

TNF-α and TNF-β are the oldest known members of the TNF/TNF receptor superfamily and are encoded on human chromosome 6 in the MHC gene cluster. TNF-α and TNF-β are also known under the old names cachexin and lymphotoxin-α, respectively. Their amino acid sequences have a homology of 36 %. Today, TNF-α is scientifically denominated as tumour necrosis factor ligand superfamily member 2 (TNFSF2). This multifunctional cytokine is involved in local and systemic inflammation. It is predominantly produced and released by activated dendritic cells, macrophages, and B and T lymphocytes. TNF-β is preponderantly released by T lymphocytes. The expression of the TNF-α gene is mainly induced by phagocytosed microorganisms, by binding of lipopolysaccharides and other bacterial products to TLRs (see ▶ Sect. 7.1) and by the interaction of interleukins (IL-1β) with their respective receptors on the cell surface. TNF-α is synthesized as a 26-kDa membrane-anchored protein that assembles into trimers. The metalloprotease TNF-α converting enzyme cleaves the membrane-anchored TNF-α precursor, releasing the soluble and functionally active TNF-α (51 kDa).

TNF-α and TNF-β bind to the same receptors (TNF-R1 and TNF-R2), which are present in the cytoplasmic membranes of many cells, and probably have identical biological activities. By binding to TNF-R2, TNF induces a signal transduction pathway in which TRAF2 and eventually NFκB are activated, whereby the expression of NFκB-dependent genes is induced. By contrast,

Table 8.3 Functions, properties and activities of interleukins

Interleukin	Molecular characteristics	Receptor*	Producer	Main function
IL-1 (IL-1α, IL-1β)	17 kDa	CD121α/IL1R1 (CD121β/IL1R2)	Macrophages Monocytes Endothelial cells	B-, T-, and NK-cell activation, induction of fever via hypothalamus
IL-2 (T-cell growth factor)	15,4 kDa, O-glycosylated	CD25/IL2Rα CD122/IL2Rβ CD132IL2Rγ	T cells	T-cell proliferation/ activation
IL-3 (colony-stimulating factor, multiple)	15–17 kDa	CD123/IL3Rα CD131/IL3Rβ	B and T cells Macrophages Endothelial cells	Growth factor for stem cells, important for haematopoiesis
IL-4	20 kDa, N-glycosylated	CD124/IL4R CD132/IL2Rγ	T_H2 cells Mastocytes	B-cell activation, switch to IgE synthesis, anti-inflammatory
IL-5	20 kDa, homodimer, N-glycosylated	CD125/IL5Rα CD131/IL3Rβ	T_H2 cells Mastocytes	Growth and differentiation of eosinophils, chemotactic for eosinophils
IL-6	21.5–28 kDa, glycosylated, phosphorylated	CD126/IL6Rα CD130/IL6Rβ	Macrophages T cells	Induction of synthesis and release of acute-phase proteins in hepatocytes, proinflammatory
IL-7	17.4 kDa	CD127/IL7Rα CD132IL2Rγ	Bone marrow Stromal cells	Proliferation of precursor cells of B and T cells, proinflammatory
IL-9 (mastocyte growth factor)	14 kDa, N-glycosylated	CD129/IL9R	T_H cells	Proliferation of mast cells
IL-10	30 kDa, homodimer	CD210/IL10Rα CDw210B/IL10Rβ	T_H2 cells $CD8^+$ T cells Macrophages Monocytes	Inhibits macrophages, inhibits TH1 cells, anti-inflammatory
IL-11	23 kDa	IL10Rα	Bone marrow Stromal cells	Induction of synthesis and release of acute-phase proteins in hepatocytes, induction of proliferation in haematopoietic precursor cells
IL-12	70 kDa, heterodimer of 40 kDa (IL-12–p40) and 35 kDa (IL-12–p35), glycosylated	CD212/IL12Rβ1 CD212/IL12Rβ2	B and T cells Dendritic cells Macrophages	Induction of differentiation of $CD4^+$ T cells, activation of NK cells, stimulates the production of IFN-γ

(*continued*)

Table 8.3 (continued)

Interleukin	Molecular characteristics	Receptor*	Producer	Main function
IL-13	13 kDa	IL13R	T_H2 cells Mast cells NK cells	Proliferation und differentiation of B cells, inhibits synthesis of proinflammatory interleukins of macrophages
IL-15	14 kDa, glycosylated	IL15Rα	Mononuclear phagocytes Macrophages	Proliferation of mastocytes and NK cells
IL-17	30 kDa, homodimer, glycosylated	CDw217/IL17Rα CDw217/IL17Rβ	$CD4^+$ T cells	Production of proinflammatory cytokines in endothelial and epithelial cells as well as fibroblasts
IL-18	18 kDa	CDw218a/IL18R1	Macrophages Monocytes Dendritic cells	Proinflammatory

NK natural killer, T_H T helper
*Receptors are indicated according to the CD-nomenclature, as far as known, and to the alternative designation

the interaction of TNF with TNF-R1 leads to the activation of caspases 8 and 10, and as a result, to the induction of apoptosis. Endothelial cells are important interaction partners of TNF, and play a key role in inflammatory processes. In them, TNFs induce the synthesis and release of proinflammatory cytokines such as IL-1, IL-6, granulocyte–macrophage CSF (GM-CSF) and platelet-activating factor. Furthermore, endothelial cells react with increased production of MHC class I antigens, tissue thromboplastin, different adhesion proteins such as intercellular adhesion molecule 1, vascular cell adhesion molecule 1 and endothelial leucocyte adhesion molecule 1 and inducible NO synthase. This enzyme leads to the production of increased levels of nitric oxide, which expands blood vessels, and thus is likely responsible for TNF-mediated hypotension. All these processes lead to an intensified adhesion of lymphocytes, and to an increased permeability of vascular walls.

Moreover, TNFs regulate the activity of immunologically active cells: macrophages are induced to produce increased amounts of TNF-α and IL-1 and to release elevated quantities of oxygen radicals, which exert a direct cytotoxic effect. A similar reaction is shown by show neutrophils, which exhibit increased phagocytosis under the influence of TNF. Furthermore, TNFs seem to stimulate the proliferation of B lymphocytes, and thus to exert an influence on the antigen-specific immune response. Natural killer cells are stimulated to synthesize TNFs. TNFs induce the production of acute-phase proteins in the liver, and the cells of the

endocrine system react with the synthesis of glucocorticoids and adrenocorticotropic hormone. Fibroblasts release IL-1, IL-6, collagenase and prostaglandin E_2. The influence of prostaglandin leads to a rise in body temperature that is further elevated by the subsequently increased expression of IL-1. Like IL-1, TNFs induce the release of chemokines in macrophages and endothelial cells, among other cells. The effect of TNFs on a variety of cells and the cytokine network explains their participation in such diverse processes as the defence against viral, bacterial and parasitic infections and the control of tumours.

8.1.4 Chemokines

The chemokine family (the name stands for chemotactic cytokines) encompasses a number of structurally very similar secretory proteins with a molecular mass of only 6–14 kDa. Over 50 different chemokines have been identified, many of which show a similar activity spectrum, and thus are redundant in their effects. On the basis of an amino acid motif at the amino terminus, chemokines are divided into four subgroups: CC, CXC, XC and CX3C chemokines. The number and arrangement of the conserved cysteine residues in their sequences are determining. CC chemokines possess two directly adjacent cysteine residues at their amino terminus; in CXC chemokines, the two cysteines are separated by one amino acid of another type; in CX3C chemokines the two cysteines are separated by three other arbitrary amino acids. In addition, the chemokines are numbered and designated with the letter "L", as they act as ligands for their respective receptors. The chemokines CCL5 (also still known under the former name RANTES for "*r*egulated upon *a*ctivation, *n*ormal *T*-cell-*e*xpressed and presumably *s*ecreted), CCL2 (also known as monocyte chemoattractant protein 1), CCL3 (also known as macrophage inflammatory protein 1α) and CCL4 (also known as macrophage inflammatory protein 1β) belong to the CC chemokines, whereas IL-8 (CXCL8) is a classic CXC chemokine. Fractalkine (CX3CL1) is only the known member of CX3C chemokines, whereas two isoforms of lymphotactin belong to the XC chemokines. Chemokines act by binding to specific receptors that are located on the surfaces of immunologically active cells. These are G-protein-coupled receptors of the rhodopsin family. The nomenclature of chemokine receptors follows that of chemokines that bind to them. CCR1 to CCR9 are receptors for CC chemokines, which are also serially numbered (Table 8.4).

Chemokines can frequently bind to several different receptors. A receptor can also recognize several chemokine ligands; therefore, the chemokine system is redundant and overlapping in its effect. In their active form, most chemokines are composed of homodimeric, homotetrameric or homooligomeric complexes; however, they can also interact to form heteromeric complexes composed of different chemokines, thus reciprocally modulating their activities. This enhances their redundant mode of action.

Table 8.4 Functions, properties and receptor binding of the most important inflammatory chemokines

Chemokine systematic/ alternative name	Producer	Receptors	Chemotactic effect
CCL1	Activated T cells	CCR8	Monocytes, dendritic cells, NK cells
CCL2/MCP-1	Activated monocytes, endothelial cells	CCR2, CCR5	Monocytes, neutrophils
CCL3/MIP-1α	Activated macrophages	CCR1, CCR5	Monocytes, granulocytes, T cells
CCL4/MIP-1β	Activated macrophages	CCR5	Monocytes, granulocytes, T cells
CCL5/RANTES	Activated T cells, platelets	CCR1, CCR3, CCR5	Granulocytes, T cells
CCL6/C10/MRP-1	Activated macrophages, neutrophils	CCR1	Macrophages
CCL7/MCP-3	Activated macrophages some tumour cell lines	CCR1, CCR2, CCR7	Monocytes, macrophages
CCL8/MCP-2	Activated macrophages, osteosarcoma cell line MG63	CCR2, CCR3, CCR5	Monocytes
CCL9/MIP-1γ	Activated macrophages, myeloid cells	CCR1	Dendritic cells
CCL11/eotaxin	Activated endothelial cells	CCR2, CCR3, CCR5	Eosinophils
CCL13/MCP-4	Activated macrophages	CCR1, CCR2, CCR3	Monocytes, T cells, eosinophils
CCL16/monotactin-1	Liver, spleen	CCR1, CCR2, CCR3, CCR5	Monocytes, T cells
CCL19/MIP-3β	Thymus, lymph nodes	CCR7	Dendritic cells, B cells
CCL20/MIP-3α	Lymphocytes, lymph nodes liver	CCR6	$CD4^{+}/CD8^{+}$ T cells
CXCL1/NAP-3	Activated macrophages, neutrophils	CXCR2	Neutrophils
CXCL2/MIP-2α	Activated monocytes, macrophages	CXCR2	Neutrophils, haematopoietic precursor cells
CXCL4/PF-4	Aggregating platelets	CXCR3	Neutrophils, monocytes
CXCL6/GCP-2	Activated monocytes	CXCR1	Neutrophils
CXCL8/IL-8	Activated monocytes, endothelial cells	CXCR1, CXCR2	Neutrophils
CXCL9/MIG	IFN-γ-stimulated monocytes and endothelial cells	CXCR3	Monocytes, T cells
CXCL10/γ-IP10	IFN-γ-stimulated monocytes and endothelial cells	CXCR3	Monocytes, T cells

(*continued*)

Table 8.4 (continued)

Chemokine systematic/alternative name	Producer	Receptors	Chemotactic effect
CXCL12/SDF-1	Bone marrow stromal cells	CXCR4	Monocytes, haematopoiesis
XCL1, XCL2/lymphotactin	Spleen, thymus, peripheral lymphocytes	XCR1	T cells
CX3CL1/fractalkin	Macrophages, endothelial cells	CX3CR1	T cells, monocytes

GCP granulocyte chemoattractant protein, *γ-IP* INF-γ-induced protein, *MCP* monocyte chemotractant protein, *MIG* monokine induced by INF-γ, *MIP* macrophage inflammatory protein, *MRP* macrophage-inflammatory-protein-related protein, *NAP* neutrophil-activating protein, *PF* platelet aggregation factor, *RANTES* regulated on activation, normal T-cell-expressed and presumably secreted, *SDF* stromal-cell-derived factor

Chemokines are produced by many tissue and immune cells. They trigger a directed migration in motile cells, which have receptors that are specifically bound by chemokines. The cells move along a concentration gradient to the site of highest chemokine concentration. In regard to their function, chemokines can be divided into two subgroups: Inflammatory chemokines are mediators of the innate immune response; they are produced by infected endothelial cells, and are released into the environment of the inflammatory site during infections and/or inflammations; however, they are synthesized particularly by activated immune cells, predominantly by mononuclear phagocytes (e.g. granulocytes; see ► Sect. 7.1). Their principal function is to attract further monocytes, macrophages and neutrophils from the blood into the inflamed tissue; some of them can additionally activate immune cells. CXC chemokines act primarily on neutrophils, whereas CC chemokines preferably stimulate monocytes and macrophages to migrate to the inflammation site (Table 8.4). In contrast to inflammatory chemokines, constitutive (homeostatic) chemokines are continuously produced in the lymphoid organs. They are involved in organ development, angiogenesis and the fine localization of immune cells in lymph nodes, the thymus and other lymphoid organs.

8.1.5 Other Cytokines

8.1.5.1 Colony-Stimulating Factors

Granulocytes are divided into three subgroups: neutrophils, eosinophils and basophils. In particular, neutrophils and eosinophils are involved in the rapid immune response against microorganisms and parasites. They migrate to the inflammation site attracted by chemokines, attach to endothelial cells by adhesion molecules and pass through the vessel walls. At the site of infection, they combat free or immunoglobulin-loaded pathogens by phagocytosis. They also drain the contents of their

granules into the surrounding area and release a plethora of degrading enzymes, cytotoxic proteins and oxygen radicals. In addition, stimulated granulocytes release IL-1, CXC8/IL-8 and prostaglandins. GM-CSF, granulocyte CSF (G-CSF) and macrophage CSF (M-CSF)/IL-3 are involved in the differentiation of haematopoietic progenitor cells to granulocytes and macrophages. They exert their functions on various cell types by binding to the respective receptors. In vitro, they stimulate colony formation of granulocytes and macrophages (GM-CSF) as well as granulocytes (G-CSF) and macrophages (M-CSF). Their mode of operation is similar to that of IL-3, which, as a versatile CSF induces colony formation of all CSF-dependent cell types. CSFs are single-chain glycoproteins (14–30 kDa) without common homology. They are released by macrophages, fibroblasts and endothelial cells, which are responsive to antigens and mediators of inflammation. Furthermore, GM-CSF is produced and released by T-helper cells. CSFs induce not only the differentiation of haematopoietic precursor cells into granulocytes and macrophages, but also strengthen the cytotoxic and the phagocytosing activities of them by an autocrine mechanism, i.e. by binding to the respective receptors of the same cells that have released the corresponding factors.

8.1.5.2 Transforming Growth Factors

Transforming growth factors (TGFs) were discovered because of their proliferation-enhancing effect on fibroblasts. They have been classified into two groups: TGF-α and TGF-β. TGF-α has a function similar to that of epidermal growth factor in the regulation of cell division. By contrast, TGF-β promotes not only tumour growth, but is also involved in inflammation and the regulation of the immune response. In contrast to TGF-α, the mode of action of TGF-β is relatively well understood. There are several TGF-β subtypes (TGF-β_1 to TGF-β_5), and they have both inhibiting and stimulatory immune functions. They are produced by different cells, such as T lymphocytes, mononuclear phagocytes, platelets and endothelial cells; they also improve wound healing and inhibit both activation of macrophages and proliferation of T lymphocytes. In addition, they suppress the activity of various cytokines. The immune stimulating effect mainly concerns the promotion of immunoglobulin class switching to generate IgA. TGF-β acts chemotactically on monocytes, and stimulates proliferation of fibroblasts and epithelial cells together with IL-1, IL-2 and IL-6 as well as growth factors such as platelet-derived growth factor, epidermal growth factor and fibroblast growth factor. Tumour growth frequently relies on these factors, which are released by tissue-migrating macrophages, monocytes and T cells, and promote the proliferation of cancer cells by a paracrine mechanism. In this case, the factors bind to their receptors on the surface of cells that are located in the surrounding region, whereby the producers themselves are not influenced. Many tumour cells are able to produce such factors. In this case, they act in an autocrine stimulatory fashion. An example of this is T-cell leukaemia induced by human T-lymphotropic virus 1 that is generated by the viral Tax protein, in which the synthesis of both IL-2 and IL-2 receptor is induced autocrinously (▶ Sect. 18.1).

8.2 How Do Viral Infections Affect the Synthesis of Cytokines?

Viral infections can influence cytokine production in single cells and organs or in the whole organism. The virus initially replicates at the site of entry to the body, and can spread from there via blood or lymph to the sites at which infection is manifested (▶ Chap. 4). Everywhere, it activates the immune system, thus inducing synthesis and release of cytokines, which are associated with local inflammations. Possibly, also defective viral forms may play an important role. In 1951, Preben von Magnus initially observed that during infections with influenza viruses defective particles arise which still have haemagglutination capacity, but are not infectious (▶ Sect. 16.3). In addition, functionally active, but also defective, double-stranded RNA molecules are synthesized in infected cells. In cooperation with the defective virus, they increase the production of type I interferons. They interfere with translation and induce the synthesis of further defective particles. Abortive replication cycles without release of progeny viruses occur infrequently. Since in this way more defective particles and double-stranded RNA molecules arise, the synthesis of interferon is further increased. As a consequence, interferon-resistant variants can be selected, and these increasingly spread and contribute to establish persistent chronic infections.

Many viruses possess genes encoding proteins homologous to cytokines or cytokine receptors. They enable the pathogens to evade both the antiviral and the immune response that is stimulated by the effect of cytokines. For example, Epstein–Barr virus possesses a gene encoding a protein with homology to IL-10 (BCRF1), which influences the effect of the natural anti-inflammatory IL-10. On the other hand, the herpes simplex virus encodes a protein which acts as an IL-8 receptor (▶ Sect. 19.5). Poxviruses express receptors for several cytokines, such as IL-1β, TNF-α and IFN-γ, which intercept these inflammation-stimulating molecules (▶ Sect. 19.6). Adenoviruses and Epstein–Barr viruses produce large quantities of small, non-coding RNA molecules during the replication cycle, and these have a pronounced secondary structure. These adenovirus-encoded virus-associated RNA molecules and Epstein–Barr-virus-encoded RNA molecules prevent the activation of protein kinase R. Thus, this enzyme, which is induced by interferon, cannot exert its function as an inhibitor of protein synthesis (▶ Sects. 19.4 and ▶ 19.5). How different viruses elude the effects of cytokines will be explained in detail in the description of the pathogenesis of the individual viruses.

8.3 Can Cytokines Be Used as Therapy for Viral Diseases?

Interferons were used for antiviral and antitumour therapeutic purposes soon after their discovery. However, the initial expectation that interferon would be a general agent against cancer was not fulfilled. Today, IFN-α is used only for the treatment of hairy cell leukaemia, which can be induced, for example, by human T-lymphotropic

virus 2. However, several cytokines are used as immunostimulatory agents in combination with chemotherapy and radiotherapy in cancer treatments. Pegylated IFN-α – IFN-α bound to poly(ethylene glycol) – is approved as an antiviral agent in combination with antiviral chemotherapeutic agents for the treatment of chronic hepatitis B and hepatitis C infections (▶ Sects. 14.5.5 and ▶ 19.1.5). In combination with acyclovir, it is also used for therapy of keratitis caused by herpes simplex virus. Frequent side effects are fever, flu-like symptoms and severe depression. Imidazoquinolines (imiquimod) activate TLR7, resulting in increased IFN-α production (▶ Chap. 9). They are used in creams to treat skin warts that are induced by human papillomaviruses (▶ Sect. 19.3). In veterinary medicine, IFN-α is used in dogs and cats for the treatment of canine parvovirus infections, the cat cold and infections with feline leukaemia virus (▶ Sects. 14.8, ▶ 18.1 and ▶ 20.1).

Various interleukins and CSFs are currently being tested for treatment of viral diseases. For quite some time, G-CSF has been applied in cancer patients that were treated with chemotherapy or radiotherapy; it stimulates neutrophils and accelerates their migration from bone marrow into the peripheral blood.

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Retrospectively, the development of antiviral agents can be divided into three phases. Drugs that were originally employed as antitumour agents were examined in the early stages for inhibition of viral replication; these included compounds such as iodine deoxyuridine and cytosine arabinoside. The former was introduced in 1962 by Herbert E. Kaufman as one of the first antiviral drugs for the local treatment of keratoconjunctivitis, which is induced by herpes simplex virus. With increased knowledge of the molecular biology of the cell and that of viruses, the predominantly empirical strategies were superseded by approaches designed to improve poorly selective agents by chemical modifications. The results of that "targeted empiricism" were drugs such as adenosine arabinoside, bromovinyl deoxyuridine and acycloguanosine. Adenosine arabinoside was the first chemotherapeutic drug that showed a healing effect on herpes simplex virus induced encephalitis and herpes infection of neonates (herpes neonatorum) by systemic, i.e. intravenous, administration. The drug acycloguanosine, which was subsequently developed by Gertrude Elion in 1977, was a breakthrough in the treatment of herpesvirus infections (▶ Sect. 19.5). The currently established methods for sequencing viral genetic information, structural analysis of viral enzymes and recent new insights in molecular genetics allow the specific investigation of the targets of antiviral agents, including the development of so-called designer drugs.

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The acquired knowledge and the new insights into the pathogenesis of viral infections constitute the basis for the development of targeted, optimally configured inhibitory antimetabolites, and thus for the design of virostatic drugs. Although a series of new antiviral compounds has been developed in recent years, not least under the pressure to find ways for AIDS therapy, there are only a few effective chemotherapeutic agents today (Table 9.1), in comparison with the large number of antibiotics available to treat bacterial infectious diseases. The production of new antiviral drugs is certainly limited by the strictly intracellular replication cycle of the virus, which complicates the selection of specific targets without generating cell damage. Besides the direct inhibition of the viral replication cycle, attempts have also been undertaken to influence therapeutically the inflammation processes triggered by viral infections, by combining chemotherapeutic drugs with cytokines, as it became clear that the pathogenesis of most viral diseases is linked to viral and immunological processes (▶ Chap. 8).

All this applies only to antiviral therapy in humans. The chemotherapeutic treatment of viral infections plays no major role in veterinary medicine. Infectious diseases of livestock which are notifiable should not be treated with antiviral agents or other drugs. In this case, culling and harmless elimination of sick animals are prescribed by law. Moreover, in animals bred for purposes of food production, the question of residues is of great importance. The effectiveness of chemotherapeutic drugs developed for humans is frequently not proven for treatment of small animals. The substances often have a different toxicity spectrum in animals, or the price of the drugs renders the cost of treatment too expensive owing to the usually long application time.

9.1 What Are the Molecular Targets of Antiviral Drugs?

As obligate cellular parasites, viruses can replicate only in living cells and use many functional activities of their hosts. Therefore, antiviral drugs must target specific viral functions as selectively as possible, and should not influence cellular processes. The chemotherapeutic index, i.e. the ratio between the concentration of a substance that is necessary to inhibit viral replication and the concentration above which a cytotoxic effect can be observed, should be as high as possible. Most of the currently available chemotherapeutic drugs inhibit replication of the viral genome. If viruses use their own polymerases for replication, these generally differ from the cellular enzymes; this makes targeted and selective inhibition possible. Viral proteases, which cleave precursor proteins and/or play an important role in generating infectious virions, e.g. in hepatitis C virus and retroviruses, respectively, are also appropriate targets for virostatic drugs. The inhibition of virus-specific processes such as attachment, uncoating, self-assembly and release of the virus from the cell, is possible as well. In addition, other viral enzymes involved in nucleic acid metabolism such as ribonucleotide reductase are also theoretical targets for the

Table 9.1 The most important currently approved antiviral chemotherapeutic agents and their mode of action

Inhibitor	Field of application	Mode of action
Inhibitors of viral polymerases		
Acycloguanosine (acyclovir)	Herpes simplex virus, varicella-zoster virus	Guanosine analogue. It is monophosphorylated by the viral thymidine kinase. Cellular kinases transform it further into the triphosphate. The viral DNA polymerase recognizes the triphosphate as a better substrate and incorporates it in the newly synthesized viral genomes. The consequence is termination of the polymerization process
Adefovir	Hepatitis B viruses	Monophosphorylated adenosine analogue. Cellular kinases phosphorylate it to the triphosphate. The triphosphorylated product is recognized as a substrate by the viral reverse transcriptase, which incorporates it into the newly synthesized double-stranded DNA. The consequence is termination of the polymerization process
Azidothymidine (zidovudine)	Human immunodeficiency viruses	Thymidine analogue. Cellular kinases phosphorylate it to the triphosphate. The triphosphorylated product is recognized as a substrate by the viral reverse transcriptase, which incorporates it into the newly synthesized double-stranded DNA. The consequence is termination of the polymerization process. The integration of the provirus into the cellular DNA does not occur
Brivudine (bromovinyldeoxyuridine)	Herpes simplex virus type 1, varicella-zoster virus	Uracil analogue. It is monophosphorylated by the viral thymidine kinase. Cellular kinases phosphorylate it further to the triphosphate. The viral DNA polymerase accepts the triphosphorylated analogue as a substrate and incorporates it in the newly synthesized viral genomes. This results in termination of the polymerization process

(*continued*)

Table 9.1 (continued)

Inhibitor	Field of application	Mode of action
Cidofovir	Cytomegalovirus	Monophosphorylated cytosine analogue. Cellular kinases phosphorylate it to the triphosphate. The viral DNA polymerase accepts the triphosphorylated analogue as a substrate and incorporates it in the newly synthesized viral genomes. This results in termination of the polymerization reaction
Dideoxy-3′-thiacytidine (3TC, lamivudine)	Human immunodeficiency viruses, hepatitis B viruses	Cytosine analogue. It is phosphorylated by cellular kinases to the triphosphate. The triphosphorylated product is recognized as a substrate by the viral reverse transcriptase, which incorporates it in the newly synthesized double-stranded DNA. This results in termination of the polymerization reaction. The integration of the provirus into the cellular DNA does not occur. It also inhibits the pyrophosphorolytic activity of the viral reverse transcriptase
Dideoxycytidine (ddC, zalcitabine)	Human immunodeficiency viruses	Cytosine analogue. It is triphosphorylated by cellular kinases. The triphosphorylated product is accepted as a substrate by the viral reverse transcriptase, which incorporates it in the newly synthesized double-stranded DNA. This results in termination of the polymerization reaction. The integration of the provirus into the cellular DNA does not occur
Dideoxyinosine (ddI, didanosine)	Human immunodeficiency viruses	Guanosine analogue. It is triphosphorylated by cellular kinases. The triphosphorylated product is accepted as a substrate by the viral reverse transcriptase, which incorporates it in the newly synthesized double-stranded DNA. This results in termination of the polymerization reaction. The integration of the provirus into the cellular DNA does not occur

(*continued*)

Table 9.1 (continued)

Inhibitor	Field of application	Mode of action
Famciclovir	Herpes simplex virus, varicella-zoster virus	Guanosine analogue. It is a prodrug with improved bioavailability. After intake, deacetylation to penciclovir occurs. This is monophosphorylated by the viral thymidine kinase. Triphosphorylation occurs by the action of cellular kinases. The viral DNA polymerase recognizes the triphosphate as a better substrate, incorporating it in the newly synthesized viral genomes. This leads to termination of the polymerization reaction
Foscarnet (phosphonoformic acid)	Herpesviruses, human immunodeficiency viruses	Pyrophosphate analogue. It binds to the pyrophosphate binding domain of the reverse transcriptase as well as the viral DNA polymerase and competes with the natural substrate
Ganciclovir	Cytomegalovirus	Guanosine analogue. It is converted to the triphosphate form by viral and cellular kinases. The viral DNA polymerase recognizes the triphosphate as a substrate and incorporates it in the newly synthesized viral genome. This results in termination of the polymerization reaction
Nevirapine, delavirdine, loviride, efavirenz	Human immunodeficiency viruses	They bind to the large subunit of the reverse transcriptase and inhibit its activity
Stavudine (d4T)	human immunodeficiency viruses	Thymidine analogue. It is converted to the triphosphate form by cellular kinases. The triphosphorylated product is recognized as a substrate by the viral reverse transcriptase, which incorporates it in the newly synthesized double-stranded DNA. This results in termination of the polymerization reaction. The integration of the provirus into the cellular DNA does not occur
Inhibitors of viral proteases		
Saquinavir, nelvinavir, ritonavir, fortovase	Human immunodeficiency viruses	Peptide mimetics. They are peptide-like inhibitors that inhibit the activity of the viral protease, preventing the cleavage of the precursor proteins. The formation of the infectivity of the virus particles does not occur

(*continued*)

Table 9.1 (continued)

Inhibitor	Field of application	Mode of action
Boceprevir, telaprevir	Hepatitis C virus	Peptide mimetics. They inhibit the viral NS3 protease and are used in combination with interferon-α and/or ribavirin
Inhibitors of viral neuraminidases		
Zanamivir, oseltamivir peramivir	Influenza A virus, influenza B virus	They inhibit the function of viral neuramidase by binding to the active centre
Inhibitors of virus uptake/uncoating		
Amantadine, rimantadine	Influenza A virus	They block the proton pump function of M2 protein. Consequently, they block the uncoating process
Pleconaril	Rhinoviruses, enteroviruses	Analogue of the pocket factor. It stabilizes viral capsids. It prevents the uncoating of the viral genomes
Enfuvirtide	Human immunodeficiency viruses	Peptide-like inhibitor. It prevents the fusion of the virus and the cytoplasmic membrane, and consequently the attachment and uptake of the virus particle
Miscellaneous antiviral chemotherapeutics		
Imiquimod	Human papillomaviruses, molluscum contagiosum	Immune response stimulating agent. It activates Toll-like receptor 7 und induces the production of inferferon-α
Ribavirin (taribavirin)	Lassa virus (systemic), hepatitis C virus (systemic), respiratory syncytial virus (as aerosol)	It inhibits the cellular guanylyltransferase. This prevents the addition of the 5′-cap group to messenger RNA molecules, thus interfering with the translation process. In its triphosphorylated form, it is incorporated in newly synthesized RNA strands by the RNA-dependent RNA polymerase, causing mutations

development of specific antiviral drugs. However, solely the active viral replication can be blocked in this way. Genomes of latent viruses that exist episomally or are integrated in the host DNA would remain unaffected.

Apart from the molecular mechanism of action, the pharmacokinetic properties of a drug, its absorption, bioavailability, half-life, excretion and not least its side effects on the organism must also be known prior to application in humans. To achieve sufficient inhibition, the blood concentration of an antiviral chemotherapeutic drug should be ten to 50 times higher than the concentration that is needed to inhibit 50 % of infectious pathogens in vitro.

9.1.1 Inhibitors of Viral Replication

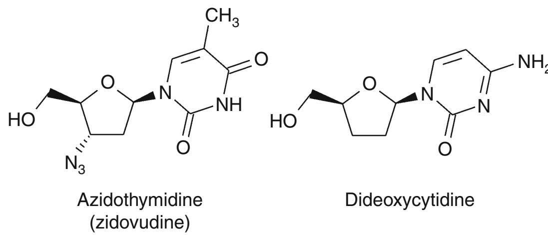
Inhibitors of viral replication can be subdivided into two groups: nucleoside analogues and non-nucleoside inhibitors (Fig. 9.1a–c). Nucleoside analogues compete with natural nucleotides, bind to the active centre of polymerases and inhibit the function of the enzyme. In addition to this form of competitive inhibition, a nucleoside analogue can also be used as a substrate by the enzyme, i.e. it can be introduced into a growing viral nucleic acid chain. If it is incorporated, it disturbs the habitual structure of DNA or RNA, which thereafter cannot be replicated and transcribed correctly, or it can lead to chain termination during replication.

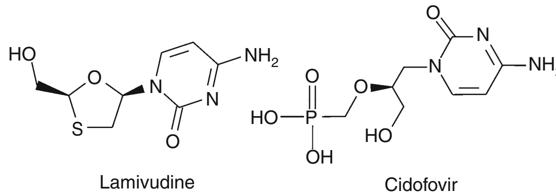
9.1.1.1 Nucleoside Analogues

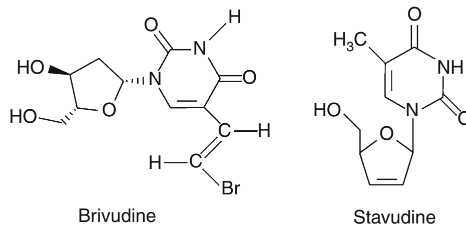
Azidothymidine – also known as 3-azido-3′-deoxythymidine and AZT – is a thymidine analogue, and is also known as zidovudin. It has an azide group instead of a hydroxyl residue at the 3′ position on the deoxyribose. The cellular enzymes thymidine kinase and thymidylate kinase convert it to the triphosphate, which is preferably used as a substrate by the reverse transcriptase of retroviruses. It causes a chain termination during transcription of the viral single-stranded RNA genome in double-stranded DNA, since no 3′-OH group is available to form the phosphodiester bond (Fig. 9.2a and ▶ Sect. 18.1). In this way, the retroviral infection cycle is stopped at a very early stage, namely before the integration of the viral genome into the DNA of the host cells. The affinity of triphosphorylated azidothymidine for reverse transcriptase is 100 times higher than that for cellular DNA polymerases α and β. Azidothymidine has been approved for treatment of infections with human immunodeficiency virus (HIV) since 1987. When azidothymidine is administered alone, it can only delay the development of AIDS symptoms, and does not cause the elimination of the virus, or even curing of the patient. Azidothymidine is usually administered in highly active antiretroviral therapy in combination with other nucleoside analogues and non-nucleoside inhibitors of reverse transcriptase as well as protease inhibitors (Sects. 9.2 and ▶ 18.1.5). In oral administration, it is well absorbed and its bioavailability is more than 60 %. Side effects are gastrointestinal problems, severe headaches and especially haematopoietic disorders (anaemia or leucopenia), which can be attributed to the cytostatic effect of azidothymidine.

Stavudine – also known as D4T and 1-[(2*R*,5*S*)-5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]-5-methyl-1,2,3,4-tetrahydropyrimidine-2,4-dione) – and dideoxycytidine – also known as ddC and zalcitabine – act like azidothymidine, but the latter is a cytosine analogue. The agents lack the 3′-OH group that is necessary for the formation of the phosphodiester bond, thus also leading to chain termination during reverse transcription of retroviral genomes. Dideoxycytidine is also approved for the treatment of AIDS and functions ten times better than azidothymidine in vitro. Its bioavailability is very good and reaches 90 %. Lamivudine – also known as 3TC and 2′-deoxy-3′-thiacytidine – is also a cytosine analogue that is mainly used in the combination therapy for HIV infections. In lamivudine, the carbon atom at the 3′ position of the deoxyribose is

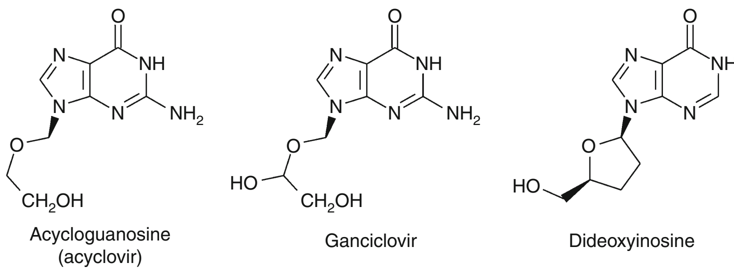
a **Pyrimidine analogues**







b **Purine analogues**



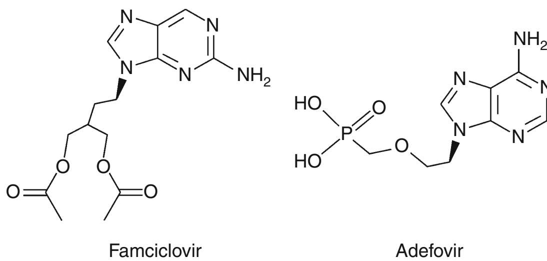
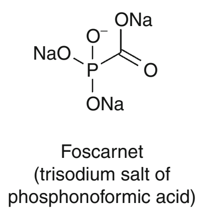
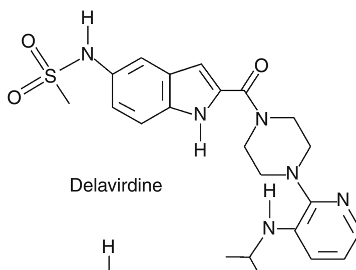


Fig. 9.1 (continued)

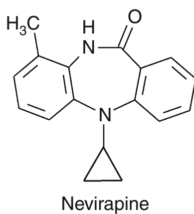
c Non-nucleoside polymerase inhibitors



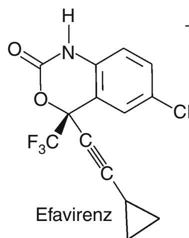
Foscarnet
(trisodium salt of
phosphonoformic acid)



Delavirdine

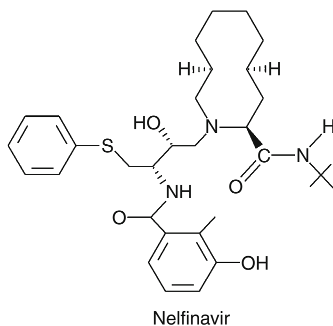


Nevirapine

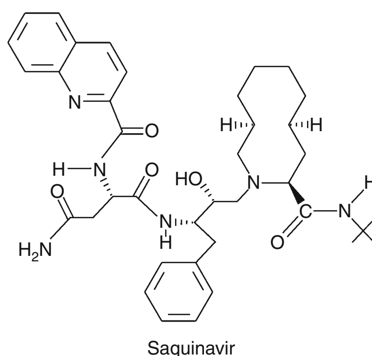


Efavirenz

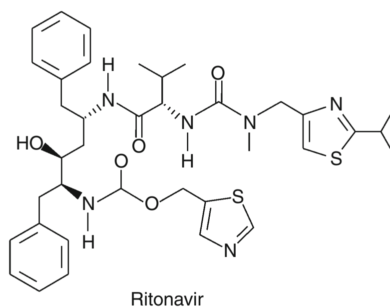
d Inhibitors of viral proteases



Nelfinavir



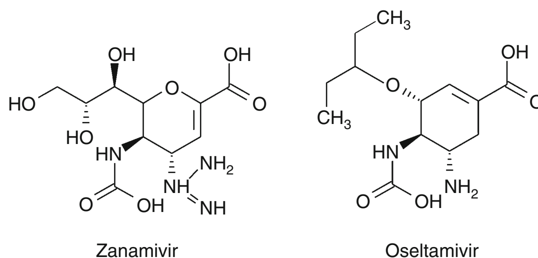
Saquinavir



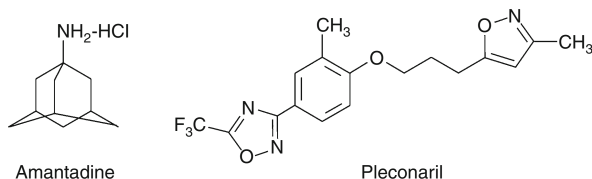
Ritonavir

Fig. 9.1 (continued)

e Inhibitors of viral neuraminidases



f Inhibitors of virus entry / uncoating



g Inhibitors of other processes

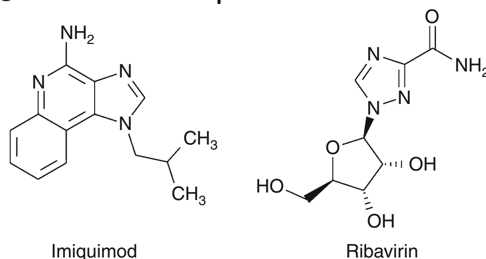


Fig. 9.1 Chemical formulas of the most important virostatic drugs

replaced by a sulphur atom, and its incorporation into a growing nucleic acid chain causes termination of the polymerization reaction. Another therapeutic agent for HIV infections is dideoxyinosine – also known as ddI and didanosine – which has a mode of action similar to that of zalcitabine. In the cell, it is phosphorylated and aminated, and it functions as an adenosine analogue. The oral bioavailability is lower than that of dideoxycytidine or azidothymidine. The advantage of dideoxyinosine resides in a low cytostatic effect on bone marrow cells. However, the occurrence of peripheral neuropathies as side effects is more frequent.

Lamivudine is used not only in treatments of HIV infections: in chronic hepatitis B infections, it also leads to a reduction of the concentration of the virus in the organism. In this case, it is generally used in combination with pegylated interferon-α (▶ Sects. 8.1 and ▶ 19.1). In addition to lamivudine, adefovir is also available for the treatment of chronic hepatitis B; it is a monophosphorylated

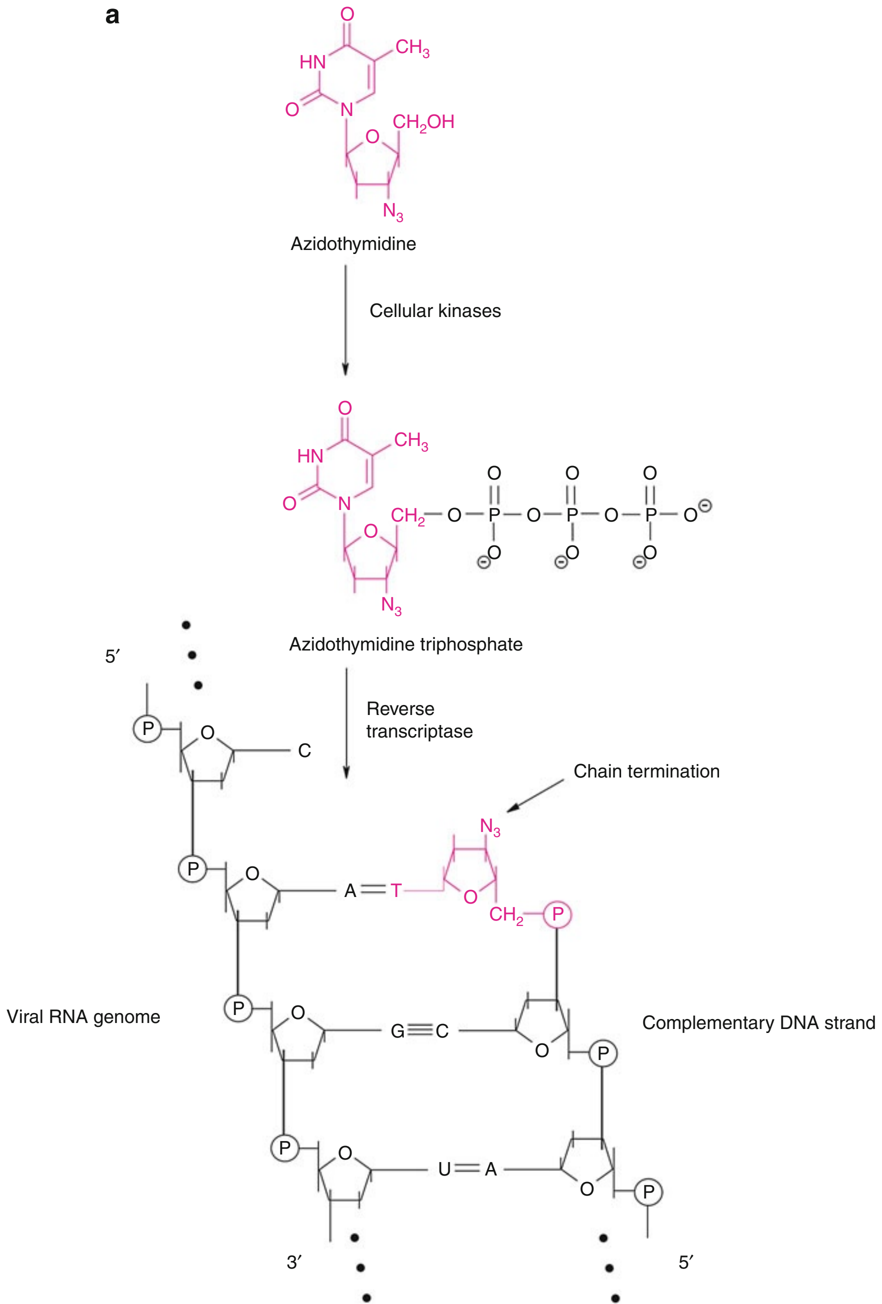


Fig. 9.2 (continued)

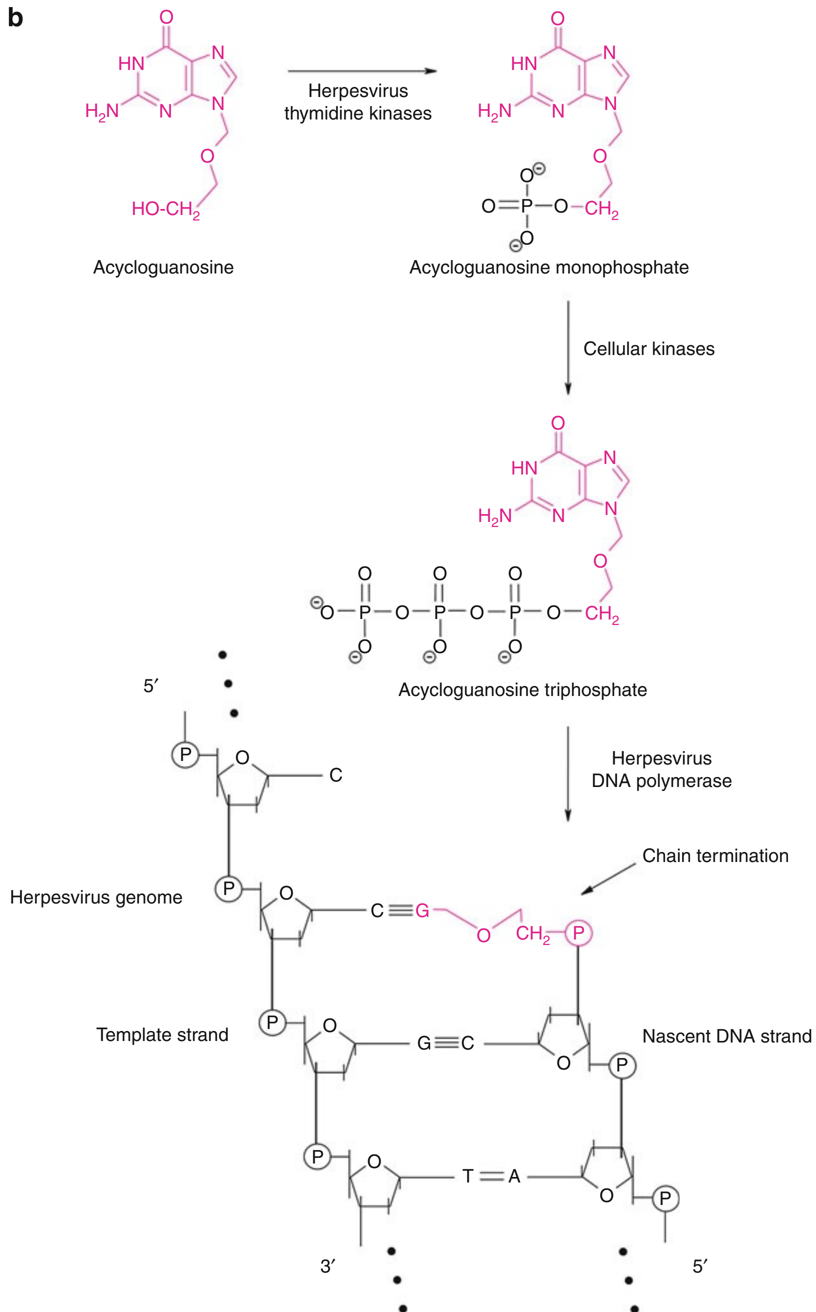


Fig. 9.2 Molecular mechanisms of antiviral chemotherapeutic agents. (**a**) Inhibition of the retrovirus reverse transcriptase by azidothymidine. Azidothymidine (*red*) is converted into the triphosphate by cellular kinases. The triphosphorylated product serves as a substrate for the

adenosine analogue – ((2-(6-amino-9*H*-purin-9-yl)ethoxy)methyl)phosphonic acid – which is characterized by an incomplete sugar moiety. It is used as a substrate by the DNA polymerase of these viruses, which also has reverse transcriptase activity; it also leads to termination of the polymerization reaction.

Acycloguanosine – also known as acyclovir and (9-(2-hydroxyethoxy) methylguanine) – is a guanosine derivative containing an acyclic sugar residue. It can be applied orally or systemically. The thymidine kinases of herpes simplex virus or varicella-zoster virus use it preferentially as a substrate and monophosphorylate it. Cellular enzymes convert it into the triphosphate. In this form, it is selectively recognized by the herpesvirus DNA polymerase, and thus is incorporated only into newly synthesized viral genomes, but not into the cellular DNA, causing termination of polymerization (Fig. 9.2b). Because of the specific activation mechanism, it is effective only in cells that are infected with herpes simplex virus or varicella-zoster virus (▶ Sect. 19.5), and thus is administered in diseases associated with these two viruses. Because of the highly selective mechanism of action, this drug is not very toxic. Acycloguanosine has been modified in various ways to increase its bioavailability (e.g. as a prodrug) by changing groups or attaching other groups. In this way, new antiviral agents have been developed, such as valylacyclovir, penciclovir, famciclovir and ganciclovir. Famciclovir is a diacetylated guanosine analogue. After its ingestion, it is converted into penciclovir in the cells. It is incorporated into the viral DNA strands by the DNA polymerase of herpes simplex virus and varicella-zoster virus, resulting in chain termination. Ganciclovir – 9-(1,3-dihydroxy-2-propoxy)methylguanine) – probably acts by the same molecular mechanism as acycloguanosine. However, it is applied in infections with cytomegaloviruses. These viruses have no thymidine kinase; the drug is phosphorylated by a viral protein kinase, presumably by the activity of the UL97 gene product. As a triphosphate, it can be used by the DNA polymerase of cytomegaloviruses during genome replication. Owing to the limited viral selectivity, ganciclovir exhibits significantly more side effects (e.g. bone marrow toxicity). Cidofovir, a monophosphorylated cytosine analogue – 1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine – is also used for treatment of cytomegalovirus infections, especially when the virus is resistant to ganciclovir. Furthermore, this drug, which has a very long half-life in the body, shows significant adverse effects, including renal toxicity. Foscarnet is still in use as an emergency drug in cytomegalovirus infections; however, it also exhibits several unfavourable effects.

Fig. 9.2 (continued) reverse transcriptase. If it is incorporated into the growing DNA strands during transcription of RNA into DNA, it causes termination of chain elongation. (**b**) Inhibition of the DNA polymerase of herpesviruses by acycloguanosine. Acycloguanosine (*red*) is monophosphorylated by the thymidine kinase of herpesviruses, and is converted into the triphosphate by cellular kinases. The triphosphorylated product is recognized by the DNA polymerase of herpesviruses as a substrate. During replication of the viral genome, it is incorporated into the newly synthesized DNA strands. This leads to chain termination

9.1.1.2 Non-Nucleoside Inhibitors

Foscarnet (trisodium salt of phosphonoformic acid; Fig. 9.1c) is systemically administered for treatment of chorioretinitis caused by cytomegaloviruses. It acts as an analogue of pyrophosphates and attaches itself not to the active centre of the viral DNA polymerase, but to the pyrophosphate binding site, which is located in the immediate vicinity. In this way, it does not compete with natural nucleotides. It is currently the only non-nucleoside inhibitor of the DNA polymerase of herpesviruses, and it also inhibits the reverse transcriptase of retroviruses such as HIV. Nevirapine, a dipyridodiazepinon, also inhibits reverse transcriptase; it binds to the larger subunit of the heterodimeric enzyme (▶ Sect. 18.1).

9.1.2 Inhibitors of Viral Penetration and Uncoating

Amantadine (1-aminoadamantane hydrochloride), which was first produced in 1964, and its derivative rimantadine (α-methyl-1-adamantanmethylamine; Fig. 9.1f) are polycyclic, aliphatic ring systems. They are inhibitors of influenza A virus. After penetration of virus particles via endocytosis, these drugs inhibit the function of the viral M2 protein, which is inserted in the envelope, and causes the acidification of the interior of the vesicles by acting as a proton channel (▶ Sect. 16.3). This prevents the fusion of the endosomal membrane with the viral envelope and the release of the genomes, so the uncoating process does not occur. Both drugs can be administered orally. Amantadine and rimantadine (not licensed in several countries, e.g. Germany) are mainly used in older or immunocompromised patients for prophylaxis and treatment of the classic flu, but only if it is induced by influenza A viruses. However, the virus develops resistance very fast. Both drugs are also used as in the treatment of Parkinson’s disease. This explains the considerable side effects in the psychiatric-neurological ambit.

Pleconaril – (3-[3,5-dimethyl-4-[3-(3-methyl-1,2-oxazol-5-yl)propoxy]phenyl]-5-(trifluoromethyl)-1,2,4-oxadiazole) – is an analogue of the pocket factor, a sphingosine-like molecule which is fitted in a hole-like cavity that is formed by the assembly of the structural proteins of picornaviruses in the capsids during morphogenesis (▶ Sect. 14.1). Pleconaril substitutes for the pocket factor, stabilizes the interaction of capsid proteins with each other and thus blocks the release of the viral nucleic acid after infection of cells. As a result, the viral infection cycle is blocked. Pleconaril could be administered as a virostatic drug for the treatment of infections with rhinoviruses (i.e. common cold) and enteroviruses (i.e. meningitis), and would significantly reduce the duration of the respective illnesses. However, no approval has been obtained for these indications until now.

Enfuvirtide is a peptidomimetic compound, which contains 36 amino acids and is identical to the amino-terminal sequence of the transmembrane protein gp41 of HIV-1. Enfuvirtide prevents the fusion of the viral envelope with the cytoplasmic membrane of the host cell, and thus hinders infection of the cell. Infection occurs

when the surface protein gp120 binds to the CD4 receptor and to a chemokine receptor. This interaction gives rise to conformational changes in the protein, whereby the amino terminus of gp41 comes into contact with the cytoplasmic membrane and mediates the fusion. Enfuvirtide prevents the conformational change by interacting with the homologue sequence of gp41, thus blocking the infection.

9.1.3 Miscellaneous Antiviral Chemotherapeutic Agents

Zanamivir – 2,3-didehydro-2,4-dideoxy-4-guanidino-*N*-acetyl-D-neuraminic acid (Fig. 9.1d) – and oseltamivir – (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-pentan-3-yloxycyclohexene-1-carboxylic acid (Fig. 9.1d) – are inhibitors of the influenza virus neuraminidase; they are applied as an aerosol spray or orally. Peramivir – (1*S*,2*S*,3*S*,4*R*)-3-[(1*S*)-1-acetamido-2-ethylbutyl]-4-(diaminomethylideneamino)-2-hydroxycyclopentane-1-carboxylic acid – acts as a transition-state analogue inhibitor of the influenza virus neuraminidase and is licensed for emergency use in pandemics in Japan and South Korea. Neuraminidase is a viral enzyme anchored in the envelope of influenza viruses. It is produced in infected cells, and is responsible for the degradation of terminal *N*-acetylneuraminic acid (sialic acid) residues on viral and cellsurface proteins, as these are used by viruses as receptors during attachment. This is mediated by haemagglutinin, another viral envelope protein. By destroying the receptors, neuraminidase prevents both the reciprocal aggregation of newly formed influenza viruses and the interaction with sialic acid residues in cellular-membrane-associated and cell-surface-associated proteins. Therefore, the virus can effectively spread in the organism. If neuraminidase is inhibited as early as possible during infection, it prevents dissemination of the virus (▶ Sect. 16.3). Therefore, these drugs diminish both the symptoms and the duration of the disease.

Ribavirin (1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Fig. 9.1b) is structurally related to guanosine. It is phosphorylated by cellular kinases to monophosphates, diphosphates and triphosphates and has a broad inhibitory spectrum against different viral infections. Ribavirin monophosphate inhibits the cellular inosine monophosphate dehydrogenase, and thus causes a decrease of intracellular GTP concentration. The triphosphate, which is particularly responsible for the virostatic effect, inhibits guanylyltransferase, which synthesizes the cap group at the 5′ termini of messenger RNA (mRNA) molecules. Uncapped mRNA molecules are not able to bind to the cap-binding complex and are not translated. Ribavivrin does not act selectively; it prevents capping of cellular and viral transcripts. Therefore, massive damage of non-infected cells also occurs during treatment. Recently, it has been found that the triphosphorylated form of ribavirin is used as a substrate and incorporated into newly synthesized viral genomes by the RNA-dependent RNA polymerases of RNA viruses. This partially causes lethal mutation in the viruses. Ribavirin is used in severe cases

of Lassa fever or respiratory syncytial virus infections in immunocompromised patients (▶ Sects. 15.3 and ▶ 16.1). In addition, it is applied in combination with interferon-α and interferon-β for the treatment of chronic hepatitis C infections (▶ Sect. 14.5). Mainly to avoid the destructive effect of ribavirin on red blood cells, taribavirin – 1-[(2*R*,3*R*,4*S*,5*S*)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1,2,4-triazole-3-carboximidamide – a prodrug of ribavirin, is currently being studied in clinical trials. Taribavirin is converted into ribavirin the liver; thereby the drug displays an elevated tropism for this organ. Therefore, it may be used preferentially for treatment of viral infections causing hepatitis, e.g. hepatitis B, hepatitis C and yellow fever.

Protease inhibitors were initially developed and used to inhibit the protease encoded by HIV and are mainly used in combination with reverse transcriptase inhibitors for treatment of AIDS. In the still immature released viruses the retroviral enzyme cleaves the Gag and Gag/Pol precursor proteins and is essential for the development of infectivity of the virions (▶ Sect. 18.1). The inhibitors are principally derived from peptides that mimic the protease cleavage sites of the precursor proteins (peptidomimetics). They are marketed, for example, under the generic names saquinavir, indinavir, nefilnavir; fosamprenavir and ritonavir. In 2011, further protease inhibitors (boceprevir, telaprevir) were licensed for treatment of hepatitis C virus infections in combination with pegylated interferon-α and ribavirin. These agents inhibit the viral NS3 protease that cleaves the viral polyprotein precursor.

Imiquimod – 1(-2)-methylpropyl-1*H*-imidazole-[4,5*c*]-quinoline-4-amine (Fig. 9.1g) – is administered locally for the treatment of warts and condylomata, which are caused by papillomaviruses (▶ Sect. 19.3). It has also a similar effect in the treatment of molluscum contagiosum, a skin disease that is triggered by molluscipoxviruses (▶ Sect. 19.6). At the application site, imiquimod induces expression of interferons and cytokines by activating Toll-like receptor 7. This causes an inflammatory reaction, which relieves the skin disease.

9.2 How Can Viruses Become Resistant to Antiviral Inhibitors?

Antiviral drugs exert a strong selection pressure on the pathogens; therefore, virus variants are increasingly selected that are resistant to the different compounds. This applies especially to viral species with an RNA genome (quasispecies). Polymerases such as RNA-dependent RNA polymerase, reverse transcriptase, but also the cellular RNA polymerases, which are involved in genome replication of different viruses (▶ Chap. 3), cannot check whether the newly synthesized sequences accurately match the original template, as they have no proofreading activity. Thus, mutations occur with a statistical probability of 10^{-4} during viral genome replication. That means every time a viral genome with a length of 10,000 nucleotides is replicated, it will statistically contain at least one erroneous incorporated nucleotide. The use of chemotherapeutic agents leads to selection of

virus variants that are able to replicate despite the presence of inhibitors. Such resistant viruses have been found especially in AIDS patients who were treated with reverse transcriptase inhibitors (▶ Sect. 18.1). In addition, influenza viruses also generate resistant virus mutants a short time after patients have been previously treated with amantadine or with neuraminidase inhibitors for treatment of H1N1 or H5N1 infections (▶ Sect. 16.3). The underlying mutations primarily affect just those regions of the enzyme which interact with the inhibitors. They alter the protein in such a way that the inhibitors are no longer accepted as substrates or binding partners. Inasmuch as the interaction sites of the various inhibitors of reverse transcriptase, such as azidothymidine, ideoxycytidine, dideoxyinosine and lamivudine, differ from each other, a change of drug is performed after the first occurrence of azidothymidine-resistant HIV variants in patients. Today, combinations of at least three or four inhibitors with differing molecular targets are used in highly active antiretroviral therapy at the beginning of therapy. In this way, it should be impossible for the virus to mutate all protein regions that are necessary to develop a possible multiple resistance. In doing so, it is expected that the enzyme function and hence survival and virulence of the virus will be severely impaired. Therefore, it is now recommended to employ even at the start of therapy, for example, a combination of the inhibitors azidothymidine, dideoxycytidine and saquinavir. If resistant virus mutants are found after a while, dideoxycytidine is usually replaced by dideoxyinosine or one of the other nucleoside analogues. In influenza A virus as a pathogen with an RNA genome, resistances against amantadine and rimantadine were observed very soon (▶ Sect. 16.3). Therefore, and also because of the much severer adverse side effects, the neuraminidase inhibitors are now clearly preferred, particularly since these are also effective against influenza B viruses and the highly pathogenic influenza A virus strains such as H5N1. Since resistant virus mutants have also been found during therapies with neuraminidase inhibitors in H5N1 infections, a combination of amantadine with neuraminidase inhibitors is currently being discussed in cases of bird flu in humans.

Nevertheless, even herpesviruses, whose DNA polymerase has a proofreading activity, develop mutants that are resistant to the inhibitor after treatment with acycloguanosine. The mutations affect principally thymidine kinase, which phosphorylates the inhibitor to monophosphate. Other mutations are localized in the polymerase gene after treatment of cytomegalovirus with ganciclovir. To what extent the mutant viruses are viable in vivo has not been finally resolved. In animal experiments, they have proven to be avirulent; however, they could induce latent infection forms (▶ Sect. 19.5). Mutations in the DNA polymerase occur less frequently, probably because they impair the enzyme function. Under consideration is also the simultaneous administration of antiviral chemotherapeutic drugs with various cytokines such as interferon-α, tumour necrosis factors or colony-stimulating factors. The latter are intended to compensate primarily for the cytostatic effect of inhibitors on the peripheral blood cells and should facilitate their rapid regeneration (▶ Chap. 8).

9.3 What Therapeutic Expectations Do We Have for Ribozymes, Antisense RNA and RNA Interference/Small Interfering RNA?

Currently, attempts are being made to develop new antiviral inhibitors by using different experimental approaches. In addition to placing emphasis on developing compounds that block the attachment of viruses to their cellular receptors in order to prevent infections from the beginning, great hope was placed on ribozymes. These are small RNA molecules with a pronounced secondary structure which function as sequence-specific RNases. Ribozymes are derived from corresponding structures in the RNA genomes of viroids. After replication, such autocatalytic RNA structures cleave the newly synthesized concatemeric genomes into individual units. It is now possible to synthesize ribozymes that recognize and cleave specific sequences in viral RNA genomes or in their respective mRNA species, to prevent viral replication or viral gene expression. However, it is difficult to introduce a sufficient amount of ribozymes into infected cells.

Another way to block viral replication is the use of antisense RNA. These RNA species are complementary to specific viral mRNA molecules. They hybridize with the mRNA to form a double-stranded RNA molecule, which cannot be translated. This procedure has proven to work well in vitro. Here again, the main problem is the introduction of a sufficient amount of antisense molecules into the cells. In addition to topological applications, such as the eye, gene therapy approaches might possibly represent a way in which genes coding for ribozymes or antisense RNAs may be introduced into infected cells as components of vectors. A first system based on this mechanism is fomivirsen, a 21-nucleotide-long deoxyoligonucleotide phosphorothioate that is complementary to the mRNA of the immediate early proteins of cytomegalovirus. It is administered for treatment of cytomegalovirus-induced retinitis.

Wide therapeutic applications are expected to emerge from small interfering RNA technology (RNA interference), which can be applied in a more targeted fashion and more effectively than antisense RNA. Apart from acute infections such as influenza, many attempts are also being made with respect to persistent infections with HIV, hepatitis B virus and hepatitis C virus. However, the well-known problems such as bioavailability, transport to the destination site and toxicity are also pertinent issues. Moreover, many viruses have developed effective strategies to avoid or circumvent attacks on such targets during co-evolution.

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10 Vaccines

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Vaccines are predominantly used for prevention; that means they should establish a protection in immunized people or animals which will protect them from a possible infection and the subsequent illness when they come into contact with the respective pathogens. Fundamentally, there are two kinds of immunization: active and passive. The latter is based on the administration of immunoglobulin preparations that can neutralize a specific virus. Therefore, passive vaccination is applied only in special cases, such as when the person to be protected recently had verifiable contact with a specific virus (postexposure prophylaxis), or if the risk of exposure to pathogens cannot be ruled out in the following weeks and an active vaccination is not possible, as in short-term planned trips to Third World countries (exposure prophylaxis). An example is the administration of antibodies specific for hepatitis B virus in cases of contamination with blood from people who have an acute or chronically persistent

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infection with this virus, and thus have high concentrations of infectious particles in the blood. Such accidents occur primarily in medical personnel by needlestick injury (▶ Sect. 19.1). In certain cases, the administration is performed in combination with an active vaccination (active–passive immunization). Specific immunoglobulin preparations are also administered when people have been bitten by animals that may be infected with the rabies virus (▶ Sect. 15.1). In the case of early application (together with an active vaccination), the antibodies can neutralize the virus, and impede its spread in the body. Since the time between contact with the virus and its spread in the organism is often very short, passive immunization is limited to a period shortly before or after exposure to the infective agent (usually within 4 days). Therefore, it is reserved for cases in which the contact with the potential pathogen is well documented and the type of infection is known, and when an appropriate immunoglobulin preparation is available. The protection afforded by antibody preparations lasts just a few weeks, as immunoglobulins are rapidly degraded in the organism. Therefore, postexposure administration of active vaccines is increasingly preferred, e.g. in the context of outbreak-control vaccination. In veterinary medicine, passive immunization is employed occasionally in young animals which were born in a flock with high infection pressure. This approach is applied, for example, in kennels when infections occur with canine parvovirus (▶ Sect. 20.1.6). However, its value is controversial, as the immunoglobulins administered hinder the more advantageous active immunization.

On the other hand, the active vaccine produces a long-lasting protection against infection, which can even last for life. In this case, a protecting immune response is induced. Ideally, it consists of a combination of neutralizing antibodies and cytotoxic T cells (▶ Chap. 7). The active immunization can be done in two ways: with live vaccines or by using inactivated vaccines. The various methods which are now used to develop vaccines or which are being attempted are illustrated schematically in Fig. 10.1.

10.1 How Do Live Vaccines Work?

Live vaccines contain attenuated, replication-competent pathogens that can replicate in the vaccinated person, i.e. they are able to infect certain cells and initiate the synthesis of viral proteins and particles, but without triggering the respective clinical picture. These viral components are recognized by the immune system of the vaccinee as foreign (exogenous), which induces the production of specific neutralizing antibodies and cytotoxic T lymphocytes (▶ Chap. 7). Neutralizing antibodies are predominantly directed against viral surface structures, and can bind to the surface of the virus, thus preventing both attachment of the virus to target cells and infection. The immune complexes of antibodies and virus particles activate the complement system, or are phagocytosed by macrophages and neutrophils. Cytotoxic T cells recognize infected cells by the T-cell receptor on

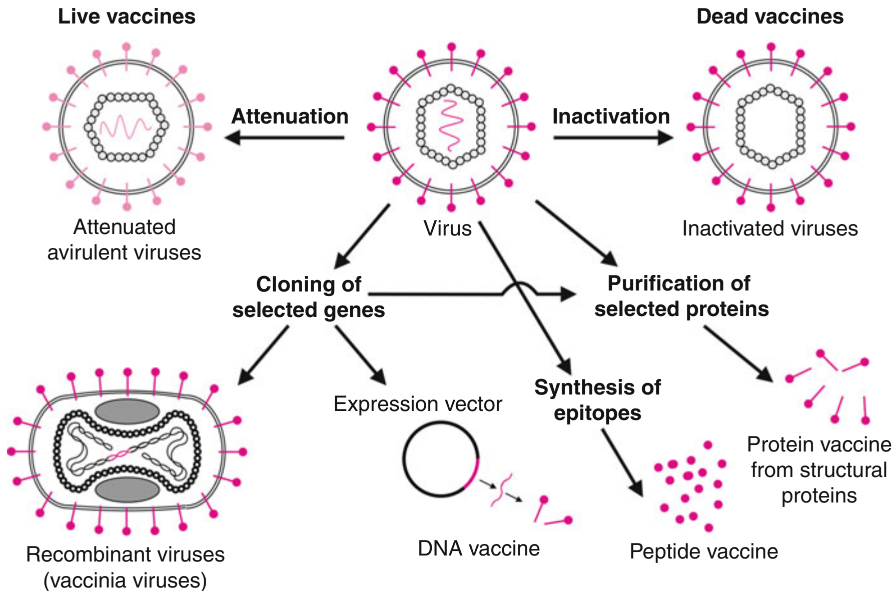

Fig. 10.1 Different ways to develop vaccines

their surface; these infected cells synthesize viral (foreign) proteins and present peptide fragments derived from them in complex with MHC class I antigens. Recognition leads to the destruction of infected cells, and thus to the elimination of the virus from the organism. Live vaccines can develop a very effective protection against a number of viral pathogens, as they effectively activate both the humoral and the cellular mechanisms of the immune system. Therefore, live vaccines are generally the ideal vaccine candidates. However, the implementation of their application requires refrigeration systems for storage, which can lead to difficulties in some developing countries.

10.1.1 Attenuated Viruses

Attenuated viruses are similar to pathogenic viruses with respect to structure, protein composition and infection behaviour. However, they clearly differ from them with regard to virulence (▶ Chap. 4). In comparison with the wild-type virus, they usually cause just a limited or weakened infection, which in most cases does not cause clinical symptoms, and can easily be controlled by the immune response of the organism. The proteins produced during the infection are ideally identical to those of the virulent virus strains, or they are at least very similar to them. The fact that viral polypeptides are synthesized in the cells during an attenuated infection leads to the emergence of virus particles which induce the generation of neutralizing antibodies and cytotoxic T cells. Therefore, the immune response that is

triggered by attenuated viruses is suitable to induce a long-lasting, effective protection against infections with the respective pathogen.

The molecular bases of attenuation are mutations in the genome of wild-type viruses. Different genes may be affected in the various vaccine viruses. Frequently, it is not even known why they generate an attenuation of the wild type. One way to attenuate virus strains is their continuous cultivation and passaging in cell culture. In this way, virus mutants are selected which are optimally adapted to the cell culture conditions. Occasionally, they lose their virulence during this process. This method made possible, for example, the isolation of attenuated polio, measles and yellow fever viruses, which do not cause diseases in humans, in contrast to wild-type infections (Table 10.1, ► Sects. 14.1, ► 14.5 and ► 15.3). Another example is the vaccine that was successfully applied to protect pigs against infections by classical swine fever virus (► Sect. 14.5). In this case, the virulent virus was attenuated by continuous passages in rabbit cells. Subsequently, these lapinized viruses were no longer able to trigger diseases in swine. Meanwhile, the application of this originally very successful vaccine has been banned. The justification for this lies particularly in trade. On the one hand, infections with the attenuated vaccine virus could not be distinguished from infections with classical swine fever virus in blood tests; on the other hand, following a vaccination, an export ban is imposed for a limited period, which, however, has economically adverse effects. Instead, it is attempted to interrupt the infection chain and to eliminate the virus from the population by culling all herds in which classical swine fever occurs (► Chap. 11, ► Sect. 14.2). By transgressing the species barrier, the infection can occasionally adopt a weakened character. Similarly, vaccinia viruses that were originally used to produce a protective immune response against smallpox virus induced local infections in humans, which in very rare cases had a generalized or fatal course. Because of this problem, further attenuation of vaccinia virus was sought by repeated passages in chicken cells. In fact, this approach resulted in modified vaccinia Ankara virus (► Sect. 19.6), which exhibits fewer adverse side effects, and is currently being tested as a vector system for expression of recombinant vaccines which are to be used, for example, in human immunodeficiency virus (HIV) patients in the context of therapeutic vaccinations.

Vaccinations with attenuated live viruses usually confer very good protection, which remains for a long time. Booster vaccinations at relatively long intervals of up to 10 years can provide continuous protection. However, attenuated viruses entail the risk that they can lose their reproduction capability and thus their efficiency because of inadequate refrigeration systems, or they can mutate back to the wild type during the weakened infection. Therefore, attention is now paid to the fact that attenuation should be based on multiple independent mutations, which largely excludes back mutation to the pathogenic wild type. However, attenuated vaccine viruses should be used only in immunologically healthy people. In immunocompromised individuals, these types of viruses can persist for a long time, and result in symptomatic infections. Since attenuated live vaccine viruses can sometimes be transmitted from vaccinees to other people in their immediate environment (e.g. polio vaccination; ► Sect. 14.1), this restriction also applies to

Table 10.1 Important vaccines for prevention of viral infections. The vaccines listed are licensed and distributed worldwide. In addition, several vaccines exist for use in humans or animals that are exclusively used in some countries. These vaccines are able to induce protection against infectious agents that not ubiquitous but are restricted to defined regions and states. For further information, the respective public health authorities should be contacted

	Live vaccines		Killed vaccines				
	Attenuated viruses	**Recombinant viruses**	**Inactivated viruses**	**Protein components**	**Peptides**	**DNA**	**Marker vaccines**
Humans	Poliovirus (Sabin)[a]	Recombinant vaccinia virus (in trial phase)	Poliovirus (Salk)[b]	Hepatitis B virus	In trial phase	In trial phase (e.g. against human immuno deficiency virus)	
	Yellow fever virus		Influenza virus	Human papillomaviruses (6, 11, 16, 18)			
	Measles virus		Hepatitis A virus	Influenza virus			
	Mumps virus		Tick-borne encephalitis virus	Human immunodeficiency virus (in trial phase)			
	Rotavirus		Rabies virus				
	Rubella virus						
	Vaccinia virus[c]						
	Varicella–zoster virus						
Animals	Canine parvoviruses	Rabies virus (recombinant vaccinia virus, f.e. France)	Rabies virus	Classical swine fever virus (emergency vaccination)	In trial phase	In trial phase	Bovine herpesvirus type 1
	Feline parvoviruses	Feline leukaemia virus (recombinant canary poxvirus)	Feline leukaemia virus	Feline leukaemia virus			Porcine herpesvirus
	Canine distemper virus		Canine parvoviruses				
	Canine adenovirus 2		Feline parvoviruses				

(*continued*)

Table 10.1 (continued)

	Live vaccines		Killed vaccines				
	Attenuated viruses	**Recombinant viruses**	**Inactivated viruses**	**Protein components**	**Peptides**	**DNA**	**Marker vaccines**
Animals	Rinderpest virus (plowright)[d]		Rinderpest virus (recombinant vaccinia virus)				
	Feline caliciviruses		Canine adenovirus 1				
	Feline herpesvirus		Feline herpesvirus				
	Feline coronavirus		Bovine viral diarrhoea virus				
	Bovine viral diarrhoea virus		Avian infectious bronchitis virus				
	Avian infectious bronchitis virus		Bovine respiratory syncytial virus				
	Bovine respiratory syncytial virus		Equine herpesviruses 1 and 4				
	Bovine parainfluenza virus		Equine influenza viruses				
	Equine herpesviruses 1 and 4		Porcine influenza viruses				
	Porcine respiratory and reproductive syndrome virus		Porcine respiratory and reproductive syndrome virus				
			Foot-and-mouth disease virus (emergency vaccinations)				
			Feline calicivirus				
			Canine parainfluenza virus				

[a]Oral vaccination, no longer recommended in countries without reported polio
[b]Recommended in countries without reported polio
[c]Smallpox vaccination was stopped worldwide on the recommendation of the World Health Organization in 1979
[d]Rinderpest vaccination was stopped world wide on the recommendation of the World Health Organisation for Animal Health in 2006

immunosuppressed people in the family and the surroundings. Furthermore, live vaccines should not be used during pregnancy.

It is also important to emphasize that attenuation is defined only for the affected host species in which it was tested, and application of a live vaccine in species other than that in which the evaluation occurred is not permitted. This has to be considered for vaccinations of zoo and wild animals, and the unquestioned use of live vaccines should be avoided as far as possible.

In particular, the use of live vaccines is prohibited when infections with the pathogenic wild type are absent or occur very rarely in a population. This status has been achieved by successful vaccination programmes, e.g. worldwide for smallpox virus and in Europe and North America for poliovirus. In these cases, the risk associated with vaccination is greater than that of being infected with the wild-type virus and of becoming ill. Therefore, in the case of smallpox virus, vaccination has been stopped, and to protect against poliovirus, an inactivated vaccine is being used today (▶ Sects. 14.1 and ▶ 19.6). Every active vaccination entails a certain rate of known harmless side effects. However, real complications (such as permanent nerve damage) are very rare events that occur with a prevalence of approximately one in a million vaccinations in adults. Therefore, every vaccination must be accompanied by corresponding obligatory medical information and documentation.

10.1.2 Recombinant Viruses

Today, recombinant viruses represent a controversially discussed variant of live vaccines. It is being attempted to modify well-explored, less pathogenic viruses (e.g. adenoviruses) and vaccine viruses that were used successfully in the past (usually vaccinia viruses) by using genetic engineering methods in such a way that they encode proteins of other viral species, in addition to their own gene products necessary for infection and replication (▶ Sects. 19.4 and ▶ 19.6). After viral inoculation, these foreign genes are expressed by recombinant viruses along with their own genes in an organism during infection. This induces an immune response against both the vaccinia virus or adenovirus proteins and the heterologous polypeptides. These recombinant, replication-competent viruses offer all the advantages of live vaccines (antibodies and cell-mediated immune response) and are, therefore, also increasingly used in therapeutic immunizations, i.e. in cases where an infection has already occurred. Vaccination should stimulate the immune response to influence the progression of the disease favourably.

Large DNA fragments cannot be cloned into the genome of vaccinia virus despite its size. Therefore, it is essential to know exactly which proteins of the virus are important for the elicitation of a protective immune response in order to produce a vaccine against the viral infection. The pertinent gene is cloned into the genome of the vaccinia virus in such a way that it is under the control of an early vaccinia virus specific promoter. Such a vaccine cannot generate the entire spectrum of an immune response that arises during an infection with the wild-type virus or its attenuated variant. In recombinant vaccine viruses, the immune response

is confined to a specific protein. Such vaccines have been extensively employed in North America (USA, Canada) and Europe (Belgium, France, Spain) to protect raccoons and foxes from rabies. The genome of the recombinant vaccinia virus harbours the genetic information coding for the G protein of rabies virus (▶ Sect. 15.1). With use of these recombinant viruses, meat-based artificial baits are prepared and scattered for foxes. The animals bite the virus-containing plastic capsule when devouring the bait, whereby they become infected with the vaccine virus, and develop an immune protection against rabies virus infections. A vaccine based on recombinant vaccinia viruses or adenoviruses has not been approved for human use yet, but is being discussed for prevention or treatment of infections with HIV, and is being tested experimentally.

10.2 How Do Inactivated Vaccines Stimulate the Immune System, and what Types Are in Use or in Clinical Trials?

According to their definition, inactivated vaccines are not able to proliferate in the vaccinated organism. They induce predominantly antibody responses. Because of the lack of active viral protein synthesis, a cytotoxic T-cell response occurs rarely. When inactivated vaccines are used, an initial immunization (two or three vaccinations) and booster vaccinations are necessary to maintain the immune protection. To enhance the immune response, these vaccines have to be applied with an adjuvant, which facilitates the migration of macrophages, monocytes, and B and T lymphocytes to the inoculation site. Aluminium hydroxide, aluminium hydroxyphosphate sulphate in combination with deacylated monophosphoryl lipid A components and certain toxoids (e.g. tetanus toxoid) are adjuvants approved for use in humans. Other adjuvants are also used in animals. A widely used adjuvant is Quil-A, a saponin that is also used for the production of immune-stimulating complexes. However, fibrosarcomas have recently been observed at the injection site in the application of inactivated vaccines (rabies virus and feline leukaemia virus) in cats. Whether this is elicited by the adjuvant or by the trauma of injection has not been conclusively elucidated. For experimental immunizations, only incomplete Freund's adjuvant is used in animals.

10.2.1 Inactivated Pathogens

The simplest form of an inactivated vaccine is a preparation of wild-type viruses that have been effectively killed by treatment with chemicals. This inactivation is usually done with aldehydic or alcoholic agents. Frequently, β-propiolactone is also used. The protein components must not be denatured to the extent that they lose their native configuration and are no longer similar to the genuine viral structures. Since the viral nucleic acid is per se frequently infectious and can lead to the production of progeny viruses, methods must be used to eliminate the infectivity which result in the degradation of the nucleic acid of the virus. For example,

the currently commonly used vaccines against influenza virus or hepatitis A virus infections (Table 10.1) are based on inactivated pathogens.

10.2.2 Use of Selected Proteins of Pathogens

Relatively new vaccines are based solely on a selected protein component of the virus. A fundamental prerequisite for the development of such a vaccine is detailed knowledge of the immunologically important components of a pathogen. If it is known against which of the viral proteins (usually surface proteins) a protective immune response is induced, the respective coding gene can be cloned into a eukaryotic expression system. From this vector, the protein can be synthesized, purified and finally administered with an adjuvant. Structural particle proteins are especially suitable for the induction of a protective immune response. An example of this is HBsAg, the surface protein of hepatitis B virus, which aggregates to vesicular particles when it is artificially expressed in yeast or other eukaryotic cells. HBsAg particles can induce, largely independently of adjuvants, the production of hepatitis B virus neutralizing antibodies and even cytotoxic T cells (▶ Sect. 19.1). Similarly, the capsid proteins L1 and L2 of papillomaviruses assemble together into particles that resemble the infectious viruses. The newly approved vaccines against infections with oncogenic human papillomaviruses (against human papillomaviruses 16 and 18) are based on such virus-like L1 particles, which – like the aforementioned HBsAg particles – are produced in yeast cells using genetic engineering methods (▶ Sect. 19.3). The particle-forming Gag proteins of HIV also seem to be very appropriate for immunization.

Another variant of this type of a subunit vaccine has been in use in veterinary medicine for many years. It is a vaccine against feline leukaemia virus, a retrovirus (▶ Sect. 18.1). It contains only the external glycoprotein (gp70) of subtype A of this virus as an immunologically relevant component, which is produced and purified in *Escherichia coli* cells in a non-glycosylated form by genetic engineering methods.

10.2.3 Synthetic Peptide Vaccines

Vaccines consisting of synthetic peptides with a length of 15–30 amino acids represent an additional form of vaccine which is currently being tested. In this case, individual epitopes of viral proteins, which induce the production of neutralizing antibodies and also activation of T cells, are selected and chemically synthesized. One advantage is that these vaccines are devoid of nucleic acids and that they can be produced in large quantities with relatively little effort. In animal studies, the protective effect of synthetic peptide vaccines has been demonstrated against infections both with foot-and-mouth disease virus (▶ Sect. 14.1) and with canine parvovirus (▶ Sect. 20.1). Prerequisite for the development of such a vaccine is also in this case detailed knowledge of the protein regions that can elicit a virus-neutralizing immune response. However, it seems rather doubtful that

a single epitope is able to do that in the long term, as most viruses have high genetic variability. Furthermore, single individuals have different abilities for immunological recognition of specific protein regions. This is interrelated with the distinct MHC phenotype of each individual. In a vaccine based on synthetic peptides, several different epitopes ought to be combined and applied with a suitable adjuvant. A vaccine based on synthetic peptides has not been approved yet.

10.2.4 DNA Vaccines

Another new vaccine type is administered as DNA. The nucleic acid contains the genes of a virus that are capable of inducing a protective immune response, i.e. usually the regions that encode surface components of a pathogen. They are cloned into a vector system together with promoter elements, which regulate their expression, and are injected intramuscularly as purified DNA. Particularly in muscle cells, the DNA can be detected over long periods as an episome. Apparently, it is degraded very slowly there. If the corresponding genes are expressed, the organism can apparently develop both a humoral and a cellular immune response. This type of vaccine has been tested predominantly in animal systems. In animal studies, a protective effect of these vaccines has been demonstrated against a variety of different animal pathogenic viruses, such as paramyxovirus, parvovirus and herpesvirus. However, large amounts of DNA and a number of booster injections are necessary. A faster degradation of the nucleic acid has also been observed. However, the inherent possibility of integration of DNA sequences which were applied with the vaccine into the cellular genome and the resulting endangerment to the vaccinee are additional problems to consider in the discussion of the harmlessness of DNA vaccines.

10.3 Reverse Genetic Methods: An Innovation in Vaccine Development

Viruses with RNA genomes present a special challenge for the development of vaccines using genetic engineering methods. Inasmuch as the techniques for targeted introduction of mutations and expression of foreign genes in prokaryotic and eukaryotic systems are based on DNA as the initial nucleic acid, the genetic information of RNA viruses must first be transcribed into double-stranded DNA. Only then does it become accessible for genetic engineering manipulations. Then, targeted mutations can be selectively introduced into the nucleic acid sequence of such DNA constructs, e.g. to generate attenuated vaccine viruses. In this case, the genome of RNA viruses, which were previously transcribed into DNA, must be present in a form that subsequently allows the synthesis of infectious viruses. This process describes the methods of reverse genetics, by which it is being attempted, for example, to construct attenuated live vaccines against respiratory syncytial virus and some other paramyxoviruses (▶ Sect. 15.3). Viruses with a segmented RNA

genome, such as influenza viruses, represent a particular challenge. In this case, the genetic information of all segments of the viral RNA genome must be transcribed into DNA and cloned into appropriate eukaryotic expression vectors. After the combined synthesis of all viral proteins and the production of new genome segments, recombinant viruses can subsequently be produced and cultivated in appropriate cell culture systems. An attenuation of the virus is possible by targeted mutagenesis. In the future, this will facilitate faster adaptation of new vaccine strains to new influenza viruses (▶ Sect. 16.3). In this case, it is also being attempted to develop novel vaccines rapidly by using prefabricated genomic libraries or universal vaccines in order to cover all new influenza virus subtypes before they have pandemic dimensions.

10.4 Marker Vaccines

Marker vaccines are of great importance in veterinary medicine. They allow the distinction of vaccinated animals from field-virus-infected animals (wild-type virus) by using simple serological methods (▶ Chap. 13). These vaccines are also referred to as DIVA vaccines (for "differentiating between infection and vaccination"). In principle, a differentiation is made between negative and positive marker vaccines. In the first case, the vaccine virus lacks a gene and the respective protein, so the vaccinated animals do not develop immunological reactions against the respective protein. The distinction of field-virus-infected animals is performed by determining the specific antibodies directed against this protein. Prerequisites for the success of this approach are that the missing protein does not exert essential functions during viral replication, and that sufficient specific antibodies against the relevant protein are produced during an infection with wild-type viruses. In veterinary medicine, such negative marker vaccines are administered in the control of two economically important herpesvirus infections, namely Aujeszky's disease in swine and infectious bovine rhinotracheitis in cattle (Table 10.1, ▶ Sect. 19.5).

Positive marker vaccines are vaccine viruses that are characterized by a unique marker (nucleic acid sequence). In cases of vaccination failure, i.e. in cases in which an infection is established in spite of vaccination, positive marker vaccines facilitate the easy identification of vaccine viruses.

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Epidemiology

11

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The term "epidemiology" was originally used for the science of major, humanity-threatening diseases. Today, it refers to the science of all transmissible and non-transmissible diseases in a population, irrespective of whether they occur frequently in time or space. In the field of microbiology, epidemiology deals with diseases which are caused by transmissible agents such as bacteria, viruses or prions, and in particular with the spread and consequences of infections. Therefore, epidemiological studies are very important for the health of the world population, and are the basis for general and veterinary measures such as quarantine or vaccinations to prevent and control pandemics and epidemics. Furthermore, they allow the development of guidelines and regulations for vaccinations and other measures that prevent infections.

Exo-epidemiology is confined to the investigation of the dissemination of the pathogen after its release from the organism. It includes their survival in the environment, their whereabouts in certain reservoirs and their retransmission to humans or animals. In contrast, endo-epidemiology deals with the way and manner in which pathogens spread within the organism and where they may persist and mutate. Epidemiology records not only the incidence of infections, diseases and deaths, but also attempts to obtain data on the disease pattern in which the aetiological relationship to a specific pathogen is not known (disease in search of virus), and this leads to the identification of diseases caused by a known virus (virus in search of disease). It extends the knowledge of the cause of infectious diseases by monitoring epidemics or pandemics and assessing the course of infectious diseases and their consequences for a population. If clusters of specific symptoms result,

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this can be an indication of the appearance of new viruses or particularly virulent strains. The survey of epidemiological data also includes the influence of living standards, social structure and human behaviour patterns, such as sexual promiscuity or drug abuse, but also veterinary relevant parameters such as stock densities, animal transport, animal markets, animal shows, seasonality, climate, vector density and mating season on the transmission frequency and infection time. The migrations of populations because of eviction or rural exodus and the associated negative social consequences, such as poor hygiene, and the declining effectiveness of public health authorities can facilitate the dissemination of infectious diseases. In antiquity, diseases such as plague, smallpox, influenza and measles spread along the caravan routes and trade routes. The worldwide growth of tourism resulted in increased contacts with previously unknown pathogens that were imported by infected people in a naive (for the pertinent pathogen) population of a country. Whereas these processes were long-lasting even a few decades ago, viruses are now spread worldwide within a few days or weeks. This was clearly demonstrated by the rapid spread of the infection with SARS coronavirus in 2003 from Southeast Asia to Europe and North America (▶ Sect. 14.8). The change of production processes in industry and agriculture and trade in consumer goods and waste as well as human interferences with the natural environment cause new distribution patterns of infections. Until a few years ago, animal pathogenic viruses, such as avian influenza virus, spread worldwide by bird and animal migrations (▶ Sect. 16.3). In addition, pathogens are today a worldwide problem through the international trade in farm animals, which often extends beyond continental borders.

Some brief explanations of the basic concepts commonly used in epidemiology for specific parameters or infectious forms follow. An epidemic is a temporally and spatially limited occurrence of an infectious disease in a population. Examples are rubella, measles and chickenpox. By contrast, a pandemic is defined as a global, time-limited or unlimited accumulation of infections, as is found in influenza or acquired immunodeficiency syndrome (AIDS). Endemic diseases are sporadic, spatially and temporally limited or unlimited appearing infectious diseases which can occur between epidemic phases. Morbidity describes the number of ill individuals in relation to the population size, and mortality is defined as the number of deaths that occur from a particular infection within a population; both are usually referred to 10^4 or 10^5 people or animals. By contrast, lethality is defined as the number of deaths in relation to the total number of people affected by a particular infectious disease. Excess mortality is the temporary accumulation of deaths as a result of an infection, such as flu or AIDS, which surpasses the long-term average mortality. Herd immunity is defined as the immunological protection that exists against a virus (measles, rubella and influenza viruses) in the population at a given time, regardless of whether it is caused by infection with the pertinent pathogen or by vaccination. The herd immunity of a population varies with the different viruses. It depends on transmission mechanisms, environmental conditions such as temperature and humidity and the population density, including vaccination discipline. To attain effective herd immunity, a vaccination rate of over 90 % of the population is usually considered necessary (e.g. measles, mumps and rubella).

11.1 How Are Viral Infections Transmitted?

Horizontal transmission refers to all types of infection of an organism by another organism, i.e. the transmission of a pathogen between hosts of a generation. This includes the homologous transmission of a virus from person to person, as well as the heterologous transmission from animals to humans (zoonosis) and vice versa. Pathogens spread by horizontal transmission in a population during epidemics, pandemics or endemics. This can be directly, for example, by virus-containing aerosols or droplets that are released when sneezing or coughing during infectious diseases of the upper respiratory tract (such as infections with influenza viruses, coxsackieviruses, adenoviruses and paramyxoviruses; ▶ Sects. 14.1, ▶ 15.3, ▶ 16.3 and ▶ 19.4), or indirectly. Indirect transmission is known, among others, for rhinoviruses (▶ Sect. 14.1), which are transmitted by droplet infection, and also via contaminated hands, doorknobs or similar household items commonly used by infected and uninfected people such as towels. Dirt and smear infections contribute especially to the dissemination of virus-induced gastrointestinal or renal diseases. Here, the pathogen is excreted in the faeces or urine, and is transmitted by contamination with this material because of inadequate hygiene. Canine parvovirus has been spread very efficiently worldwide by the soles of shoes which were contaminated with viruses from dog excrements. In regions with a low hygienic standard, sewage is discharged untreated into rivers and seas. This practice and also fertilization of plants with human faeces, which is widespread in some countries, can lead to contamination of food with polioviruses, enteroviruses, hepatitis A viruses (▶ Sect. 14.1) and caliciviruses (▶ Sect. 14.2) – an important cause of epidemics. Mussels thrive particularly well in such polluted waters and simultaneously enrich hepatitis A viruses. Hantaviruses and arenaviruses (▶ Sects. 16.1 and ▶ 16.2) are excreted in the faeces of infected rodents, and thus are disseminated into the ground. In the case of contact with such contaminated earth, there is a risk of infection with the relevant pathogens. Similarly, organ transplants can lead to transmission of such viruses and also herpesviruses (Epstein–Barr virus or cytomegalovirus; ▶ Sect. 19.5). In the worst case, all viruses which have a more or less pronounced viraemic phase or are situated in the organ at the time of graft removal can be transmitted by transplants. In fact, lymphocytic choriomeningitis and rabies have been transmitted in this way (▶ Sects. 15.1 and ▶ 16.1). Many viruses are present in the saliva and can be transmitted to babies by kissing or mouth-to-mouth feeding. In other cases, the virus is present in the semen or the cervical secretions and can be transmitted by sexual intercourse. This is particularly true for human immunodeficiency virus (HIV), papillomaviruses and some herpesviruses (▶ Sects. 18.1, ▶ 19.3 and ▶ 19.5). Viruses that are present in the blood during the disease (hepatitis B and hepatitis C viruses, HIV, parvovirus, cytomegalovirus), are transmitted by smeared blood, for example, perinatally during the birth process from the infected mother to the neonate, but also through contaminated blood products or stored blood (▶ Sects. 14.5, ▶ 18.1, ▶ 19.1 and ▶ 20.1).

Some viral species are transmitted by arthropods (ticks, mosquitoes). The animals are infected with the pathogen when it is taken up with the blood when

stinging or biting. After reproduction in ticks or insects, the virus can be transmitted to other organisms through biting or stinging. This form of heterologous transmission is found, for instance, in yellow fever virus, dengue virus, tick-borne encephalitis virus, togaviruses and orbiviruses, and in some bunyaviruses (▶ Sects. 14.5, ▶ 14.6, ▶ 16.2 and ▶ 17.2). On the other hand, rabies virus enters the wound and thus the organism through the bite of infected vertebrates (▶ Sect. 15.1). The geographic distributions of viruses that are transmitted by arthropods are mostly identical to those of the mosquitoes and ticks that they use as vectors. Shifts and changes in climate conditions, as are to be expected because of global warming, can lead to the occupation of new regions and niches by insects and the viruses that are associated with them.

Dissemination of viruses among people who live in close contact with each other is known as nosocomial infection. Such infections are commonly observed in nursing homes, kindergartens and hospitals. In these communities, many pathogens spread swiftly. The concept of iatrogenic infection refers particularly to the spread of pathogens by medical procedures such as organ transplants and blood transfusions, and by improperly performed medical interventions, such as the use of contaminated equipment, syringes or needles. Iatrogenic transmissions have frequently been observed in bovine leukaemia virus infections in which the virus was transmitted through the use of insufficiently disinfected needles from animal to animal and from flock to flock.

The transmission of an infection between generations (from mother to fetus) is referred to as vertical transmission. This happens when pregnant women are acutely infected with certain viruses (rubella virus, cytomegalovirus, parvovirus B19; ▶ Sects. 14.6, ▶ 19.5 and ▶ 20.1), and the pathogen is transplacentally transmitted to the fetus. Similarly, this also applies when the viral genome is inherited by the next generation as an integrated part of the genetic information of the cell (e.g. of the egg or sperm cell). This vertical transmission from parents to offspring is known primarily for endogenous retroviruses (▶ Sect. 18.1) or for bovine and porcine pestiviruses (▶ Sect. 14.2).

11.2 Where Do Human Pathogenic Viruses Reside?

Where viruses reside in the time between their epidemic occurrences is often unclear. Sporadic cases of apparent infections occur repeatedly; in such cases, the virus is probably transmitted by healthy people with asymptomatic (inapparent) infections. Some viral species are able to survive in the environment for considerable time before they infect their hosts again; these include parvoviruses and rotaviruses (▶ Sects. 17.2 and ▶ 20.1), picornaviruses (▶ Sect. 14.1) and also poxviruses (▶ Sect. 19.6). Even now, poliovirus is occasionally reintroduced from countries that are not free of poliovirus infections (e.g. India and several African countries), and it proliferates in sero-negative people until a developing herd immunity or specific outbreak-control vaccinations eliminate the virus from the corresponding population. In contrast, other pathogens such as rubella virus and

most paramyxoviruses (▶ Sects. 14.6 and ▶ 15.3) are instable in the environment, and do not infect animals. They are completely eliminated from the organism by the immune system during the course of infection. Only little is known concerning their whereabouts during the periods between sporadically occurring infections or epidemics. The first appearance of such a viral species must have been linked to human urbanization because such a virus would disappear from small, isolated human populations; therefore, it must be a phylogenetically relatively new virus, which could maintain its infection chains only in populations with a relatively high density. The term "emerging virus diseases" includes infectious diseases in which the viruses have been transmitted to a new host (such as SARS coronavirus; ▶ Sect. 14.8) or appear again as re-emerging viruses after long periods of time, as is the case for some hantaviruses (▶ Sect. 16.2).

In contrast, herpesviruses show a completely different behaviour. They remain latent in the body after infection, and the lifelong latency with recurrent excretion phases facilitates the dissemination of the virus even after long intervals (▶ Sects. 18.1 and ▶ 19.5). HIV infections also appear clinically unnoticed for a long time, although in this case infectious viruses are always present in the blood. The same applies to viruses that produce persistent infections and are present over long periods in the blood and are excreted or released from the skin, such as hepatitis B virus, adenoviruses and papillomaviruses (▶ Sects. 19.1, ▶ 19.3 and ▶ 19.4). Therefore, they are considered as one of the phylogenetically old viruses, which are well adapted to humans and can survive even in small populations. However, the new highly sensitive methods for detection of viral nucleic acids show that, besides these forms of chronically persisting or latent infections with relapsing recurrence or reactivation, there is also latency of viral genomes in which neither reactivation of the viral genetic information nor the associated synthesis of virus products is observed. This DNA latency in the cells of different tissues has particularly been described for parvovirus B19 (▶ Sect. 20.1).

Sometimes, viruses infect certain animal species, and these are used as reservoirs for these pathogens, whence they can be transmitted to humans on certain occasions, or accidentally. Influenza viruses infect various bird species, which excrete them in the faeces. They can be transmitted to swine, just as the human virus types. In the case of double infections in swine, genome segments of both virus types can be recombined. In this manner, new reassortants of influenza viruses arise, which are occasionally pathogenic for humans and can spread pandemically in the population (▶ Sect. 16.3). Other viruses can bridge larger periods by having long incubation times. These include rabies viruses, which use foxes, badgers and bats as reservoirs. In this case, human infections represent the end of the infection chain, and a further dissemination is generally not possible (▶ Sect. 15.1). Even other viruses are able to leave their natural reservoir, and can be transmitted to humans: different animal poxviruses, hantaviruses, Ebola virus, the pathogen of Lassa fever and flaviviruses, including tick-borne encephalitis virus (▶ Sects. 14.5, ▶ 15.4, ▶ 16.1, ▶ 16.2 and ▶ 19.6). They reach the human organism by contact with animals as well as by tick or mosquito bites, thus leaving their natural hosts, rodents or monkeys.

11.3 To what Extent Are Most Viruses Optimally Adapted to their Hosts?

The currently predominant viral infections are the result of evolutionary processes between hosts and parasites which have taken place over very long time periods. Frequently, viral species related to human pathogens are found in various animal species. It is thought that these have adapted to humans during their evolution to become human pathogens, which remain permanently in the population after a certain period of time. If they are highly pathogenic for humans, then they will eliminate themselves from the population by destroying their hosts immediately. Therefore, the ideal situation is a balance between survival of the host and a more or less optimal viral replication with minimal damage. Examples for the optimal adaptation to a host are some arenaviruses (▶ Sect. 16.1), among them lymphocytic choriomeningitis virus and the causative agent of Lassa fever. Both viruses are highly adapted to their hosts, namely wild mice. These viruses are transmitted vertically during pregnancy to offspring, in which they induce an immune tolerance. These animals are healthy virus carriers, and excrete large amounts of viruses for life. However, if they infect humans, this frequently leads to severe diseases. Occasionally, new viral infections emerge in humans. This is particularly the case when contact occurs with rare and reclusive species which transmit the adapted pathogens to humans. An example in this context is the SARS epidemic of 2003. It is assumed that bats are the animal species that serves as the natural host of this very dangerous virus for humans (▶ Sect. 14.8). Other examples are human Nipah and Hendra infections, which are also transmitted by bats. Poliomyelitis was also a relatively well adapted human virus infection under living conditions with low hygiene standards. Here, the pathogen was usually transmitted during the first 6 months of life. Protective maternal IgG antibodies are still available in the infants at this time. Sequelae from the disease and occasionally paralysis are found only in infections occurring at an older age (▶ Sect. 14.1).

11.4 What Methods Does Epidemiology Use for Studying Viral Diseases?

The epidemiological methods which are especially used for viral diseases include survey and collection of data on the occurrence of specific disease clusters. This anamnesis constitutes the basis of all studies. Detection of specific antibodies, viral genomes or viral proteins in blood or tissue samples makes possible the diagnosis of acute or previous viral diseases (▶ Chap. 13).

Frequently, the aetiological role of a virus can no longer be evidenced by the fulfilment of the Henle–Koch postulates. These require the pathogen always be detectable in the patients, isolable from them, cultivable in pure culture and able to generate the disease after inoculation in a susceptible host (▶ Chap. 1). According to these criteria, many viruses cannot be characterized as pathogens of the infectious diseases caused by them, not least because many viruses cannot be propagated

Table 11.1 The Evans postulates

The Evans postulates
The prevalence of the disease should be significantly higher in populations exposed to the putative disease-causing agent than in control populations that have not been so exposed
Exposure to the pathogen is more frequent in the population with the disease than in the population without the disease
In prospective studies, the incidence of the disease should be significantly higher in populations exposed to the putative disease-causing agent than in those not exposed to it
In a region with exposure to the pathogen, the incubation periods should follow a normal distribution
All exposed individuals should react with an immune response, which can exhibit a biological variance
A measurable immune response should regularly appear after exposure to the putative pathogen either as a new response in those lacking this before exposure or as an increased response in terms of the corresponding parameters
Experimental reproduction of the disease should occur with higher incidence in animals appropriately exposed to the putative pathogen than in those not exposed to it
Elimination of the putative pathogen should result in a significant reduction of the incidence of the disease
Vaccination should lead to a decrease of the incidence of the disease in a population
The overall picture of the postulates should be plausible in a biological and epidemiological sense

in cell culture. Therefore, it is reasonable to extend these definitions. Helpful in this context are the so-called Evans postulates. They assess the association of a pathogen with a disease, even using indirect criteria. Essential aspects of the Evans postulates are summarized in Table 11.1.

Nucleic acid sequencing and determination of restriction enzyme patterns of DNA sequences render it possible to compare the virus strains or variants that emerge during an epidemic or pandemic. They make possible molecular epidemiology, which allows one to identify certain people, animals or livestock as a source of infection. In this way, molecular pedigrees can be established, which allow conclusions to be drawn on the origin of the pathogen.

Another field of modern epidemiology is so-called modelling, which is the theoretical construction of the development of a viral infection in the population. By modelling, one can estimate the worst-case scenario by variation of individual parameters. For many questions this is an important factor.

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Viral Evolution

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Viruses are ideal objects for studying evolutionary processes because of their short generation time, high numbers of offspring that they produce during infection and not least because of their simple structure. Viruses must continuously adapt to the conditions of their host or their host populations, so selection mechanisms are accessible to experimental approaches. In this context, different criteria play an important role, such as the antigenic diversity, the extent of virus excretion, and the degree of virulence. The complete adaptation of a virus to its host, which leads to a minimization of virulence of the infectious agent, is for both parties the desirable consequence: i.e. a problem-free coexistence and survival. For example, hepatitis G virus (GB virus C) which was initially isolated from patients with liver inflammation, seems to persist in many people without causing illnesses. A similar situation is observed with torque teno viruses (▸ Sects. 14.5 and ▸ 20.2). Spumaviruses are also found in many animal species and humans without causing symptomatic infections (▸ Sect. 18.1). For many viruses, the maximum exploitation of genetic variability is not always useful. Viruses reach a limit at which a greater variance is no longer advantageous: the proportion of non-infectious virus variants among the progeny becomes too high, whereby the potentially possible error limit is reached.

12.1 How Do Mutations Lead to the Emergence of Novel Viruses?

The members of the various virus families use different replication strategies. Consequently, the evolution of all viruses differs. For the replication of their

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genome, RNA viruses are reliant on the activity of viral RNA-dependent RNA polymerases, which do not have exonucleolytic proofreading activity. This 3′–5′ exonuclease activity is associated with cellular DNA polymerases and verifies the correct addition of the corresponding complementary nucleotide at the 3′ end of an elongating DNA chain during the synthesis of a new DNA strand. If a mismatching nucleotide is erroneously incorporated, the subsequent polymerization step is transiently blocked, and the mispairing nucleotide will be excised by the associated proofreading 3′–5′ exonuclease activity. This error-avoidance mechanism essentially contributes to the high accuracy of cellular DNA replication, which has an average error rate of 10^{-9}; i.e. one erroneous mispairing nucleotide per billion synthesized nucleotides. Therefore, many more mismatched nucleotides are found in most RNA viruses, which exhibit an error rate of 10^{-3}–10^{-4}. Assuming that a virus with an intact genome has a length of about 10,000 nucleotides, the genomes of progeny viruses will differ from the genome of the parent virus by one to ten nucleotides. In fact, one is not dealing with a uniform virus population but, strictly speaking, with a population of very closely related viruses, a phenomenon that is referred to as the concept of quasispecies formation. This is known especially for hepatitis C virus and human immunodeficiency virus (HIV) (▶ Sects. 14.5 and ▶ 18.1). Basically, the same also applies to DNA viruses, in which the phenomenon is considerably less pronounced, as the mutation rate is lower by a factor of at least 10^2. Polyomaviruses, papillomaviruses and parvoviruses (▶ Sects. 19.2, ▶ 19.3 and ▶ 20.1) use cellular DNA polymerases for replication of their genetic information, and are accordingly genetically much stabler than RNA viruses. By contrast, the complex DNA viruses such as herpesviruses and poxviruses (▶ Sects. 19.5 and ▶ 19.6) have their own DNA polymerases with 3′–5′ exonuclease activity for checking for and correcting erroneously incorporated nucleotides. Therefore, the generation of quasispecies does not play a major role in these viruses.

Besides the selection of individual virus variants due to mutations that confer on them a selective advantage in the offspring generation, sometimes there is a simultaneous selection of independent mutations, which are per se not advantageous for the emerging viruses, but accidentally arise as hitchhiking (passenger) mutations together with other beneficial mutations. This situation is particularly complex in viruses whose genes are encoded by overlapping reading frames, as is the case in hepatitis B virus (▶ Sect. 19.1). Permanently evolving viruses are real consequences of all these evolutionary processes. If the mutations are associated with changes of pathogenic properties, they can have decisive consequences for the infection process in their hosts and their survival.

The emergence of "successful" new viruses is based on two independent mechanisms as well as combinations of them:

1. The genetic alteration (mutation) of a virus and its selection
2. The change of the social structures and/or the living and environmental conditions of the host population.

Mutations of the viral genetic information can be manifested in various ways. The consequence of mutations in the genes that code for viral surface proteins, and

thus are subject to selection pressure by the immune system, is called antigenic drift. This antigenic drift is especially pronounced in RNA viruses, such as the caliciviruses, orthomyxoviruses and retroviruses. It is associated with the formation of quasispecies and has a considerable pathogenetic significance (▶ Sects. 14.2, ▶ 16.3 and ▶ 18.1). Like the selection pressure of the immune system on viral surface proteins, antiviral chemotherapy can also exert a selection pressure on mutations in polymerase genes, for example, leading to the formation of therapy-resistant virus variants (▶ Sect. 9.2).

Furthermore, a permanent emergence of new viruses which arise independently of the selection pressure of the immune system or antiviral chemotherapy is also observed. A well-studied example is canine parvovirus, which arose from the pathogen of feline panleucopenia by only a few mutations (▶ Sect. 20.1.6). Canine parvovirus emerged initially in dog populations in Europe in 1978, and was disseminated to all continents in a few months during a pandemic (▶ Chap. 11). That pandemic was associated with high mortality, so millions of dogs died of the haemorrhagic gastroenteritis caused by the virus. Today, it is known that mutations in the genome of the well-known feline panleucopenia virus are responsible for the emergence of canine parvovirus. Only three amino acid substitutions in the capsid protein of feline panleucopenia virus were sufficient for the emergence of the canine virus with an altered host tropism. These mutations alter the receptor binding site of the virus, and enable the new pathogen to bind to canine cells. Pedigree analysis of different viral genomes provided evidence that canine parvovirus did not emerge directly from feline panleucopenia virus, but that arose from feline panleucopenia virus via infection of wild carnivores, especially European red foxes.

Moreover, there is good evidence that the great diversity of picornaviruses (▶ Sect. 14.1) has emerged from a common ancestor virus and has evolved by point mutations during evolution. Because of the high error rate of the viral RNA-dependent RNA polymerase, whose activity is essential for these RNA viruses, mutations arise during viral genome replication with the above-mentioned rate of 10^{-3}–10^{-4}. In addition, recombination processes also play an important role in the generation of new RNA viruses and many other pathogens (Sect. 12.2).

12.2 How Do Viruses Gain New Genes and Functions?

In addition to mutations, viruses with segmented genomes, such as orthomyxoviruses, bunyaviruses, arenaviruses, birnaviruses and reoviruses (▶ Chaps. 16 and ▶ 17), can also undergo profound genetic changes, known as genetic reassortment. This refers to the exchange of one or more genome segments between two related viruses which have infected a cell simultaneously. If the genetic redistribution leads to exchange of genome segments that encode viral envelope proteins, the newly emerging viruses gain a new antigenic pattern, which is called antigenic shift. Well-documented examples of this are the classic pandemics which were triggered in the last century by influenza A virus. Under the names Spanish flu,

Asian flu and Hong Kong flu, they have influenced world history and caused millions of fatalities (▶ Sect. 16.3). The pandemic viruses usually represent genetic reassortants of human and avian influenza A virus subtypes. Swine infected with both subtypes are a "mixing jar" for the emergence of novel influenza viruses. Productive double infections can arise in them, as they are susceptible not only to porcine influenza viruses, but also to avian and human influenza viruses. In contrast, avian influenza A virus subtypes can infect humans generally only in exceptional cases by very close contact, as poultry is not susceptible to human influenza virus subtypes. These relationships confer on porcine influenza A virus infections a specific and potential zoonotic significance, since they can transmit new virus variants to humans.

Besides the known generation of reassortants in influenza A viruses, this process is also known among reoviruses. The ubiquitous occurrence of rotaviruses in calves and piglets and the potential threat of genetic reassortment with human strains put these infections under a particular spotlight (▶ Sect. 17.2).

Nevertheless, even viruses with non-segmented genomes are able to interchange large gene regions. This mechanism is known as genetic recombination. It is enabled by changing the use of the template strand during nucleic acid synthesis, a process that may occur when certain cells of an organism are infected with two different but related virus types. Genetic recombination has been described in a variety of viruses and is particularly documented in different RNA viruses. Classic examples are togaviruses from the group of the New World equine encephalitis viruses (▶ Sect. 14.6). Western equine encephalitis virus, which causes acute encephalitis in horses and humans, originated by genetic recombination between eastern equine encephalitis virus and a Sindbis-virus-like isolate. Today, Sindbis virus is detectable only in the Old World; however, a similar virus must originally have coexisted with eastern equine encephalitis virus on the American continent before it was displaced by the newly emerged western equine encephalitis virus.

Genetic recombination is possible not only between two related viruses, but also between viral and cellular nucleic acid molecules – a process that often has great pathogenetic importance. Well-studied examples are the oncogenic retroviruses, which have incorporated a cellular oncogene in their genetic information, and thus are able to generate tumours in their hosts (▶ Chap. 18). Avian Rous sarcoma virus and feline sarcoma virus are examples.

Not only cellular oncogenes have been integrated into viral genomes, genetic recombination events have also been described with other cellular genes. For example, bovine diarrhoea virus, a flavivirus, has integrated the ubiquitin gene in its RNA genome by recombination with cellular messenger RNA during evolution. This gene has been incorporated in the region that encodes the viral non-structural proteins. In this way, a new cleavage site is created within the polyprotein, which is recognized by the cellular ubiquitin hydrolase. This process is associated with an alteration of the phenotype: starting from an originally non-cytopathic virus, a pathogen with high virulence has emerged which causes a fatal disease in chronically infected cattle, which is known as mucosal disease (▶ Sect. 14.2).

12.3 What New Infectious Agents Have Emerged Recently?

The origin of HIV-1 and HIV-2 can be explained by repeated host changes (different monkey species in West Africa) and their transmission to humans. In Africa, monkeys are infected with different species-specific variants of simian immunodeficiency virus (SIV); however, they do not become sick. HIV-1 probably originated from a chimpanzee virus (SIVcpz) that was transmitted to humans (▶ Sect. 18.1.5). In contrast, HIV-2 has developed from a type of SIV found in sooty mangabeys (SIVsmm) which has been repeatedly transmitted from monkeys to humans. During an adaptation phase in the new host, humans, the virulence was increased and virus variants were selected that were able to spread effectively from person to person. The pandemic-like character of HIV infections could be developed only by a radical change in living conditions and social structures in Africa. Trade, urbanization and global tourism have to be mentioned in this context. Since the emergence of HIV-1, different subtypes have arisen by mutations, and these have different geographical distributions. These processes are additionally influenced by genetic recombination between the different subtypes. Epidemiologically important in this context is that the type of HIV-1 (HIV-1B) that is found in Europe and in North America differs from the types that are found in Africa (HIV-1A, HIV-1C) in the efficiency of their transmissibility: HIV-1B specifically infects cells in the intestinal mucosa, which is considered as a result of the selection of transmission by homosexual practices.

Chikungunya virus, a togavirus that is transmitted by mosquitoes, causes a highly febrile human disease in tropical countries, in southeastern Africa and on the Indian subcontinent (▶ Sect. 14.6). In 2007, Chikungunya virus infections were observed for the first time in northern Italy because the tiger mosquito has become endemic in southern Europe and the virus found its way to Europe through infected mosquitoes or patients. If this pathogen can adapt to other European native mosquito species by mutations in the coming years, then this tropical infectious disease will possibly spread also in central Europe. Toscana virus (▶ Sect. 16.2), which is transmitted by sandflies, is another pathogen that causes meningitis and feverish diseases, and might also spread from southern to northern Europe because of global warming.

Bluetongue virus, a ruminant pathogenic virus (cattle, sheep, goat, deer), is ubiquitous in Africa and in some regions of southern Europe. In 2006, bluetongue virus (serotype 8) was described in Germany for the first time. Starting from a first outbreak in Belgium, bluetongue virus spread swiftly by infected culicoids (midges). At the end of 2007, the Benelux countries and Germany were extensively affected. In addition, there were also outbreaks in France and the UK. It is not known how the virus was introduced. Similarly, the role of global warming on viral development in arthropod hosts is also not clear.

SARS virus was first described as a clinically very severe pneumonia in Southeast Asia in the autumn of 2002 (▶ Sect. 14.8). Presumably, it was transmitted by contact of people with certain species of civet cats, which are traded in markets, and then spread to the human population very fast. Some rigorous national and

international measures have been taken to control the outbreak of this per se animal pathogenic virus, and to prevent its adaptation to humans as a host organism. Similarly, it is feared that the widespread and highly pathogenic avian flu virus H5N1 may change by continuous mutations and adapt to humans as its preferred host (▶ Sect. 16.3). Initially it infected only poultry in Southeast Asia, but now also populations of migratory birds are affected, and these have transported and spread this highly pathogenic virus strain from Asia to Europe and Africa. However, in a few exceptional cases people can be infected who do not transmit the virus further. Nevertheless, there is concern that the virus could change by mutations during its reproduction in infected patients. This may create new variants of the H5N1 virus which would be adapted better to humans, spread in the human population and might lead to the emergence of a novel very dangerous influenza pandemic. Therefore, drastic measures have also been taken in this case, e.g. culling of infected livestock when the first suspected cases occur, in order to control the emergence of the H5N1 virus in commercial poultry and to minimize the risk of transmission to humans. Unlike H5N1 virus, the H1N1 influenza A virus subtype, which caused flu in humans for the first time in Mexico in April 2009, exhibits a relatively low pathogenicity. Therefore, Mexican flu spread worldwide within a few months and became a new pandemic. The development of a new reassortant in swine is considered as causal for the emergence of the novel subtype.

Further examples of new zoonotic transmissions are Nipah virus and Hendra virus in Southeast Asia and Australia. Dramatically changed living conditions in the habitat of the host, fruit bats (flying foxes, *Pteroptus giganteus*), caused by human interventions in the form of massive land clearing and deforestation also plays a crucial role. This forced the bats to find new habitats, namely habitats shared with swine (Nipah virus) and horses (Hendra virus). Swine and horses may be infected via pasture or feed contaminated with the saliva of infected bats. From these species the viruses found their way to humans in a second step.

The occurrence of bovine spongiform encephalopathy is an example of the impact of industrialization processes in agriculture on the emergence of a new infectious disease. By feeding cattle, which are physiologically genuine herbivores, with inadequately inactivated animal proteins originating from animal waste of sheep and cattle carcasses, the heat-stable agent was transmitted to this species and caused the emergence of bovine spongiform encephalopathy, an animal and human pathogenetically and economically (variant Creutzfeldt–Jakob disease in humans) highly significant disease.

Therefore, considering the ubiquitous and fast processes that occur in the evolution of viruses, special caution is required. Factors that can promote and support the accidental emergence of a new virus should be avoided whenever possible. These include the imprudent use of live vaccines, particularly those that can establish persistent infections in the vaccinees, and especially the use of viruses as biological weapons to reduce or eradicate certain hosts, as occurred during the control of the rabbit plague in Australia by administration of rabbit haemorrhagic disease virus and myxoma virus (▶ Sects. 14.8 and ▶ 19.6). The exposure of naive populations of potential hosts to a novel virus can cause a non-calculable biological

disaster, just as we have seen in the infections with the new canine parvovirus (▶ Sect. 20.1). Of course, the use of viruses as a weapon for bioterrorism must be prohibited as well. Last but not least, such use of pathogenic agents can contribute, in addition to catastrophic pandemics, to the emergence of new virus types, which would also have fatal effects on their developers and users.

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Laboratory Methods for Detecting Viral Infections

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The first methods for detection of bacterial infections were available around 1880. After staining, bacterial pathogens were recognized in the light microscope because of their size and could be cultivated in culture media. Viruses evaded this approach, as they are significantly smaller, and as obligate parasites are not able to multiply in cell culture media. Although some viral infections could be associated with specific cellular changes and certain depositions in the infected tissue around the turn of the century, e.g. Negri inclusion bodies in nerve cells during rabies, a specific diagnosis was only possible through the development of cell culture methods and modern molecular biology. Today, viral infections can be detected directly by determining the agents, individual viral proteins, or their genetic information, or other materials in the blood of infected people or animals by using appropriate methods. Direct detection of viruses is possible, with the exception of latent or persistent infection forms, only during the acute phase of the disease. In some cases, the pathogens are present in the infected organism only before the symptomatic phase, so the direct

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detection of the virus is frequently not successful. Therefore, infections or contact with pathogens is usually demonstrated in virus diagnostics indirectly by characterization of the developing specific immune response.

13.1 How Can Viruses Be Detected Directly?

13.1.1 Viral Cultivation and Derived Detection Systems

For the cultivation and propagation of most viruses, continuously growing cell lines are available today. The preferentially sterile patient material to be investigated, such as blood, serum, pharyngeal lavage or urine, is freed from raw impurities and incubated with the cells in a small volume. After attachment of the virus particles to the cells, antibiotic-containing medium is added to the cultures to prevent the growth of bacteria that may be present as contamination in the test material. In the following days, the cells are microscopically checked for morphological changes, such as the appearance of cytopathic effects, plaques in the cell layer due to cell death, inclusion bodies and giant cells (▶ Chap. 5), which allow an initial inference on the replicating virus, and also serve as evidence that infectious viruses were present in the starting material.

The classic form of viral cultivation usually involves a relatively long incubation period of 1–4 weeks. A shortened version is the shell vial assay. In this method, the susceptible cells are incubated with the material that contains the suspected virus in the wells of a microtitre, a 24-well plate or on glass slides in centrifuge tubes and are centrifuged at low speed. This probably alters the fluidity of the cytoplasmic membrane of the cells, thus leading to faster penetration of the pathogens. After incubation for 1–2 days, viral proteins can be demonstrated in the cells by means of immunofluorescence or similar procedures.

Primary cell lines are barely used for cultivation of human pathogenic viruses today. They have only limited division capacity; therefore, they must be repeatedly established. An exception is foreskin fibroblasts, which are occasionally used for cultivation of herpes simplex virus. Particularly, the routine use of embryonic stem cells, which possess increased division capacity, is highly regulated. However, for other primary human cells it must also be demonstrated that they are free of viruses after cultivation, in particular they must not contain any chronic hepatitis viruses (B, C, D) or human immunodeficiency virus (HIV). In veterinary medicine, primary cell lines are commonly used in exceptional cases for the cultivation of certain pathogenic viruses in poultry virology. In certain cases, they are even used for vaccine production as well. This is the case, for instance, for porcine parvovirus.

The formerly widespread viral cultivation in embryonated chicken eggs is no longer used routinely. It is only used in certain cases, such as the cultivation of new influenza virus isolates or for vaccine production (▶ Sect. 16.3). By ultracentrifugation of culture supernatants or broken cells, the viruses can be concentrated and rendered available for further investigations. This includes, among other things, electron-microscopic determination of virus particles. Further analyses of viral proteins or genomes serve to characterize the type of virus.

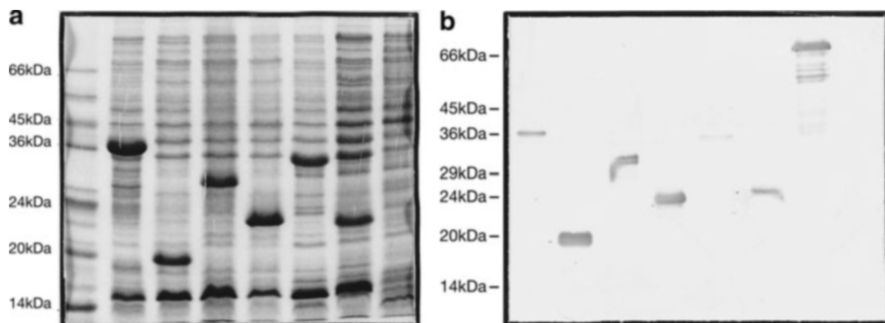


Fig. 13.1 Example for the detection of viral proteins. (**a**) Sodium dodecyl sulphate–polyacrylamide gel. Protein extracts from *Escherichia coli* bacteria expressing different regions of a protein of parvovirus B19 were electrophoretically separated and stained with Coomassie blue. All proteins present in the preparation exhibit a blue colour (here in *black*). (**b**) Western blot. The protein bands of the sodium dodecyl sulphate–polyacrylamide gel shown in (**a**) were transferred to a nitrocellulose membrane (Western blot) and then incubated with rabbit polyclonal antibodies that specifically recognize the parvovirus protein. After a wash procedure, the membrane was treated with secondary antibodies that are conjugated with horseradish peroxidase (immunoglobulins from swine, which are directed against the Fc region of rabbit antibodies) and specifically bind to the bound primary antibodies. Subsequently, the membrane was incubated with diaminobenzidine solutions. In the area of protein bands to which the antibody complexes have bound, a brown precipitate is produced indicating a positive response (Courtesy of Andreas Gigler, Institute of Medical Microbiology, University of Regensburg, Germany)

13.1.1.1 Protein Detection

Western Blotting

One way to determine the type of virus is the identification of specific viral antigens in Western blot tests. For this purpose, the proteins of infected cells or virus particles which were previously pelleted by ultracentrifugation are separated by electrophoresis on a sodium dodecyl sulphate–polyacrylamide gel. Their pattern and molecular masses can provide evidence for the type of virus. However, a final assignment is only possible serologically in the Western blot (Fig. 13.1). For this purpose, the separated proteins are transferred from the polyacrylamide gel to a nitrocellulose or nylon membrane (actual Western blot) and then incubated with antisera containing immunoglobulins, which specifically react with the viral antigens of interest (primary antibody). Optimal are preparations of mouse monoclonal antibodies that recognize defined epitopes of a viral protein. Sera from people who have withstood an infection with the corresponding virus type, and thus have produced specifically binding antibodies, can also be used. The nitrocellulose membranes are then treated with other so-called secondary antibodies, which are specific to the Fc regions of the primary antibody; e.g. with immunoglobulins from swine, which were inoculated with Fc regions of murine antibody molecules, and have developed an immune response against murine antibodies. Human antibodies are usually detected with rabbit immunoglobulins, which bind to the Fc regions of human IgG. The different enzymes necessary for the further detection reactions are

covalently bound to the secondary antibody, e.g. horseradish peroxidase. These secondary antibodies interact with the antigen–antibody complexes on the membranes, and in the next step, they are detected by adding the appropriate substrate, in this case diaminobenzidine. In the positive case, brownish protein bands arise (Fig. 13.1). Alternatively, the use of alkaline phosphatase for labelling produces a blue colour.

Antigen ELISA

This variant of ELISA is employed for detecting viral proteins or particles as an alternative to the analytical Western blot. The test is usually performed in microtitre plates which contain 96 wells and are made from specially treated polystyrene. Murine monoclonal antibodies against a specific viral protein are bound to the surface of the plastic wells. Thereafter, suspensions which contain the virus in question or the viral proteins of interest are added the wells. If the relevant antigens are present, they will interact with the polystyrene-bound immunoglobulins. The antigen–antibody complexes can be detected in the next step by addition of a further antibody, which binds to a different epitope of the same viral protein. These immunoglobulins are covalently conjugated with horseradish peroxidase, so the complex can be visualized by the addition of *o*-phenylenediamine as a soluble substrate. Photometric measurement of the intensity of this chromatic reaction makes possible a quantitative or semiquantitative determination of the viral antigen, which was present in the starting material (Fig. 13.2a). Examples of antigen ELISA applications in human medicine are infections with adenoviruses, influenza viruses, parainfluenza viruses and respiratory syncytial viruses in respiratory secretions and rotaviruses, adenoviruses and noroviruses in faeces. In veterinary medicine, antigen ELISA is used to detect rotaviruses, coronaviruses and parvoviruses in various animal species.

Immunofluorescence

Direct immunofluorescence is used to investigate whether virus proteins are produced in infected cells. The cells are dropped on slides, fixed and treated with alcoholic solvents to render cell membranes permeable. Thereafter, they are incubated with immunoglobulins directed against the viral proteins to be detected. The following treatment with secondary antibodies, which are directed against the Fc region of the previously used immunoglobulins and linked with fluorescent compounds (e.g. fluorescein isothiocyanate), allows one to visualize viral proteins in different compartments, such as the nucleus, the cytoplasm and the cell membranes (Fig. 13.3).

Detection of Virus Properties

Some viruses encode specific enzyme activities, which can be detected as characteristic properties in infected cells, or associated with viral particles in the culture supernatant. These include, for example, the determination of reverse transcriptase activity, which is produced by human immunodeficiency viruses and is a component of the resulting virus particles (▶ Sect. 18.1). On the basis of the amount of this

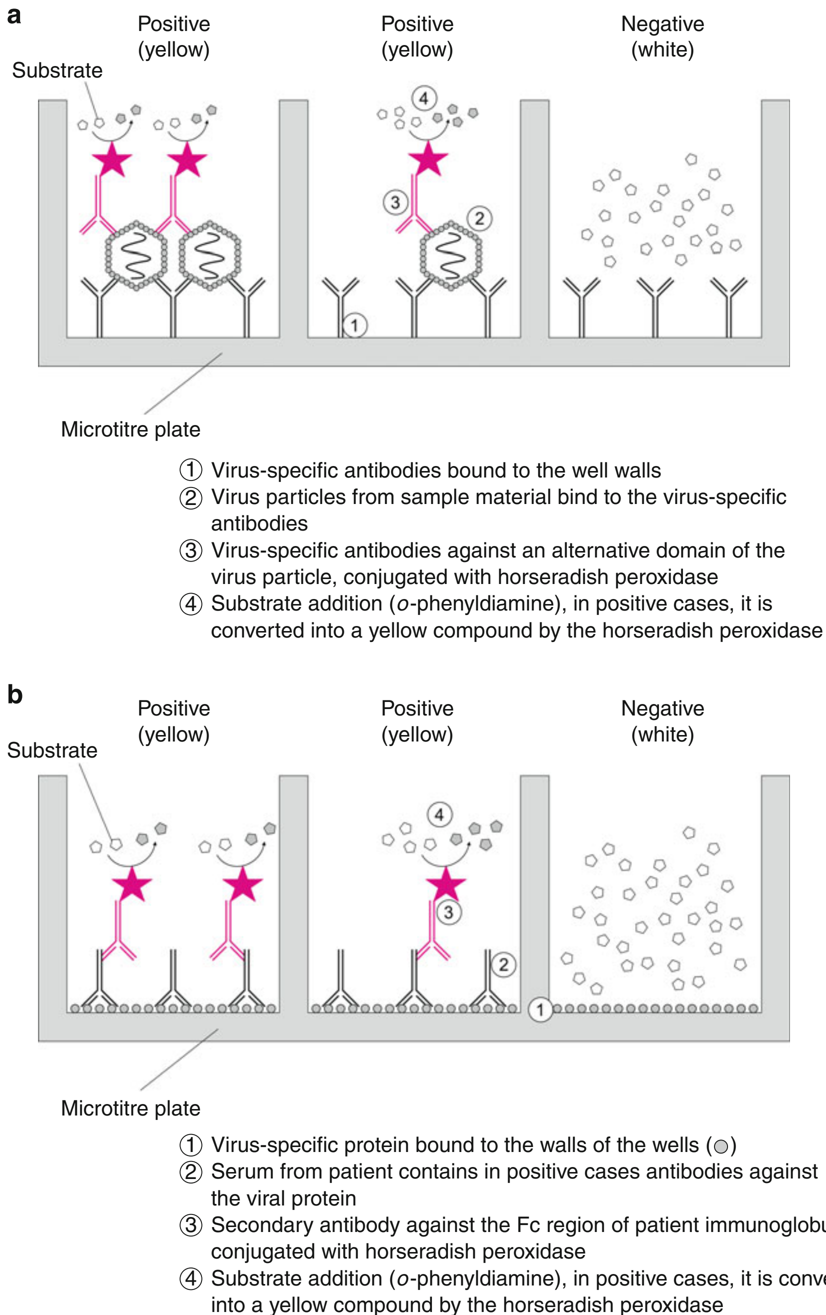
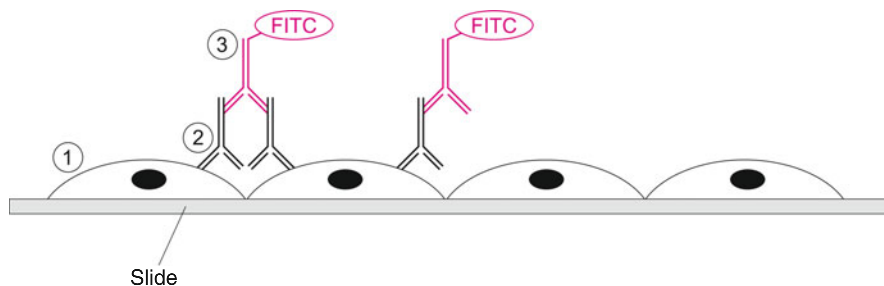


Fig. 13.2 The reaction steps in different types of ELISA. (**a**) Antigen-capture ELISA for detecting viral proteins or particles. (**b**) ELISA to detect specific antibodies (Sect. 13.2)



① Infected, fixed cells

② Specific antibodies, e.g. in sera from patients

③ Secondary antibody against the Fc region of human immunoglobulins; if they are bound, the cellular structures that are recognized by the complex are stained in green because of the fluorescein isothiocyanate (FITC) labelling

Fig. 13.3 The reaction steps in the immunofluorescence test

enzyme detected in the culture supernatant, the number of virus particles produced can be quantitatively determined. Other viruses are able to agglutinate erythrocytes. This haemagglutination capacity is found both in human pathogenic and in animal pathogenic viruses. It is associated with viral envelope proteins, and hence with the virions. Therefore, haemagglutination tests can be performed, among others, with paramyxoviruses and orthomyxoviruses (▶ Sects. 15.3 and ▶ 16.3) as well as with flaviviruses, togaviruses, coronaviruses and parvoviruses (▶ Sects. 14.5, ▶ 14.6, ▶ 14.8 and ▶ 20.1). Erythrocytes of appropriate species are used and mixed with the virus-containing suspensions; if red blood cells agglutinate, this indicates the presence of viruses. If the reaction can be inhibited by adding virus-specific antibodies, then this so-called haemagglutination-inhibition test allows determination of the virus type in the starting material. However, this method has become obsolete in routine diagnosis. Nowadays, the haemagglutination-inhibition test is only performed to evidence rubella-virus-specific antibodies in the context of statutory maternity care (▶ Sect. 14.6).

13.1.1.2 Detection of Viral Nucleic Acids

Alternatively to proteins or enzyme activities, viral nucleic acids can be isolated from infected cells and specifically analysed by Southern blot, Northern blot or dot-blot tests. The purified DNA is cleaved by restriction enzymes. The resulting fragments are separated according to their size by agarose gel electrophoresis. Subsequently, DNA fragments are transferred from the gel to a nitrocellulose or nylon membrane (Southern blot); the same is done for detection of viral RNA (Northern blot), but omitting cleavage with restriction endonucleases. The DNA or RNA preparations can also be spotted directly on the membrane (dot blot). In the case of double-stranded nucleic acid molecules, a denaturation step is required to

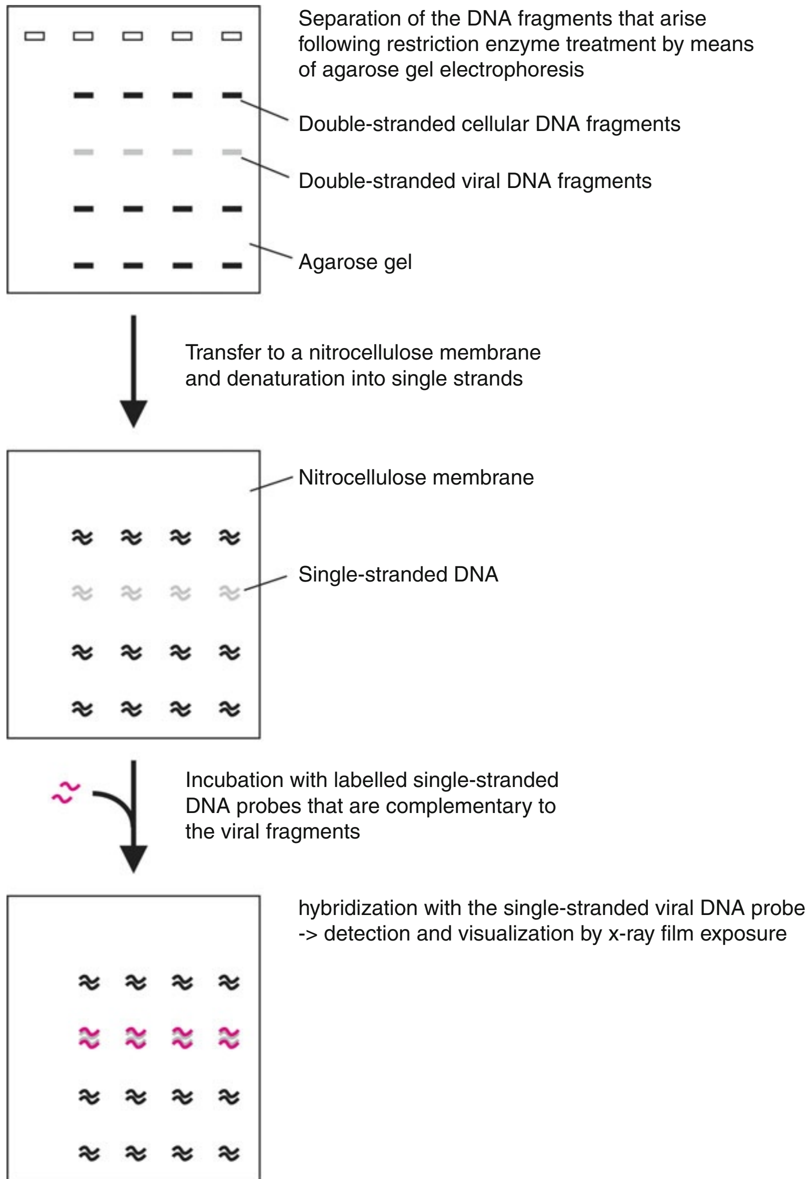


Fig. 13.4 Principle of the Southern blot test

generate single-stranded molecules. Subsequently, the nitrocellulose or nylon membrane is incubated with labelled single-stranded DNA or RNA probes which are complementary to the nucleotide sequences examined and hybridize with them, forming double-stranded molecules (Fig. 13.4). Whereas the hybridization reaction was formerly done using mainly radioactive nucleotides containing ^{32}P or ^{35}S,

non-radioactive systems are now predominantly used. Usually, modified nucleotides are incorporated into the DNA probes, which were labelled with biotin or digoxigenin molecules. After hybridization with the viral nucleic acids, the nitrocellulose or nylon membrane is incubated with streptavidin- or digoxigenin-specific antibodies. These reagents are covalently linked with enzymes that facilitate detection by a colorimetric reaction. Besides the afore-mentioned horseradish peroxidase (see Sect. 13.1.1.1.1), alkaline phosphatase conjugated reagents are increasingly used. Through incubation with X-phosphate (5-bromo-4-chloro-3-indolyl phosphate) and 4-nitro blue tetrazolium chloride, a deep-purple precipitate is formed as evidence of enzyme activity. The intensity of the colour is directly proportional to the quantity of viral nucleic acid on the blot. Alternatively, the detection reaction can also be performed by chemiluminiscent reagents. As these methods are clearly inferior concerning sensitivity and specificity in comparison with modern polymerase chain reaction tests, these methods are only rarely employed for diagnostic purposes today.

13.1.2 Direct Detection of Viruses in Patient Material

Many of the diagnostic tests used are so sensitive that they can also be used for detection of viruses in sera or other materials from patients without previous in vitro cultivation and propagation of the pathogens. This applies especially to infections in which large amounts of virus particles or specific proteins are produced and released into the blood, such as HBsAg in hepatitis B virus infections (▶ Sect. 19.1), the capsid protein p24 in acute HIV infections (▶ Sect. 18.1) and the capsids of parvovirus B19 (▶ Sect. 20.1). In veterinary medicine, the following pathogens are also detected by that means: feline leukaemia virus in the blood of infected cats (▶ Sect. 18.1.6) and canine parvovirus and bovine rotaviruses and coronaviruses in the faeces of the animals (▶ Sects. 14.8.6 and ▶ 17.2.6). The presence of proteins or virus particles can be verified using the methods described in Sect. 13.1.1. Commonly, antigen ELISA is used.

In veterinary medicine, so-called rapid or stick tests have been developed for a number of applications. In such tests, viral proteins are detected by immunochromatographic methods. These tests are based on the principle of an ELISA, but the antibodies are fixed on gold or latex beads (diameter 20 μm) and can be performed in the veterinarian's office. Antibody-loaded beads bind virus particles and/or proteins and migrate, owing to capillary forces, on the membrane, which is submerged in the solution. Antibodies are also fixed on the membrane, and these stop the antigen-loaded beads. These are then visualized as a dark band. Control reactions using immunoglobulin-specific antibodies, which are also bound to the membrane, complement the test (Fig. 13.5). Such rapid tests have been developed, among others, for the detection of various gastroenteritis pathogens in animals (such as parvoviruses, rotaviruses and coronaviruses; ▶ Sects. 14.8, ▶ 17.2 and ▶ 20.1). In human medicine, such simple rapid tests are increasingly used for the diagnosis of influenza virus infections, since treatment with neuraminidase inhibitors follows immediately after a positive result.

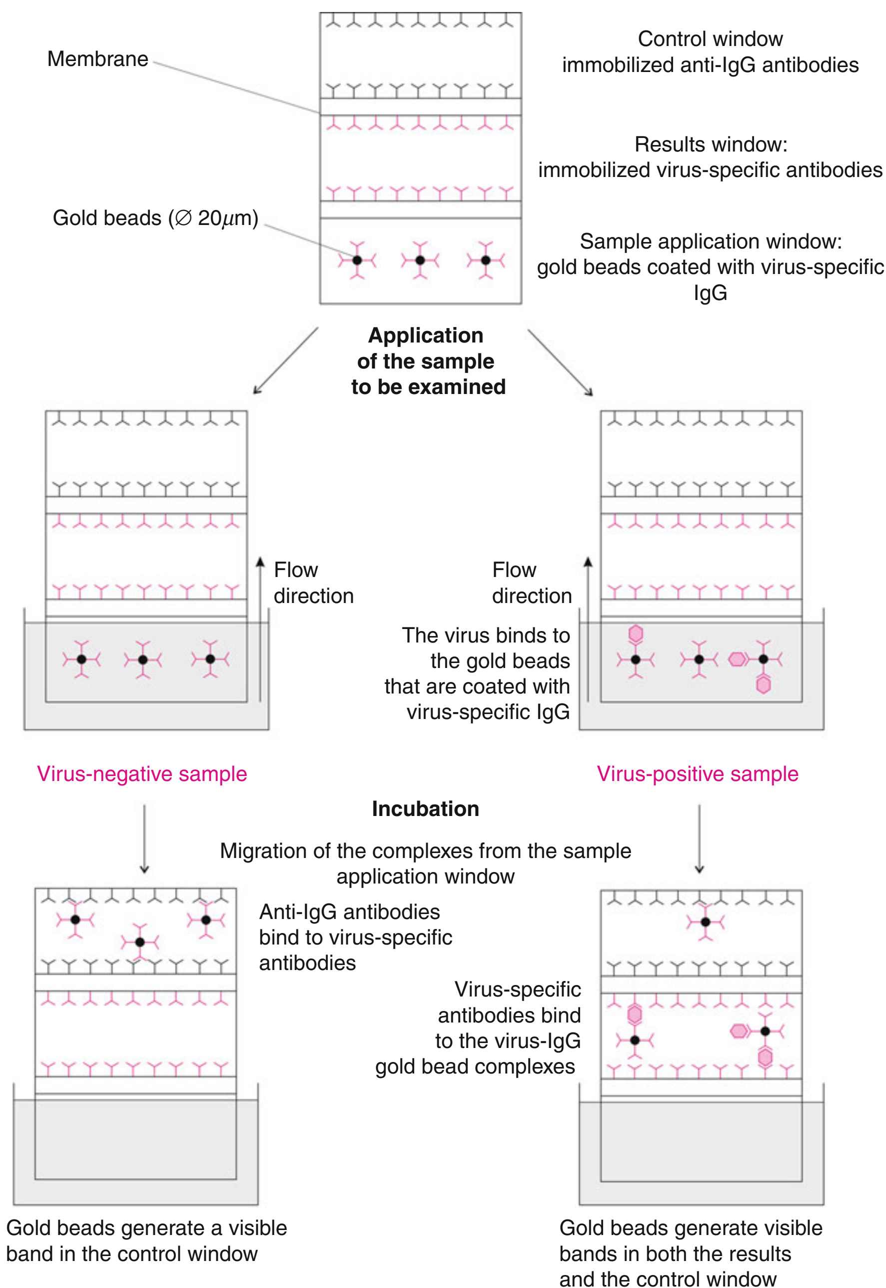


Fig. 13.5 Immunochromatography (rapid test for detection of viruses in patient material). This test is aimed at detecting viruses in liquid patient materials, such as serum, sputum and stool samples. Membranes are used (nitrocellulose or similar) which are coated with specific reagents in different sections (windows). *Sample application window*: large quantities of gold

In recent years, highly sensitive and quantifiable systems have been developed primarily for the detection of viral nucleic acids in the blood or infected fluids and tissues; they have replaced many of the antigen tests, and will be briefly discussed below.

13.1.2.1 Polymerase Chain Reaction

The polymerase chain reaction (PCR) allows the amplification of very small quantities of viral genomes or transcripts directly from patient material. Theoretically and practically, it is possible to detect even a single nucleic acid molecule in the test sample. Initially, the viruses have to be broken up (e.g., using chaotropic agents) and the proteins that are present in the sample must be removed, as they can interfere with the subsequent reactions. They are normally degraded with proteases, or removed by phenol extraction and then precipitated and eliminated from the samples by centrifugation. Two oligonucleotides (primer) must be selected (15–20 nucleotides in length) that are complementary to each strand of the double-stranded DNA, flanking a region of 200–400 bases (in real-time PCR also significantly shorter). The DNA is converted into single strands by heat denaturation (usually at 94 °C). Subsequently, the primers are added in high molar excess, and they hybridize with the respective DNA strands during annealing and form short double-stranded regions (annealing, usually at 50–60 °C). The reaction mixture also contains a heat-stable form of DNA polymerase (usually *Taq* polymerase from the thermophilic bacterium *Thermus aquaticus*) and the four nucleoside triphosphates dATP, dGTP, dCTP and dTTP in appropriate concentrations and buffer systems. The hybridized oligonucleotides function as primers. They provide the necessary free 3′-OH ends, onto which the *Taq* polymerase synthesizes the complementary DNA sequence (elongation or chain extension, usually at 72 °C). This step completes the first cycle. As a result, two double-stranded DNA molecules are present in the reaction mixture, which are separated again by a short heat denaturation step, which initiates the second cycle. During the following annealing,

Fig. 13.5 (continued) beads (diameter 20 μm) contain covalently bound IgG antibodies on the surface, which bind specifically to epitopes on the surface proteins of the pertinent virus. *Results window*: IgG molecules are covalently bound to the membrane, and can bind to the same or different epitopes of the virus to be detected. *Control window*: this contains anti-IgG antibodies covalently bound to the membrane. The test principle is as follows. The membrane is incubated with the biopsy material in the area of the application window and placed in a container with buffer solution. If the material contains the virus in question, then the virus will bind to the IgG antibodies on the gold beads. Virus–IgG gold bead complexes and unloaded beads migrate with the buffer front into the results window. IgG antibodies, which are covalently linked to the membrane, bind to free epitopes on the surface of the virus. In this way, the migration of virus–IgG gold beads is stopped, and they form a golden (*dark*) band within the results window. The unloaded (virus-free) IgG gold beads migrate further with the buffer front into the control window, where their migration is stopped, as they react with the immobilized anti-IgG antibodies. As a result, a second band arises in the control window. From the strength of the bands and their ratio, it is possible to infer the amount of virus in the starting material. If the test material does not contain any viruses, only one band will be formed in the control window

oligonucleotides hybridize again with the single strands and serve as a primer for the synthesis of further double strands: thus, the original DNA molecule has been amplified in a chain reaction to four double-stranded molecules. The cycles are repeated as often as desired, achieving a logarithmic amplification of nucleic acid molecules (2^n, n is the number of cycles; Fig. 13.6). After about 30–40 cycles, the logarithmic amplification phase ends owing to depletion of reagents and enzyme; the PCR amplification products can be separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. For analytical purposes, such as cloning of amplification products, it is also possible to design the primers in such a way that they contain unique restriction enzyme cleavage sites. If the amplified double-stranded DNA is cleaved, the resulting fragment can be identified according to its size after separation of the reaction mixture by agarose gel electrophoresis, or by a subsequent Southern blot assay; or it can be processed further. To increase the diagnostic sensitivity, but also the specificity, two PCRs can be combined by a second, inwardly shifted primer pair (nested PCR). Since DNA is very stable over long periods of time, its sequences can be detected in older and even in fixed tissue samples. Therefore, viruses can be detected in very old, formaldehyde-conserved samples and even in embalmed mummies.

The use of the PCR technique also allows one to investigate how certain virus types, e.g. influenza viruses, have changed and developed in the past centuries. This may provide more precise insights into viral evolution.

If the original nucleic acid is RNA, it is primarily converted into single-stranded DNA using a reverse transcriptase and an appropriate primer. This is followed by the amplification reactions described above. Both reactions can be done in the same reaction tube and using identical buffer conditions. *Taq* polymerase can also be modified so that it can catalyse both reactions. With use of appropriate control standards it is also possible to determine the quantity of nucleic acid in the starting material, e.g. in sera. A disadvantage is often the high sensitivity of the PCR method, particularly nested PCR: since it is possible to amplify even a single DNA molecule, minimal contamination in the reaction mixture is sufficient to generate false-positive results. Besides this carry-over contamination, a primary contamination can occur by infiltration within the laboratory when very high amounts of virus are present in the material (e.g. norovirus or parvovirus B19). This risk is especially high in diagnostic laboratories, where the pertinent materials are routinely handled. Therefore, depending on the PCR application, all solutions, buffers and reaction mixtures which are used for the PCR must be prepared and pipetted in separate rooms (spatial compartmentalization); furthermore, negative controls have to be performed in all tests to indicate possible contamination.

Nowadays, there are automated test systems that allow quantitative determination of viral nucleic acids in the starting material (real-time PCR). For this purpose, the DNA or RNA molecules are amplified as described above. A defined set of single-stranded probes which have a length of 25–40 nucleotides and are complementary to sequences of the amplified regions are added to the reaction mixture. These sequence-specific probes are labelled with a fluorescent group at the 5′ terminus (e.g. 6-carboxyfluorescein). Conversely, they carry a different chemical

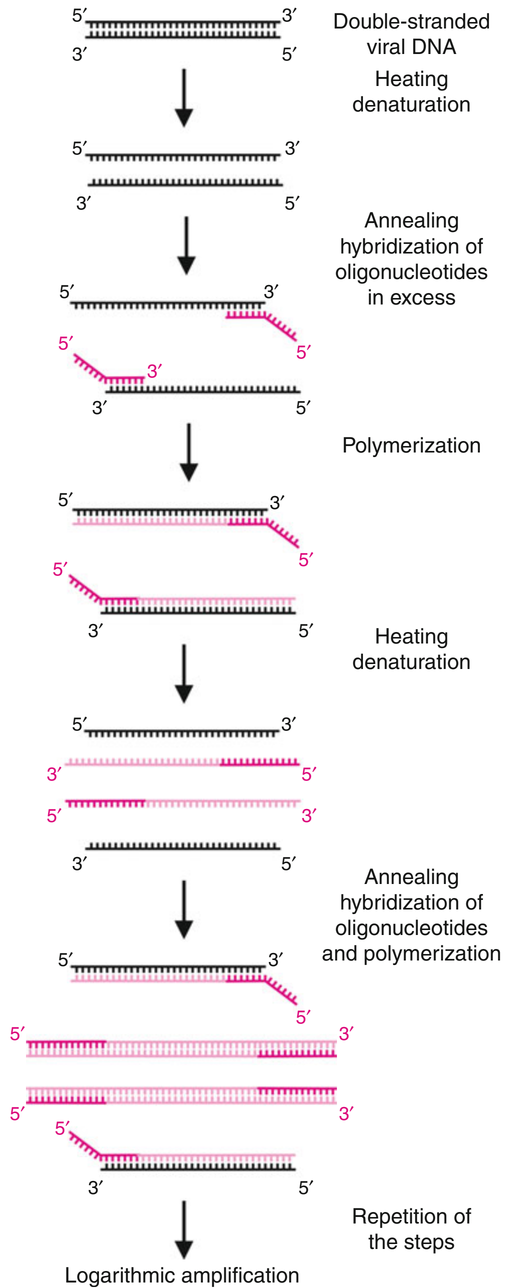


Fig. 13.6 Principle of the polymerase chain reaction

group at the 3′ end (e.g. 6-carboxytetramethylrhodamine) which suppresses the fluorescence of 6-carboxyfluorescein. Prerequisite for this quenching effect is that the two groups are located close to each other, e.g. at the two ends of each probe (fluorescence energy resonance transfer technology). If viral genomes are present in the sample, then the nucleotides of the probe are degraded by the exonuclease activity of *Taq* polymerase during the polymerization process. This step leads to separation of the two fluorescent groups. The quenching effect is abolished by this step and the fluorescence of 6-carboxyfluorescein can be measured in real time. The more viral nucleic acid was in the starting material, the higher is the fluorescence intensity. Common procedures have a logarithmic amplification efficiency of six to eight logarithmic magnitudes. With use of several primer pairs and differentially labelled probes, various amplification products can also be detected specifically and quantitatively in a single assay (multiplex PCR). Thus, internal controls are possible for checking both the DNA extraction efficiency and possible internal inhibitions. Another advantage of real-time PCR is the significantly reduced risk of end-product contamination.

Alternatively, the amplified DNA sequences can be quantitatively detected in an ELISA-like hybrid capture assay. The amplification products are extracted from the solution by a specific probe that is bound to the polystyrene microtitre plates. If biotinylated primers or nucleotide derivatives are added to the amplification step, then they can be detected by peroxidase-conjugated antibodies.

13.1.2.2 In Situ Hybridization

In frozen sections of infected cells or tissues, such as in pathology, viral DNA and RNA can be detected by in situ hybridization with specific, labelled DNA or RNA probes that are complementary to the target sequence. Usually, the probes are labelled with ^{3}H-thymidine or biotinylated nucleotide derivatives. In the first case, after hybridization, the frozen sections are coated with a film emulsion and subsequently developed, whereby a granular blackness can be observed in the infected cells by microscopic examination. This method can be combined with PCR, so even minute amounts of viral nucleic acids can be detected in frozen sections (in situ PCR).

13.1.2.3 Branched DNA Detection

This test system is suitable for the detection of nucleic acids in sera of patients. It was developed to determine the quantity of DNA genomes of hepatitis B virus as well as the amount of RNA genomes of HIV and hepatitis C virus. The proteins that are present in the samples are degraded by protease treatment, and DNA as well as RNA molecules are concentrated by centrifugation. DNA oligonucleotides are added to the solution as primary probes, which are partially complementary to viral genome sequences and form with them short double-stranded regions; the non-complementary part of the oligonucleotide remains as a single strand. This nucleic acid complex is bound to microplates containing oligonucleotides as capture probes

in polystyrene wells and that are complementary to other regions of the viral genomes In the next step, DNA molecules are added, and they hybridize through their ends with the single-stranded regions of the primary probes and have about 20 branches, whose sequences can hybridize with further complementary probe oligonucleotides. These probes are labelled with alkaline phosphatase. The quantitative detection is performed by addition of substrates which release chemoluminiscent molecules during the enzymatic reaction. Inasmuch as the original sequence is not amplified in the branched DNA detection method, but the signal is, it is less vulnerable to contamination.

13.2 How Are Specific Immune Reactions Used for the Indirect Detection of Viral Infections?

As already mentioned, viruses are present and can be detected often only for a short time in patients. Therefore, a diagnosis must frequently be made indirectly, i.e. by determining the immune response that is elicited against a given pathogen during infection. Usually, patient antibodies are detected which specifically bind to specific viral proteins in the serum, and sometimes in the cerebrospinal fluid. IgM antibodies generally indicate an acute or recent infection. By contrast, if IgG antibodies against a specific virus are detected, a past or former infection can be inferred. They are also indicative of an immune status which protects the person from a new infection with the same pathogen (▶ Chap. 7). Especially for diagnosis of acute infections, it is important to determine the concentration of IgM and IgG antibodies during infection. Occasionally, one also tests for IgA antibodies. All antibodies can be detected by Western blot, ELISA or indirect immunofluorescence analyses. Sometimes, the haemagglutination-inhibition test is also used. If specific functions are to be associated with immunoglobulins, such as their ability to neutralize the corresponding virus, then one examines whether the immunoglobulins can inhibit the infection in vitro. For this purpose, specific quantities of infectious virus particles are incubated with antibodies before the mixture is added to the cells of a tissue culture (Sect. 13.1). Neutralization tests are still routinely used for detecting antibodies against poliovirus and in veterinary virology and for the detection of antibodies against canine distemper virus.

13.2.1 Western Blotting and ELISA

Western blotting and ELISA differ from the tests described in Sect. 13.1 in one point: in this case, the antigen is predefined, i.e. certain viral proteins or particles. They are separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Western blot). In ELISA, the protein preparations are bound to the polystyrene of microtitre plates or other matrices. The patient sera in which the antibodies in question are to be detected are incubated with the nitrocellulose membrane or pipetted into the wells of microtitre plates.

In this case, the detection reactions are also performed with enzyme-conjugated secondary antibodies that specifically recognize and bind to the Fc region of human IgM or IgG (Fig. 13.2b). ELISA does not only allow an answer to the question of against which viral antigen the antibodies are directed, but also an answer to the question of the concentration of immunoglobulins, as serial dilutions of sera can be analysed. Until a few years ago, viruses cultivated and enriched in cell culture were commonly used as antigen material in Western blotting or ELISA. However, viral proteins are preferentially used today, and they are produced using genetic engineering methods. For this purpose, viral antigens are chosen against which virus-type-specific antibodies are produced during infection and that are also conserved and hence not easily mutable. Usually, they are viral structural proteins. The genes that code for these proteins are cloned and expressed in *Escherichia coli* or yeast under the control of bacterial promoters. These bacteria produce large amounts of the respective viral proteins, which can be purified and used as antigens in Western blotting or ELISA. This approach has proven to be cheaper and less hazardous than the cultivation of large amounts of infectious pathogens. Is also known that antibodies are regularly produced against specific epitopes in the course of infection; these protein regions can be synthesized chemically as peptides and used as antigens in ELISA.

The use of purified protein preparations makes the electrophoretic separation of proteins dispensable. In these cases, they are directly applied on the membranes with a brush or a fountain pen. In these line-blot tests, the same membranes are used as are employed in Western blot tests.

13.2.2 Indirect Immunofluorescence Tests

For this purpose, in vitro infected culture cells are deposited and fixed on slides. The serum dilutions to be tested are added to the cells and bound antibodies are detected by using fluorescein isothiocyanate conjugated immunoglobulins that, depending on the question to be answered, are directed against IgG or IgM of the species examined.

13.2.3 Test Systems for Detection of the Cellular Immune Response

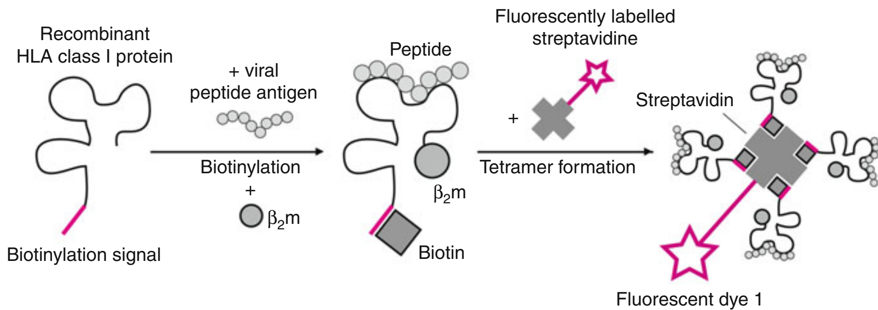
For several years, attempts have been made to obtain additional diagnostic or prognostic information by detecting specific cellular immune responses. The test systems used are considerably more complex than those employed for the detection of specific antibodies. In the first step, T lymphocytes must be isolated from the blood of the subjects by density gradient centrifugation (e.g. Ficoll gradient) or enriched by lysis of erythrocytes. The further purification of the different cell populations is performed by binding of cells to magnetic beads coated with specific

antibodies (e.g. directed against CD4 or CD8 receptors on the surface of T-helper cells and cytotoxic T lymphocytes). Alternatively, the cells can be labelled with specific antibodies directed against certain surface proteins and then isolated by fluorescence-activated cell sorting (FACS). In the second step, the respective T-cell populations are analysed in tests.

In addition to intracellular staining for detection of cytokines in the cells by FACS analyses, the tetramer test is used when specific T cells which recognize viral peptides in complex with MHC class I antigens are to be detected. The procedure relies upon recombinantly produced MHC class I or MHC class II proteins which are linked with biotin molecules in their carboxy-terminal domains. After their purification, the MHC class I protein preparations are incubated with β_2-microglobulin and peptides (in the case of MHC class II proteins only with peptides) that are derived from the viral antigens against which the T lymphocytes react that one wants to detect. In the next step, the peptide–MHC–biotin complexes bind to avidin, which is labelled with fluorescent dyes (e.g. fluorescein or phycoerythrin) and has four binding sites for biotin. The fluorescent avidin–biotin–peptide–MHC complexes are then incubated with purified T lymphocytes, which were isolated from the blood of the subjects examined (Fig. 13.7). If T cells whose T-cell receptors specifically bind to the MHC–peptide complexes are present in the cell preparation because of a past infection, then the fluorescent complexes will bind to the surface of T cells, which can be quantitatively detected by FACS analyses.

The lymphocyte proliferation or stimulation test is used for detecting specifically reactive lymphocyte populations. In this procedure, the isolated lymphocytes are incubated with the antigens in question or with antigen-presenting cells. Lymphocytes, which recognize the antigen in question, begin to proliferate. Lymphocyte proliferation can be detected by addition of ^{3}H-thymidine to the culture medium and can be followed by measuring the incorporation of the radioactive nucleotide into the cellular DNA. Alternatively, secreted proteins (such as certain interleukins), which are released as a result of lymphocyte recognition, can be detected by ELISA in the culture supernatant or by means of enzyme-linked immunosorbent spot tests at the level of individual cells.

The enzyme-linked immunosorbent spot test is used for the quantitative determination of lymphocytes, which produce and secrete certain proteins (such as interleukins, chemokines and antibodies) as a result of specific antigen recognition (or repeated stimulation). If the detection of interleukins or chemokines is desired, then antibodies which specifically bind to the respective proteins are attached to the polystyrene material of microtitre plates; if specific antibodies are to be detected, then the microtitre plates are coated with the corresponding proteins. The lymphocytes to be examined, which were isolated from the blood of subjects, are added to the wells of the microtitre plate so that they form a thin layer on the floor. During an incubation period at 37 °C, the lymphocytes secrete the products in question, which bind to the interleukin-specific antibodies or to the protein antigens on the floor of the microtitre plate.



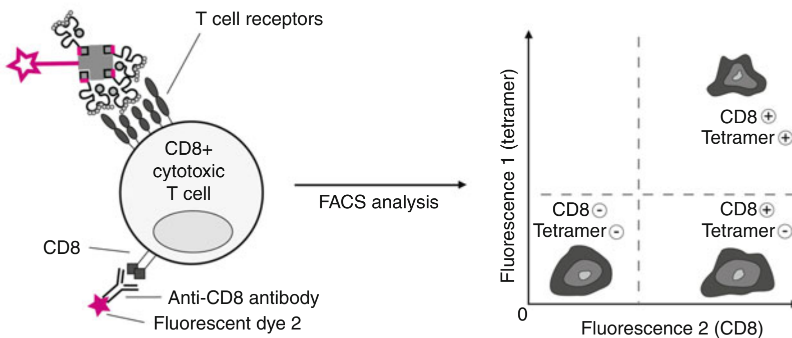


Fig. 13.7 Course of the tetramer test for detection of specific reactive T lymphocytes. (**a**) Production of tetrameric molecules. Recombinantly produced HLA class I antigens are biotinylated at a carboxy-terminally attached biotinylation domain and incubated with a virus-specific peptide antigen (T-cell-specific epitope) and recombinantly produced β_2-microglobulin (*β_2m*). Four biotinylated HLA class I/peptide/β_2-microglobulin complexes bind to streptavidin molecules, which were labelled with fluorescent dye 1, forming "tetramers". (**b**) Detection of virus-specific T cells by flow cytometry. $CD8^+$ T lymphocytes are isolated from the blood of patients and incubated with CD8-specific monoclonal antibodies, which are labelled with another fluorescent dye. The labelled $CD8^+$ T cells are incubated with the tetrameric complexes. If T lymphocytes are present which recognize the viral peptide antigen, they will bind to the tetrameric complexes using their specific T-cell receptor. On the basis of their fluorescent labelling, the T cells that are linked to the tetrameric complexes can be measured, quantified and distinguished from those without complexes by fluorescence-activated cell sorting (*FACS*) analyses

Subsequently, the lymphocytes are removed and the plates are washed. A semisolid agar layer is added containing dye-labelled antibodies, which recognize the complexes formed. Each of the relevant antibodies or interleukin-producing lymphocytes is depicted in a perceptible colour point on the floor of the plate. Counting is usually performed by appropriate automated camera and evaluation systems.

13.3 What Important New Methods for Detection of Viruses Have Been Developed in Recent Years?

13.3.1 Multiplex Reactions and Genotyping

In contrast to the general detection of bacterial infections, e.g. by amplification of bacterial 16S ribosomal RNA, no general screening test is available for detecting viral infections. This implies searching specifically for each potential pathogen. With regard to practicality and costs, the ability to detect multiple viruses using a single PCR would be a solution. Ideally, all possible pathogens for diarrhoea or meningitis could be detected in a single assay. The principle of multiplex PCR takes this into account, as several primer pairs and probes can be mixed together. However, this approach is generally associated with a loss of sensitivity. An elegant solution is when a conserved region can be amplified with a primer pair, in which the determination of subtypes or genotypes is possible by different probes with a sufficient number of sequence differences. This principle has been successfully applied in determining the high-grade and low-grade malignant subtypes of human papillomaviruses. On the basis of microarray chip technology, efforts are currently being made to develop miniaturized chip tests for viral diagnostics.

13.3.2 Resistance Tests

In the treatment of persistent viral infections, such as with HIV, hepatitis B virus, hepatitis C viruses, herpes simplex virus and cytomegalovirus, we are confronted with the problem of the development of resistant viruses. It is often important to search specifically for the presence of well-known mutations which determine the respective resistance. In the case of HIV, phenotypic tests were initially applied in which the virus infecting the patient was cultivated in cell culture and examined for sensitivity to the corresponding drugs. Later, it was attempted to insert the tested genes in recombinant viruses. But, it is more practical to determine the predominant genotype of the virus, e.g. by PCR and subsequent sequencing. In the case of HIV, the genes encoding the reverse transcriptase complex and the viral protease are examined. Owing to the increasing prevalence of already resistant viruses in new infections, this can also be important before the first therapy.

13.3.3 Biosensors

In recent years, biosensors have been increasingly discussed and tested as rapid and sensitive test systems for the detection of various classes of substances and molecules. The immunosensors allow the measurement of specific antigen–antibody complexes; they are based on different principles, which can be addressed herein only briefly. For use as chemical sensors in liquids, such as sera, volume resonators seem to be particularly appropriate today. These include oscillating quartz crystals,

which (depending on the test principle) are coated with antigenic proteins or monoclonal antibodies on a specially treated surface. If an alternating electric voltage is applied to these quartz crystals, the crystal becomes excited, resulting in elastic oscillations, whose amplitude reaches a maximum when the electrical frequency matches one of the natural mechanical frequencies of the respective quartz crystal. These oscillations can be measured by appropriate measuring systems. If a quartz crystal coated with antigens is placed in a solution containing specifically binding antibodies, then they bind to its surface and alter its mass. This changes the oscillation frequency, indicating positive antibody detection. Besides these piezoelectric immunosensors, attempts are being made to develop measuring techniques that work similarly to the potentiometric electrodes of pH metres. In this case, the objective is to determine the potential change that arises owing to the antigen–antibody complex formation on a thin, equilibrated silica gel layer on the surface of the pH glass membrane. Ion-sensitive field-effect transistors are a subgroup of potentiometric immunosensors; they have the fastest response time (only a few seconds) among all the systems mentioned, thus making possible extremely fast measurements.

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Part II

Viruses with Single-Stranded, Positive-Sense RNA Genomes

14

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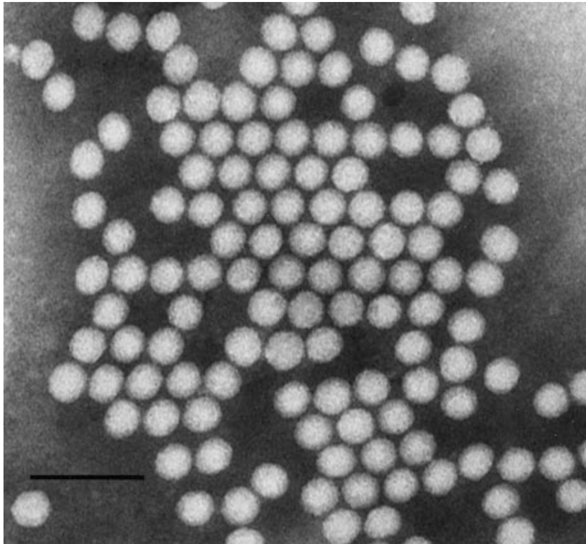
Eight virus families whose members infect vertebrates are currently known to possess single-stranded, positive-sense RNA genomes: the families *Picornaviridae*, *Astroviridae*, *Caliciviridae* and *Hepeviridae* have non-enveloped capsids, whereas the families *Flaviviridae*, *Togaviridae*, *Arteriviridae* and *Coronaviridae* are characterized by enveloped capsids. They all have in common the property of using their own genome as messenger RNA (mRNA), from which they synthesize one or several polyproteins that are subsequently cleaved into individual proteins by viral or cellular proteases. These viruses possess the genetic information for the synthesis of an RNA-dependent RNA polymerase. This enzyme transcribes the positive RNA strand as well as the complementary negative RNA strands, which arise as intermediate products of genome replication. In the course of this process, the new genomic RNA molecules are generated from the second transcription step. The classification into the different taxonomic families depends on the number, size, position and orientation of viral genes in the RNA molecule, the number of different polyproteins that are synthesized during viral infection and the existence of an envelope as a virion component.

14.1 Picornaviruses



The prototypic member of picornaviruses was discovered in 1898 by Friedrich Loeffler and Paul Frosch, who described the pathogen of foot-and-mouth disease (FMD; also referred to as hoof-and-mouth disease) as a filterable agent, thus demonstrating the existence of animal pathogenic viruses for the first time. Karl Landsteiner and Emil Popper published an article in 1909 which reported the identification of a virus as the pathogen of poliomyelitis, a disease that was described for the first time by Jacob von Heine in 1840 and later also by Oskar Medin. That these viruses are able to cause a cytopathic effect in cultures of human embryonic cells was demonstrated by John F. Enders, Thomas H. Weller and Frederick C. Robbins in 1949; however, the actual characterization as poliovirus was ascertained by Herdis von Magnus and co-workers only in 1955.

Coxsackievirus was discovered by Gilbert Dalldorf in 1947, when he infected newborn mice with virus-containing material and observed paralysis in the treated animals. The virus was named after the town Coxsackie in the federal state of New York, where it was isolated from a patient. Owing to different paralysis characteristics that they cause in newborn mice, coxsackieviruses have been subdivided into two groups, A and B. In addition to these viruses, there are also human pathogenic picornaviruses which in contrast to coxsackieviruses do not cause any signs of paralysis in newborn mice, but are associated with a lethal outcome within a few days: echoviruses and parechoviruses. The eponymous acronym "echo" stands for some viral properties: enteric, cytopathogenic, human, orphan; the last term indicates that no disease could be associated with the viral infection at that time. The multifarious symptoms that are caused by these viruses were discovered later and include diarrhoea, eczema and in rare cases also encephalitis and meningitis.

Another human pathogenic picornavirus was characterized much later: hepatitis A virus, the pathogen of an epidemic form of liver inflammation for which the genus *Hepatovirus* was created. Epidemiologically and diagnostically, hepatitis A was distinguished at an early date from hepatitis B. The first electron-microscopic detection of hepatitis A virus was performed by Stephen F. Feinstone and colleagues in 1973. However, the successful cultivation of this virus was only achieved in 1979; it was assigned to the picornavirus family in 1982. Besides the pathogens mentioned, there are also picornaviruses that can infect humans very frequently: namely rhinoviruses as the causative agent of the common cold.

14.1.1 Classification and Characteristic Prototypes

The family *Picornaviridae* is classified into the order *Picornavirales* together with the families *Dicistroviridae*, *Iflaviridae*, *Marnavidirae* and *Secoviridae*. Whereas members of the families *Dicistroviridae* and *Iflavividae* infect various species of insects and other invertebrates, those belonging to the families *Marnaviridae* and *Secoviridae* are pathogenic in algae and plants. The family *Picornaviridae* comprises a large number of virus groups and virus types (Table 14.1) that infect vertebrates. The members of this family can cause completely different disorders in humans, and partially also severe diseases. The name "picorna" is an abbreviation that alludes to two molecular properties of the virus family, i.e. small (*pico*) viruses with an RNA genome. The classification of picornaviruses into 17 genera is based on their respective molecular-biological properties, sequence homologies as well as on the diseases they induce: *Enterovirus*, *Parechovirus*, *Hepatovirus*, *Cardiovirus*, *Sapelovirus*, *Teschovirus*, *Kobuvirus*, *Aphthovirus*, *Erbovirus*, *Avihepato*virus, *Tremovirus*, *Senecavirus*, *Aquamavirus*, *Cosavirus*, *Dicipivirus*, *Megrivirus*, and *Salivirus*. More than 150 known human pathogenic viruses that belong to the genus *Enterovirus* have been newly classified on the basis of new sequence data of the viral genomes. Until recently, human rhinoviruses were also assigned to the genus *Enterovirus*, which comprises a large number of different species and types of viruses which can infect both animals and humans. Today, this genus is subdivided into 12 species, namely enteroviruses A-I and rhinoviruses A, B and C. All species differ by certain molecular properties (e.g. human enteroviruses A and B differ from human enteroviruses C and D by distinct sequence elements within the 5′ untranslated region, UTR) and/or in regard to their infection courses. Owing to its similarity to human enterovirus C, poliovirus has been assigned to this species; however, poliovirus differs from human enteroviruses by its property of causing poliomyelitis. Genome sequencing revealed that many of the different types of coxsackieviruses, enteroviruses and echoviruses have arisen by genetic recombination between various enteroviruses. For example, the animal pathogenic swine vesicular disease virus is a recombinant between the genomes of human coxsackievirus B5 – from which the coding region for the structural proteins is derived – and echovirus 9, from which the sequences encoding the

Table 14.1 Characteristic members of picornaviruses

Genus	Human virus	Animal virus
Enterovirus	**Enterovirus A**	**Enterovirus A**
	Coxsackievirus A2-A8, A10, A12, A14, A16 Enterovirus A71, A76, A89-91, A114, A119	Enterovirus A19, A43, A46, A92 (simian) Enterovirus A13 (baboon)
	Enterovirus B	**Enterovirus B**
	Coxsackievirus A9, B1-B6 Echovirus 1–7, 9, 11–21, 24–27, 29–33 Enterovirus B69, B73–75, B77–88, B93, B97, B98, B100, B101, B106, B107	Enterovirus B110 (chimpanzee) Simian enterovirus SA5
	Enterovirus C	
	Poliovirus 1–3	
	Coxsackievirus A1, A11, A13, A17, A19-22, A24	
	Enterovirus C95, C96, C102, C104, C105, C109, C113, C116-118	
	Enterovirus D	**Enterovirus D**
	Enterovirus D68, D70, D94, D111	Enterovirus D120 (gorilla)
		Enterovirus D111 (chimpanzee)
		Enterovirus E
		Bovine enterovirus/BEV A1–A4
		Enterovirus F
		Bovine Enterovirus/BEV B1–B6
		Enterovirus G
		Porcine enterovirus/PEV 9, 10, 14, 15, 16
		Ovine enterovirus/OEV 1
		Enterovirus H
		Simian enterovirus/SEV A1
		Enterovirus J
		Simian virus 6
		Enterovirus J103, J108, J112, J115, J121 (simian)
	Rhinovirus A	
	Rinovirus A1, A2, A7–13, A15, A16, A18–25, A28–34, A36, A38–41, A43–47, A49–51, A53–68, A71, A73–78, A80–82, A85, A88–90, A94–96, A98, A100–103	
	Rhinovirus B	
	Rhinovirus B3-6, B14, B17, B26, B27, B35, B37, B42, B48, B52, B69, B70, B72, B79, B83, B84, B86, B91, B93, B97, B99	
	Rhinovirus C	
	Rhinovirus C1–51	

(*continued*)

Table 14.1 (continued)

Genus	Human virus	Animal virus
Sapelovirus		**Porcine sapleovirus** (PEV 8)
		Simian sapelovirus
		Avian sapelovirus (duck)
Parechovirus	**Parechovirus**	**Ljungan virus** (rodents)
	Human Parechovirus 1–16	
Hepatovirus	**Hepatitis A virus**	
Avihepatovirus		**Duck hepatitis virus A**
Senecavirus		Seneca Valley virus (natural host unknown)
Cardiovirus	**Theilovirus**	**Theilovirus** (rodents)
	Saffold virus 1–8; Vilyuisk human encephalomyelitis virus	Theiler's murine encephalomyelitis virus; Theravirus
		Encephalomyocarditis virus Mengovirus (mice)
Teschovirus		**Porcine teschovirus** 1–13
Tremovirus		**Avian encephalomyelitis virus**
Kobuvirus	**Aichivirus A**	**Aichivirus B**
		Bovine kobuvirus
		Aichivirus C
		Aka porcine kobuvirus, canine kobuvirus, murine kobuvirus
Aphthovirus		**Bovine rhinitis Avirus**
		Bovine rhinitis B virus
		Equine rhinitis A virus
		Foot-and-mouth disease virus types O, A, C, SAT1-3, Asia 1
Erbovirus		**Equine rhinitis B virus**
Cadicivirus		**Cadicivirus A** (canine)
Cosavirus	**Cosavirus A–D**	
	Dekavirus	
Salivirus	**Salivirus A**	
	Human klassevirus 1	
Megrivirus		**Melegrivirus A**
		Turkey hepatitis virus 1
Aquamavirus		**Aquamavirus A**
		Seal picornavirus 1

The newly defined virus species are indicated in bold, benath are given the traditional names of the virus types/isolates, which are partially still in use

non-structural proteins originated. *Aichi virus*, a member of the genus *Kobuvirus*, is widely spread as a pathogen of gastrointestinal infections especially in Asia. Similar manifestations have been observed with Cosa- and Saliviruses. Human rhinoviruses are grouped into three distinct species: rhinoviruses A, B, and C.

This subdivision is based on differences in the amino acid sequences of capsid proteins. Nonetheless, many of the currently known virus types have still not been assigned to any of these species. The genera *Cardiovirus*, *Sapelovirus*, *Teschovirus*, *Erbovirus*, *Cadicivirus*, *Aquamavirus*, and *Aphthovirus* comprise primarily animal pathogenic picornaviruses that partly cause animal epidemic diseases and have considerable importance for animal breeding and food technology; e.g. FMD virus, which has been classified into the genus *Aphthovirus*. Seven different serotypes can be distinguished worldwide today. The name of this genus alludes to the blisters or cysts (aphthae) that arise in the mucosa of infected animals. The genera *Avihepatovirus*, *Megrivirus* and *Tremovirus* have been created for species that have been isolated from birds. Only one viral species has been assigned to the genus *Senecavirus*: Seneca valley virus was identified in the cultured cell line PER C6; its natural host has not yet been identified. The 5′ UTR of this new virus displays some similarities to that of pestiviruses and hepaciviruses (Sect. 14.5).

In addition to this taxonomic classification, picornaviruses can be subdivided into two principal groups according to their molecular properties that are closely associated with pathogenesis: (1) acid-stable viruses that can survive in the acid environment of the stomach without a loss of infectivity, thus infecting the host organism preferentially through the digestive tract – to this group belong enteroviruses, parechoviruses, hepatoviruses, avihepatoviruses, sapeloviruses and teschoviruses; (2) acid-labile viruses that infect the host organism preferentially via the upper respiratory tract, such as rhinoviruses, aphthoviruses and erboviruses.

14.1.2 Structure

14.1.2.1 Virus Particle

All picornaviruses have a very similar structure, which consists of a non-enveloped icosahedral nucleocapsid with a diameter of approximately 30 nm. The capsids are composed of four viral proteins: VP1, VP2, VP3 and VP4. Occasionally, a fifth polypeptide is found in inconstant quantities; it is denominated VP0 and is the precursor of components VP2 and VP4, which arise by proteolytic cleavage during viral maturation. Especially in parechoviruses, VP0 is barely cleaved; therefore, their particles contain large quantities of the precursor protein. Inasmuch as the particle structure of several picornaviruses has been elucidated by X-ray diffraction analysis, the arrangement of the different viral structural proteins in the particles is well known. The virions are composed of 60 units of each of the four single proteins VP1, VP2, VP3 and VP4, in which VP4 is localized inside the particle and in association with the RNA genome. The hepatovirus VP4 is very small (21–23 amino acid residues), and its presence in the virion has not been definitively resolved. VP1, VP2 and VP3 form the surface of the icosahedron (Fig. 14.1).

The virions of enteroviruses, parechoviruses, kobuviruses, cardioviruses, teschoviruses and hepatoviruses are very stable and cannot be compromised even by an acid environment with pH values of 3 and below. This suggests that a very

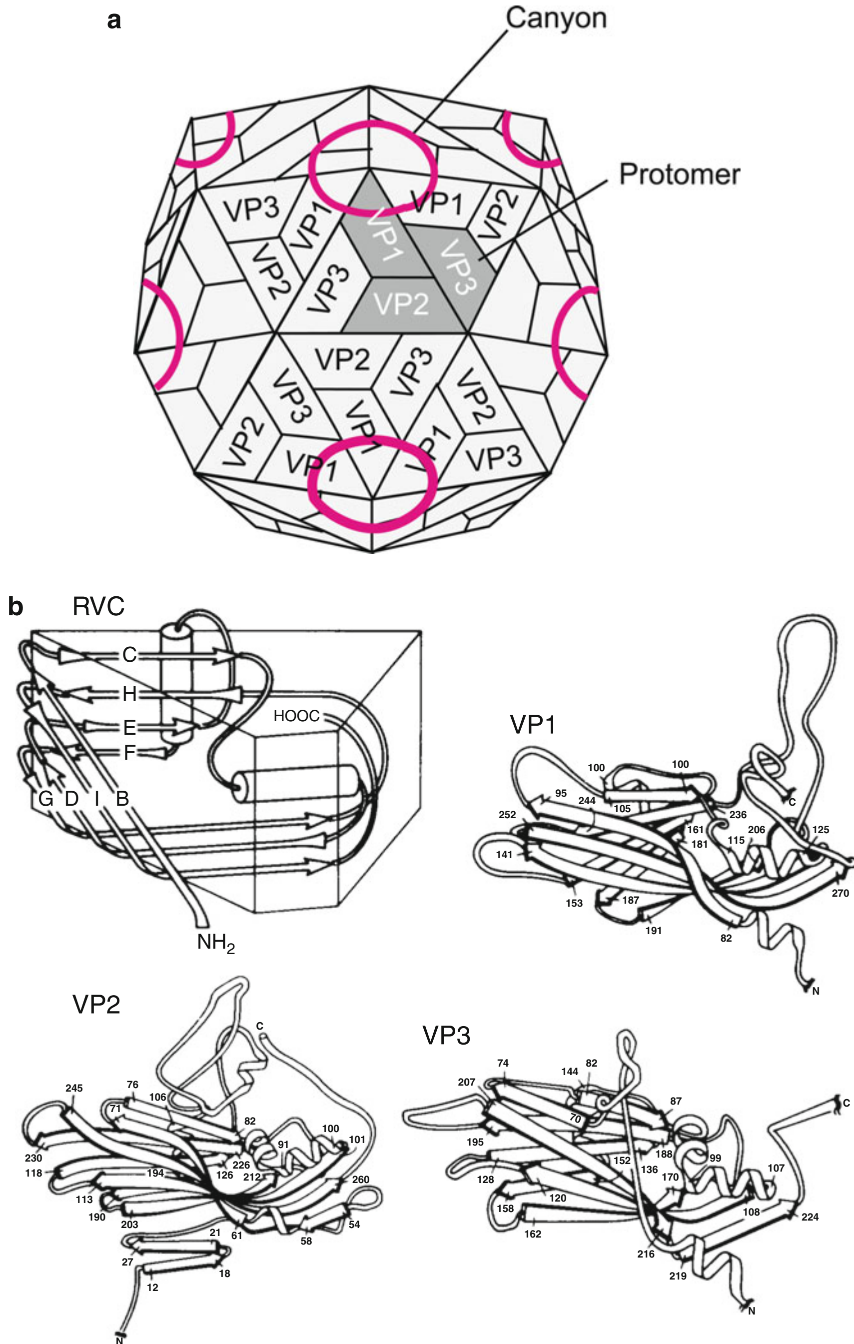


Fig. 14.1 (continued)

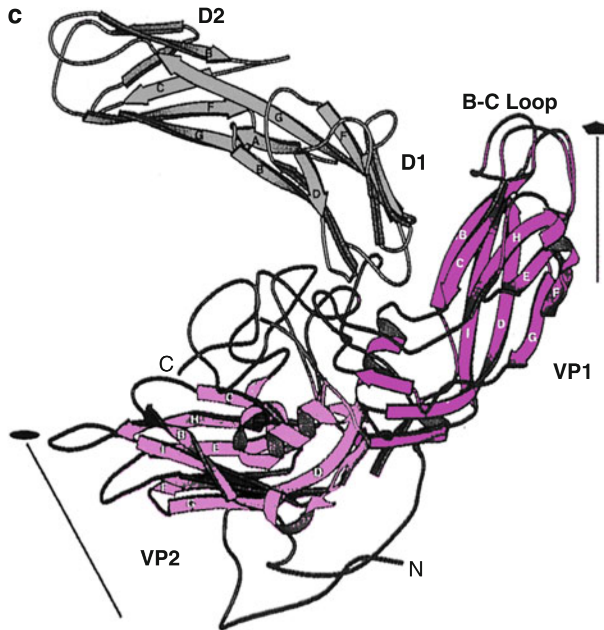


Fig. 14.1 (**a**) Structure of a picornavirus particle. The positions of capsid proteins VP1, VP2 and VP3 are shown schematically. VP4 resides on the inner side of the capsid, and is not exposed on the surface. The location of the precursor product for VP1, VP2 and VP3, the protomer, is indicated by the *grey-shaded region*. The so-called canyons, which as moat-like clefts or crevices surround the vertices of the icosahedron, are shown in *red*. (**b**) Structure of poliovirus capsid proteins VP1, VP2 and VP3. Also shown is the RNA virus capsid domain (*RVC*) of the proteins, which represents the common wedge-shaped folding pattern consisting of eight antiparallel β-sheet structures. Single β-sheets are represented by *arrows* and are indicated by *capital letters* according to their sequential order within the proteins. The α-helical regions are represented by *cylinders*. The structures of VP1, VP2 and VP3 are illustrated as ribbon models. The *numbers* refer to the amino acid positions numbered from the amino terminus of the respective capsid protein. The α-helical structures are represented by the helical folding of the ribbons. The amino- and carboxy-terminal regions are not included in the illustration for better identification of the common structural motif. (**c**) The canyon structure of human rhinovirus 14 and its interaction with the cellular receptor intercellular adhesion molecule 1 (ICAM-1). The structures of capsid proteins VP1 (*dark red*) and VP2 (*light red*) are shown as ribbon models. The antiparallel β-sheets are represented by *arrows* and are indicated by *capital letters*. The crystal structure of the amino-terminal region of CD4 was used as a basis for structure modelling of receptor domain D1 of ICAM-1 (*grey*) because of their extensive reciprocal homology (**b** From Hellen and Wimmer 1995; **c** after Olsen et al. 1993)

tight interaction must exist between the various capsid proteins within the particle of such viruses. Furthermore, these viruses also exhibit a very high resistance against detergents. Even as free virus particles, they are able to survive in the environment for relatively long periods of time.

Table 14.2 Comparison of distinct picornavirus genomes

Virus	Genome size (nucleotides)	(5′ UTR)	Polyprotein (amino acids)	(3′ UTR)
Poliovirus type 1	7,433	740	2,207	72
Human enterovirus B (coxsackievirus B3)	7,400	741	2,185	100
Hepatitis A virus	7,478	733	2,227	64
Rhinovirus (type 14)	7,209	624	2,178	47
Foot-and-mouth disease virus	8,450	1,199[a]	2,332	87

UTR untranslated region
[a]Contains a poly(C) tract of 100–170 nucleotides, depending on the virus isolate

14.1.2.2 Genome Organization and Structure

The viral nucleic acid is associated with amino acid residues on the inner side of the icosahedral particles. It consists of single-stranded RNA. The genome is between 7,212 (human rhinovirus B; type 14) and 8,450 (FMD virus) nucleotides in length (Table 14.2). The RNA has a positive sense. Accordingly, viral proteins can be directly translated from the RNA without requiring an intermediate transcription step. The 3′ end of the RNA genome is polyadenylated; the sequence of about 60 adenylate residues is coded in the viral genome. Vpg (for "viral protein genome-linked"), a small viral protein, is covalently linked to the 5′ end of the viral genome. Vpg is 22 amino acids long in poliovirus and other enteroviruses and 23 amino acids long in hepatitis A virus and rhinoviruses. It is esterified with the phosphate group of the uridine residue at the 5′ end of the genome by the OH group at position 3 in the phenol ring of a tyrosine residue.

The genome of picornaviruses contains a long single open reading frame that encodes a large precursor protein (Fig. 14.2). This experimentally not isolable protein is processed by proteolytic cleavage during its synthesis, thus leading to all the various viral components, i.e. structural and non-structural proteins as well as viral enzymes. A large untranslated sequence region (5′ UTR) is located between Vpg at the 5′ terminus of the genome and the start codon of the precursor protein; it has a length of 412 nucleotides in porcine teschovirus, 1,624 nucleotides in rhinoviruses and 1,199 nucleotides in FMD virus. The vast majority of the 5′-terminal non-coding nucleotides form intramolecular base pairings, resulting in a pronounced secondary structure (Fig. 14.3). The first 88 nucleotides immediately adjacent to the 5′ terminus form a secondary structure resembling a cloverleaf, which is involved in the initiation of genome replication for generating new positive-sense RNA molecules. The further downstream located sequence region possesses an internal ribosome entry site (IRES) that facilitates binding of ribosomes independently of the 5′-cap structure that is usually present at the 5′ end of eukaryotic mRNA molecules. Mutations in this region can strongly influence the translatability of the mRNA and virulence. Furthermore, a short untranslated sequence region is located between the stop

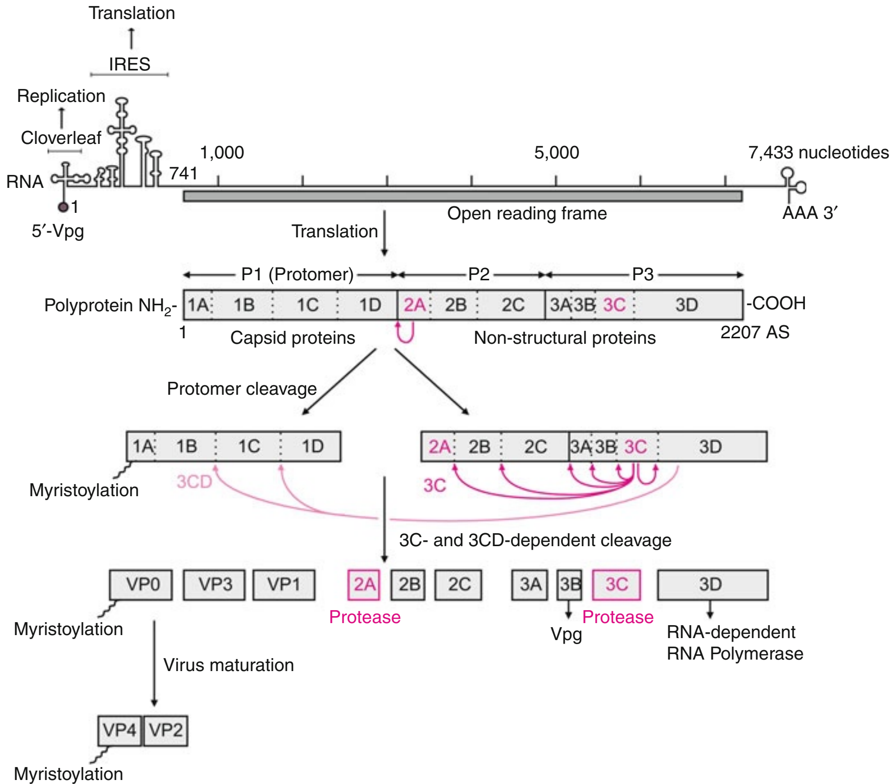


Fig. 14.2 Genome structure of picornaviruses using the example of poliovirus 1. The viral genome possesses a covalently linked protein (*Vpg*) at the 5′ terminus and is polyadenylated at its 3′ end. Adjacent to the 5′-terminal Vpg there are RNA sequences that form a cloverleaf-like secondary structure, which is essential for genome replication. *IRES* indicates the internal ribosome entry site that is necessary for binding of ribosomes to the viral RNA genome. The single open reading frame encodes a large precursor polyprotein that is processed into the distinct viral components (structural proteins, non-structural proteins and enzymes) by the proteolytic activity of 2A, 3CD and 3C proteins. The regions representing the proteases are illustrated in *red*; *red arrows* indicate the cleavage sites of the respective proteases

codon for the polyprotein and the poly(A) tail at the 3′ terminus of the genome, which comprises 47 and 100 nucleotides in rhinoviruses and coxsackieviruses, respectively.

14.1.3 Viral Proteins

14.1.3.1 Polyprotein

The open reading frame on the picornavirus genome encodes a very large continuous protein, which comprises more than 2,100 amino acids in all viruses. Its sequence contains all proteins and functions that are necessary for a successful

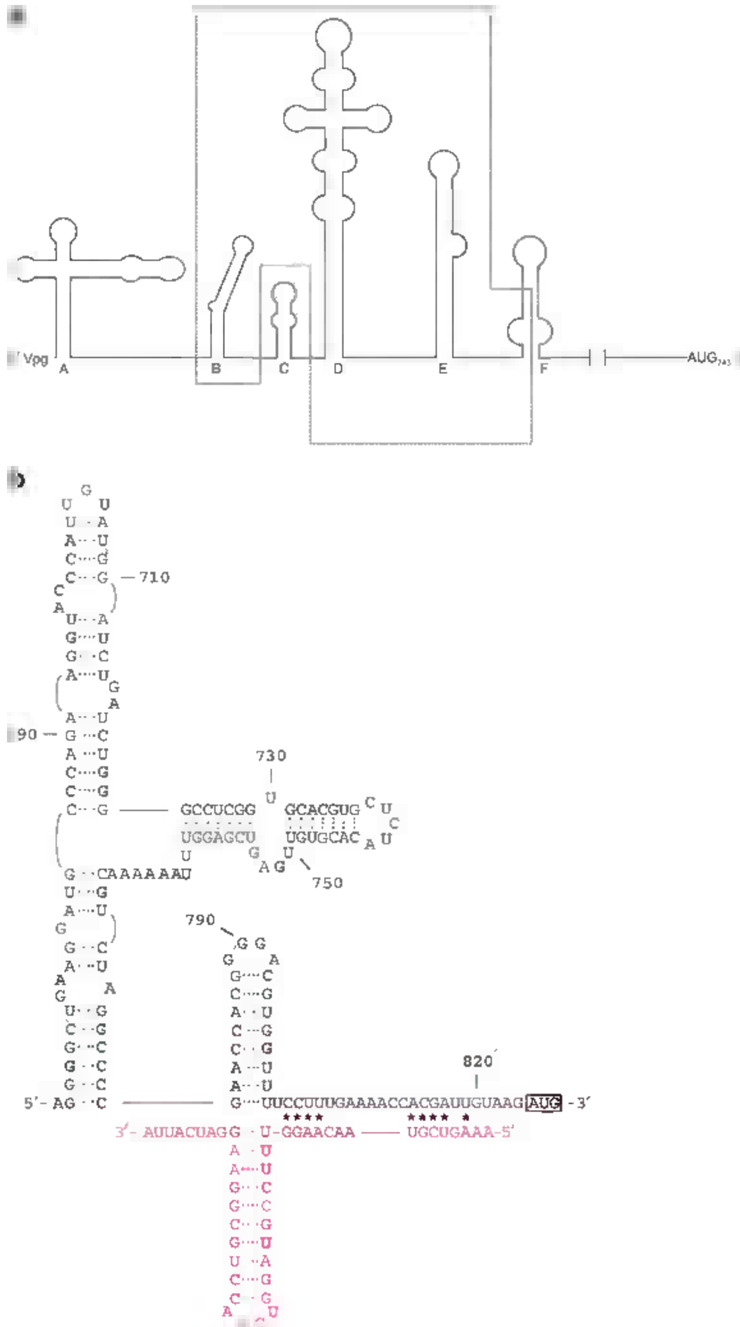


Fig. 14.3 (**a**) Computer model of the energetically favoured folding of the 5′ untranslated region (UTR) of poliovirus. The sequence elements comprising the IRES are indicated within the *frame*.

infection process. The arrangement of the proteins on the precursor polypeptide is identical in all picornaviruses (Fig. 14.2). The polyprotein is subdivided into three regions and the proteins, which arise by proteolytic cleavage, are designated with capital letters according to the respective regions of the polyprotein. The amino-terminal region contains the precursor sequences of the viral capsid proteins (1A–1D); the non-structural proteins 2A–2C, which play an important role in the adaptation of the virus to the cellular metabolism, are located in the middle of the polyprotein. Moreover, enzymatically active and further non-structural proteins (3A–3D) are generated from the carboxy-terminal regions. Usually, 11 viral proteins are generated from the precursor polyprotein. In aphthoviruses, erboviruses, kobuviruses, teschoviruses and cardioviruses, a short leader protein (L protein) is found at the amino terminus, which in mengovirus comprises 67 amino acids. In FMD virus, the L protein encompasses 205 amino acids and possesses proteolytic activity. In such cases the polyprotein does not begin with the sequences of the structural proteins, but begins with the L protein. In most picornaviruses, an amino acid sequence harbouring proteolytic activity (2A protease) is located immediately downstream of the region for the structural proteins. The 2A protease develops its proteolytic activity cotranslationally. In enteroviruses and rhinoviruses the amino acid chain between the end of the capsid proteins and the beginning of the 2A protease undergoes autocatalytic cleavage. The amino-terminal region of the precursor protein is excised from the nascent, not yet completely translated carboxy-terminal moiety, generating the protomer, the precursor of structural proteins. In aphthoviruses, erboviruses and cardioviruses, the autocatalytic cleavage reaction exerted by the 2A protease occurs not upstream but immediately downstream of its own amino acid sequences, thus leading to a polypeptide product that contains both the protomer and the 2A protease. In hepatoviruses and parechoviruses, the 2A protein does not function as a protease during the early steps of the infection cycle; instead, it develops its proteolytic activity in a late phase during the formation of new capsids. In this case, the cleavage of the structural protein moiety is performed by the 3C protease at the transition site between domains 2A and 2B. The properties and functions of all proteins that arise by cleavage of the polyprotein are summarized in Table 14.3.

14.1.3.2 Structural Proteins

The protomer encompasses the amino acids of capsid proteins, which are arranged in the order 1A, 1B, 1C and 1D; they correspond to the structural

Fig. 14.3 (continued) (**b**) IRES region of the encephalomyocarditis virus. The computer analysis prediction of the energetically favoured secondary structure of the 5′ UTR of the viral genome is shown. The *numbers* refer to the corresponding nucleotide positions starting from the 5′ terminus of the genome. By interaction with various cellular proteins, the secondary structures stabilize a nucleotide sequence located directly upstream of the start codon used for translation of the polyprotein. This sequence can form a partial double-stranded region (indicated by *stars*) with sequences at the 3′ end of the 18S ribosomal RNA (marked in *red*) within the small (49S) ribosomal subunit. The illustrated sequence ends with the start codon (AUG) of the polyprotein

Table 14.3 Comparison of the properties of picornavirus proteins

	Number of amino acid residues						
Protein	**Poliovirus**	**Coxsackievirus**	**Hepatitis A virus**	**Rhinovirus**	**Foot-and-mouth disease virus**	**Modification**	**Function**
Leader					205		Protease, performs the autocatalytic cleavage of the protomer, induces the degradation of eIF-4G
VP4	69	69	23	69	81	Myristoylated (lacking in hepatitis A virus)	Structural protein, on the inner side of the particle, interacts with RNA
VP2	271	261	222	262	218		Structural protein
VP3	238	238	246	236	221		Structural protein
VP1	302	284	274	290	212		Structural protein
2A	149	147	71	145	16		Enteroviruses, rhinoviruses: protease, performs protomer cleavage before of the 2A domain of polyprotein
							Aphthoviruses, erboviruses, cardioviruses: protease, performs protomer cleavage after the 2A domain of polyprotein, mediates the degradation of eIF-4G
							Hepatoviruses, parechoviruses: no protease, active during morphogenesis

2B	97	99	215	97	154		Influences host specificity
2C	329	329	335	330	317	NTP-binding	ATPase, initiation of RNA synthesis, chaperone activity forming ribonucleoprotein complexes?, helicase activity?
3A	87	89	74	85	154		Hydrophobic domain for anchoring the 3AB precursor on the membrane, influences uridylylation of Vpg
3B	22	22	23	23	23/24	Uridylylated	Vpg, covalently linked to the 5′ terminus of the viral genome
3C	182	183	219	182	214		Protease, performs all cleavage reactions, except for VP0 to VP4 and VP2 and for the protomer
3D	461	462	489	460	470		RNA-dependent RNA polymerase

The protein succession order in the table corresponds to the real sequence order in the polyprotein
NTP nucleoside triphosphate

proteins VP4, VP2, VP3 and VP1, respectively. Cellular enzymes modify the protomer by attachment of a myristic acid residue to an amino-terminal glycine. For this purpose, the previous autocatalytic removal of the L protein is necessary in aphthoviruses, erboviruses, kobuviruses, teschoviruses, and cardioviruses. In the other picornaviruses, the amino-terminal methionine is removed by cleavage. Myristoylation remains unaffected during the subsequent cleavage reactions and is detectable also in VP4. An exception is observed with hepatitis A virus as no amino-terminal fatty acid modification of the protomer has been found so far.

The virus-encoded 3C protease is responsible for processing the capsid precursor protein into the single components VP0, VP3 and VP1. This enzyme is located in the carboxy-terminal region of the polyprotein and develops its activity relatively late. The protomer is already folded into the domains that correspond to the four capsid proteins before cleavage. Nevertheless, these proteins change their conformation once more by folding during processing, thus leading to the mature proteins that are found in infectious virus particles. The final cleavage of the VP0 moiety to form VP4 and VP2 occurs during viral maturation; the proteolytic activity required for this process resides in the carboxy-terminal domain of VP0 within the sequences of the VP2 domain. A serine residue at position 10 of VP2, which is conserved among picornaviruses and is located close to the VP4/VP2 cleavage site, seems to be involved in conferring the serine protease activity that executes the autocatalytic cleavage. However, an additional tight interaction between VP0 and the viral RNA genome in the immature virus particle is essential for the proteolytic activity. It is assumed that for successful proteolytic cleavage of VP0 a base (acting as a proton donor) of the genome must interact with the amino acid sequences. Therefore, this enzyme is activated within the genome-containing but still immature virus particles, but not until they have been released from infected cells. In hepatitis A virus, the proteolytic cleavage of structural proteins is performed by a different mechanism: in this case, a protomer is formed that also contains the 2A domain in addition to the capsid proteins. The protomer is cleaved by the 3C protease at the transition sites between the VP2/VP3 and the VP3/VP1 regions; however, it does not recognize the cleavage site between the VP1 and 2A domains. The resulting protein, VP1/2A (also known as pX), is cleaved by a not yet characterized cellular protease at a later step in the infection cycle during morphogenesis of new virus particles.

VP1, VP2 and VP3 shape the faces of the icosahedral infectious virus particle. X-ray structure analyses of different picornaviruses revealed that these proteins exhibit very similar folding patterns among each other as well as among the proteins of different virus types. They consist of eight antiparallel β-sheet structures, which are connected by amino acid loops and are arranged in such a way that a wedge-shaped protein structure can be formed (Fig. 14.1b). Since this protein structure has proven to be a universally valid folding pattern for capsid proteins of small RNA viruses with particles of icosahedral symmetry, it has been designated the RNA virus capsid (RVC) domain. The eight β-sheets form the lateral sides of the conserved, wedge-shaped protein structure. The joining protein loops exhibit

considerable variability concerning length and sequence, however without influencing the basic wedge-shaped structure. The variable loop regions contain epitopes that are recognized by virus-neutralizing antibodies, which are generated during viral infection. In contrast, the amino-terminal regions of capsid proteins are situated on the inner side of the particles. They intertwine with each other forming an interconnected network which is responsible for the stability of the particles. The fact that VP1, VP2 and VP4 possess very similar structures suggests that the genes encoding these proteins have evolved from a common ancestral precursor by duplications.

In 1985, when Michael Rossmann, James Hogle and their respective co-workers published the protein structure of the rhinovirus and poliovirus capsids, they revealed not only the above-mentioned common characteristics, but also further structural features that result from folding and reciprocal interactions between the components. They found a ditch-like groove or crevice of approximately 25 Å in depth on the surface of rhinovirus B (type 14) particles which surrounds the vertices of the icosahedron and arises from the structures and interactions between different amino acid residues of VP1, VP2 and VP3. This structure, which Rossmann denominated "canyon", has been found in all picornaviruses, excepting in hepatitis A virus and FMD virus. The amino acids that line the walls of the canyon with their functional side groups facilitate binding of the respective virus particles to their specific cellular receptors (Fig. 14.1a, c). Owing to their size, neutralizing antibodies are not able to penetrate into the canyon to impede binding of the virus to its specific cellular receptor. However, they can bind to epitopes on the upper surface of the particles located near the entrances to the canyons, thus indirectly impeding attachment of the virus by steric hindrance. Because of antibody binding, surface regions are exposed to a selection pressure that leads to a certain variability of the exposed amino acids, and to the development of differing capsid variants. On the other hand, the actual receptor binding site within the canyon does not underlie such a selection mechanism and remains unaffected, thus preserving the cell specificity of the corresponding virus type.

The structural analyses also revealed the existence of a small hole-like cavity beneath the canyon floor. The cavity (pocket) is accessible from the canyon floor by a pore and has an additional connection to the inside of the particle. It contains a sphingosine-like fatty acid molecule, the "pocket factor". It is removed from the pocket during structural rearrangements of capsid proteins which are triggered upon receptor binding. During this process, VP4 is lost as an inside-associated protein, leading to destabilization of the virus particle, which facilitates the release of the RNA genome into the cytoplasm through the vertices of the receptor-associated icosahedron. Knowledge of the predominantly hydrophobic amino acids that line the cavity facilitated the development of therapeutically active and optimally adapted drugs such as pleconaril (see ▶ Chap. 9) that stabilize virus particles and inhibit the release of the RNA genome. Therefore, the use of such compounds can prevent or at least restrict viral infections. Nevertheless, it also soon became apparent that viruses are rapidly able to develop resistances.

The emergence of different serotypes that retain cell specificity is particularly pronounced in rhinoviruses, which comprise more than 100 different stable serotypes, which are especially characterized by the fact that neutralizing antibodies against them are strongly type-specific and cannot bind to the cell surface of other serotypes. Therefore, after a cold, people are protected against further infections with the same rhinovirus serotype, but not against other serotypes, which can continuously lead to recurrent colds. Such a plethora of different serotypes does not exist among enteroviruses. Poliovirus has three serotypes, and hepatitis A virus has only one serotype. The high genetic stability of enteroviruses, parechoviruses and hepatoviruses is probably correlated with the strong acid resistance of their capsids, which are able to survive in the acid environment of the stomach. Indeed, only a few amino acid sequences are capable of conferring such high acid stability, which, however, strongly limits the variability.

14.1.3.3 Enzymes

Proteases

Picornaviruses possess proteases, which become active in different phases of the infection cycle. The 2A protease is a cysteine protease and is localized immediately contiguous to the structural protein regions (Fig. 14.2). In the case of aphthoviruses, the 2A protease is very small, comprising only 16 amino acid residues; however, in poliovirus and rhinoviruses it is notably larger and has 149 and 147 amino acids, respectively. It develops its activity early during the infection cycle, and cotranslationally cleaves the protomer sequences from the nascent polyprotein between a tyrosine residue and a glycine residue. The cleavage site is located either immediately in front of (enteroviruses and rhinoviruses) or after (aphthoviruses, erboviruses, teschoviruses and cardioviruses) its own enzyme sequences. In hepatitis A virus and parechoviruses, the 2A protein does not possess proteolytic activity; instead, it is involved in the process of viral capsid formation at the end phase of the replication cycle.

Besides its role in releasing the protomer from the nascent polyprotein, the 2A protease also catalyses proteolytic cleavage reactions in cellular proteins. Best known is the indirect degradation of the protein p220: in this case, the 2A protease cleaves a cellular factor, which thereby becomes active as a protease. This enzyme induces the degradation of the cellular translation initiation factor eIF-4G (p220). This is a component of the cap-binding complex, also known as the eIF-4F complex, which comprises the cap-binding protein and translation initiation factor eIF-4A. The cap-binding complex is generally involved in the initiation of translation of eukaryotic mRNAs, as it interacts with the cap structure at the 5′ terminus of the mRNA and mediates binding of the ribosomes. Cleavage of the eIF-4G factor by the viral 2A protease leads to destruction of the functional activity of the complex in translating cellular mRNAs, resulting in a breakdown of cellular metabolism. This process has been denominated virus-host shutoff. In aphthoviruses, cleavage of the eIF-4G factor is mediated not by the 2A protease, but by the proteolytic activity of the L protein (206 amino acids), which resides at the amino terminus of the polyprotein. The L protein is also activated early during the infection cycle, and induces its own

cleavage from the polyprotein, leading to the release of the protomer. This process has not been demonstrated in other picornaviruses so far.

In hepatoviruses and parechoviruses, the activity of the 3C protease, which recognizes the amino acid sequence glutamine–glycine, is responsible for all proteolytic reactions in the precursor protein which lead to the generation of the single components from the polyprotein; in enteroviruses, rhinoviruses, cardioviruses and aphthoviruses, the 3C protease is also responsible for all proteolytic reactions except the cleavage of the protomer, which is performed by the 2A protease. Exceptions are the processing of VP0 to VP4 and VP2 during viral maturation by the enzymatic activity of VP2 and the cleavage of VP1/2A by a cellular protease in hepatitis A virus. The 3C protease domain is located at the carboxy-terminal region of the polyprotein and comprises 182 amino acids in poliovirus and rhinoviruses, and 217 residues in hepatitis A virus (Table 14.3). In a first reaction step, an intramolecular amino-terminal cleavage reaction occurs in front of the 3C moiety. The resulting intermediate product, $3CD^{pro}$, exhibits proteolytic activity. The 3D moiety present in $3CD^{pro}$ is indeed essential for processing the protomer into capsid proteins, because an efficient cleavage between VP3 and VP1 is only possible when the 3D domain is still linked with the 3C protease (Fig. 14.2). The cleavage of $3CD^{pro}$ into 3C and 3D occurs intermolecularly (in *trans*), i.e. active intermediate 3CD products interact and perform a reciprocal cleavage reaction.

The 3C protease is also involved in the specific degradation of cellular components. Its direct or indirect proteolytic effects seem to impair the RNA polymerase III dependent cellular transcription by which transfer RNA (tRNA) species and other small RNA molecules are synthesized. The degradation reaction is directed against both transcription factor TFIIIC and the TATA-box-binding protein. Furthermore, the 3C protease of FMD virus is also able to induce the cleavage of histone H3. The cleavage sites of proteases 2A and 3C are defined not only by the two amino acid residues between which the cleavage occurs, but also by the neighbouring amino acids and by the folding of the precursor proteins into secondary and tertiary structures.

RNA-Dependent RNA Polymerase

During the replication cycle of picornaviruses, the positive-sense RNA genome is transcribed into an intermediate, negative-sense RNA molecule, which, in turn, serves as a template for the synthesis of positive-sense progeny RNA genomes (for details, see Sect. 14.1.4). The synthesis of an RNA molecule by using an RNA template is a process that does not exist in cellular nucleic acid synthesis; hence, cells do not possess enzymes that can perform such (synthesis) reactions. For this reason, it is essential for picornaviruses to possess the genetic information encoding their own RNA-dependent RNA polymerase. This enzyme is located at the carboxy terminus of the polyprotein and is represented by the 3D region on the precursor molecule; the intermediate product $3CD^{pro}$, which is generated by proteolytic cleavage, possesses protease activity but no polymerase activity. The length of the 3D polymerase ($3D^{pol}$) ranges between 460 amino acids in rhinoviruses and

491 amino acids in hepatitis A virus. The error rate of RNA-dependent RNA polymerases is relatively high, as has been demonstrated for $3D^{pol}$ of poliovirus. On average, it causes one error per 2,200 polymerized nucleotides during synthesis of RNA chains. This implies that every newly synthesized RNA strand contains approximately four mutations.

14.1.3.4 Other Proteins

In addition to the structural proteins and enzymes already mentioned, further protein components are located within the sequence of the polyprotein, and are generated by the activity of the 3C protease during the infection cycle (Fig. 14.2). The very small 3B component was mentioned already; it constitutes Vpg, which is covalently linked to the 5′ terminus of the viral genome. The Vpg sequence is repeated three times in the polyprotein of aphthoviruses. The direct precursor of Vpg is 3AB. It is generally accepted that 3AB is anchored to intracellular membranes (e.g. endoplasmic reticulum, ER, membrane) by a hydrophobic amino acid sequence present in the 3A moiety. The tyrosine residue at position 3 of the 3B domain becomes uridylylated and functions as a primer for initiation of RNA synthesis during genome replication. Directly following uridylylation, the 3C protease cleaves the precursor at the cleavage site between 3A and 3B, leading to the release of Vpg from membrane anchoring. Mutations within the hydrophobic domain of the 3A protein inhibit uridylylation and RNA synthesis.

The 2C and 2BC proteins are involved in RNA replication. They are responsible for the formation of rosette-like membrane structures that are derived from the ER. The synthesis of new viral RNA occurs in the cytoplasm on the surface of such vesicles. In addition, there are partially contradictory reports in regard to their activities. The 2B protein comprises 97 amino acids in poliovirus and rhinoviruses, 154 residues in FMD virus and 215 residues in hepatitis A virus. It seems to be related to host specificity of the different viruses. It exerts its effect in *cis*, i.e. possible defects cannot be complemented by the concurrent introduction of wild-type proteins. Human rhinoviruses carrying mutations within the 2B-encoding region of the genome are also able to proliferate in murine cells. There are data indicating that mutated 2B proteins can interact with certain murine-cell-specific factors, thereby facilitating RNA replication in cells that are non-permissive for wild-type viruses. The 2B proteins of hepatitis A virus interfere with the antiviral responses mediated by retinoic acid inducible gene (RIG) I by inhibiting phosphorylation, and consequently activation of interferon regulatory factor 3 (IRF-3), thus preventing transcription of the interferon-β (IFN-β) gene (see ▶ Chap. 8). On the other hand, the activity of the 2B or 2BC proteins of enteroviruses and FMD virus inhibits cellular protein transport from the ER to the cell surface via Golgi vesicles. This effect causes a reduction in the concentration of MHC class I antigens on the surface of virus-infected cells, enabling them to evade the cellular immune response.

The 2C protein (length of 317 amino acids in aphthoviruses, 330 amino acids in rhinoviruses, 335 amino acids in hepatitis A virus) possesses an NTP-binding

site and ATPase activity. It appears to be involved in initiation of RNA synthesis. In poliovirus and FMD virus, mutations in the 2C gene lead to resistance against guanidine hydrochloride and benzimidazole derivatives, two effective inhibitors of viral RNA synthesis. The amino acids phenylalanine and asparagine at positions 164 and 179 (as well as their corresponding substitutions with tyrosine and glycine) seem to be important for the acquirement of resistance. Mutations within the NTP-binding site are lethal for the viruses. Furthermore, there is evidence that $2C^{ATPase}$ may act as a chaperone in the formation of ribonucleoprotein complexes, which must be formed during the replication of the viral genomes. In this connection, the 2C protein might promote the coordinated interaction of the cloverleaf structure at the 5′ terminus, with the 3′ UTR sequences, the poly(A) tail, the *cis*-responsive element and the several proteins involved in this complex.

Functions of the Protein CD155

CD155 is expressed in many cells of various tissues. The membrane-anchored variants CD155α and CD155δ are associated with proteins of the extracellular matrix, e.g. vitronectin and nectin-3. In addition, CD155α and CD155δ are associated with α_v integrins in the cytoplasmic membrane. These properties characterize CD155 as a cell–cell and cell–matrix adhesion molecule, which is linked with the cadherin adhesion system. The cytoplasmic domain of CD155α and CD155δ is associated with the protein Tctex-1, which is a subunit of the dynein motor protein complex. This protein is synthesized in all neurons, including the motor neurons of the spine. Possibly, this interaction may be important for the retrograde transport of polioviruses into the neurons, and thereby for the pathogenesis of poliomyelitis.

14.1.4 Replication

In the first step of the infection process, picornaviruses attach to specific cellular membrane proteins. On the virus side, the structures and amino acids of the canyon are the prevailing factors that mediate receptor binding. The cellular receptors of several viruses have been identified and are also molecularly well characterized; for attachment, all picornaviruses seem to use cell surface proteins that are members of the immunoglobulin superfamily (Table 14.4). In some cases, coreceptors have been identified that additionally support binding, or interact with the actual receptors.

Polioviruses bind to the glycosylated surface protein CD155, which belongs to the immunoglobulin superfamily. It possesses three immunoglobulin-like domains: an amino-terminal, variable V region, followed by two conserved C2 domains. There are four variants of CD155 that are generated by alternative splicing: CD155α and CD155δ are membrane-anchored forms and serve as receptors for polioviruses; CD155β and CD155γ are soluble isoforms. The three serotypes of poliovirus compete for the same attachment site, which is localized in the V domain

Table 14.4 Cellular receptors of various picornaviruses

Genus	Virus	Receptor	Receptor family
Enterovirus	Poliovirus	**CD155**	Ig superfamily
	Coxsackieviruses A13, 18	**CAR**	Ig superfamily
		ICAM-1	Ig superfamily
	Coxsackievirus A9	**CAR**	Ig superfamily
		Integrin $\alpha_v\beta_3$	Ig superfamily
	Coxsackieviruses A2, B1, B3; B5	**CAR**	Ig superfamily
		CD55 (DAF)	Ig superfamily
	Echovirus (types 1 and 8), Parechovirus (type 1)	**Integrin $\alpha_3\beta_1$**	Ig superfamily
	Echovirus (types 3, 6, 7, 11–13, 21, 24, 25, 29, 30, 33), Enterovirus 70	**CD55 (DAF)**	Ig superfamily
Rhinovirus	Rhinovirus (major group)	**ICAM-1**	Ig superfamily
	Rhinovirus (minor group)	**(V)LDL-receptor**	–
	Rhinoviruses A54, A89	Heparan sulphate	Heparan proteoglycan
Hepatovirus	Hepatitis A virus	**TIM-1, TIM-3**	Ig superfamily
Aphthovirus	Foot-and-mouth disease virus	**Integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$**	Ig superfamily
		Heparan sulphate	Heparan proteoglycan
Cardiovirus	Theiler's encephalomyelitis virus	**Unknown**	Sialoglycoprotein
	Subtype GDVII (neurovirulence)	Heparan sulphate	Heparan proteoglycan
	Subtype DA (low neurovirulence)	$\alpha_{2,3}$-Linked sialic acid	24 kDa Glycoprotein

The respective main receptors are highlighted in *bold*, and beneath them are the corresponding coreceptors

CAR coxsackievirus and adenovirus receptor, *ICAM-1* intercellular adhesion molecule 1, *DAF* decay-accelerating factor, *Ig* immunoglobulin *TIM* T-cell immunoglobulin- and mucin-domain-containing molecule, *VLDL* very low density lipoprotein

of the receptor. The neighbouring C2 region seems to stabilize the appropriate structures needed to facilitate receptor binding.

More than 90 % of rhinoviruses (major group) use intercellular adhesion molecule (ICAM) 1 proteins (CD54) as receptors; these glycosylated cell surface proteins also belong to the immunoglobulin superfamily and are responsible for intracellular signal transduction during inflammatory processes (Fig. 14.1c). The remaining rhinoviruses (minor group) bind to the very low density lipoprotein (LDL) receptor; however, for some viruses (human rhinoviruses 54 and 89) heparan sulphate seems to be sufficient for binding to the cell surface. In the case of hepatitis A virus, the proteins TIM-1 and TIM-3 have been identified as cellular interaction partners. The family of T-cell immunoglobulin- and mucine-domain-containing proteins (TIM proteins) represent a group of receptor proteins that also belong to

the immunoglobulin superfamily and are present in differing quantities on T_H1 and T_H2 cells and seem to regulate both the humoral and the cellular immune response.

Coxsackieviruses bind to the coxsackievirus and adenovirus receptor (CAR), also a member of the immunoglobulin superfamily, which is anchored in the cytoplasmic membrane by a hydrophobic domain in the carboxy-terminal region and exhibits two immunoglobulin-like domains. This cell surface protein, which is also used as a receptor by adenoviruses (▶ Sect. 19.4), is structurally and functionally similar to adhesion molecules. The amino-terminal domain of the CAR protein fits into the canyon on coxsackievirus capsids. Apart from the CAR protein, there are further cell surface proteins that can function as coreceptors for the various coxsackievirus types: some coxsackieviruses bind to ICAM-1 (coxsackieviruses A13, A18 and A21); coxsackievirus A9 interacts, like echovirus 9, with the vitronectin receptor, a protein that has been classified in the group of integrins ($\alpha_v\beta_3$) within the immunoglobulin superfamily. Furthermore, echoviruses 1 and 5 as well as parechovirus 1 bind to integrin $\alpha_2\beta_1$, which is also known as VLA-2. Other enteroviruses and echoviruses as well as Coxsackie B viruses use the surface protein CD55 (decay accelerating factor) as an additional interaction partner. This member of the immunoglobulin superfamily is responsible for protecting cells from lysis by the complement system. All these cell membrane proteins are expressed in different phases of leucocyte differentiation, and are involved in adhesion and recognition processes between different cell types. Another additional factor involved in the interaction between the host cell and coxsackievirus B3 is a protein with homology to nucleolin. This protein (110 kDa) is involved in the transport of ribosomal proteins from the nucleus into the cytoplasm, and is possibly also involved in attachment and penetration of adeno-associated adenovirus 2 (▶ Sect. 20.1).

However, the specific recognition of cell surface proteins is not the only feature that can account for the cell specificity of the different picornaviruses. Intracellular factors are also crucial for successful viral replication. This has been demonstrated especially in polioviruses. CD155 proteins have been found on a plethora of different cells to which polioviruses attach; however, the viral replication cycle can proceed only in a few cell types.

After attachment, structural rearrangements occur in the viruses that are bound to the cell surface. Such rearrangements are especially well studied in rhinoviruses that bind to ICAM-1 (Fig. 14.1c). A putative amphipathic α-helix within the amino-terminal region of VP1 is exposed, and interacts with lipid components in the cytoplasmic membrane. This results in a conformational change in the canyon, which leads to a tighter binding to the D1 domain of ICAM-1. VP4, which is located on the inner surface of the virions and is tightly associated with the viral genome, is released from the capsids. Additionally, the sphingosine-like molecule, the pocket factor, is also displaced during this process. The viral genome is translocated into the cytoplasm through a cylindrical orifice which is formed at the contact sites between the vertices of the icosahedron and the cell membrane. Alternatively, there are data suggesting that viral entry can also occur via endocytosis. The vesicles that are formed during this process carry abundant quantities of clathrin, a cell

membrane protein that accumulates in high concentrations at viral attachment sites. For the progression of the replication cycle, it is necessary that the viruses are released from the vesicles. An ATP-dependent proton pump located in the endocytic membrane is responsible for acidification of the interior of endocytic vesicles. This process leads to a rearrangement of the capsid in a way similar to that described above. In the next step, VP4 proteins and clathrin are released. As a result, small pores are formed at the junctions between VP1 proteins in the remodelled capsids and the vesicle membrane, through which the viral RNA is released into the cytoplasm.

Since the viral genome is a positive-sense RNA molecule that acts as mRNA, the next step consists of protein translation and synthesis of the polyprotein. The RNA does not possess the cap structure usually found at the 5′ terminus of cellular mRNAs and which is necessary for the correct binding of ribosomes to the translation initiation site. Contrarily, the viral genome contains an, in comparison with cellular mRNA species, unusual long non-coding sequence (IRES) that is located upstream of the start codon and can comprise up to 1,199 nucleotides. It possesses a strongly pronounced, stable secondary structure with intramolecular double-stranded sequence regions (Fig. 14.3). The IRES facilitates recognition of the picornavirus genome as mRNA by the ribosomal subunits, facilitating their interaction with the start codon to initiate the first steps of the translation. It is assumed that the secondary structure exerts a stabilizing effect on a nucleotide sequence located immediately upstream from the start of the translation and which is complementary to the 18S ribosomal RNA of the small ribosomal subunit. Therefore, the ribosomal RNA can hybridize with the viral genome, guiding the ribosomes to the correct start site for protein synthesis. Cellular proteins are functionally involved in initiating translation. They also bind to the IRES region, influencing thereby the cell and host specificity of the different picornaviruses. One such protein is the La protein (p52), which usually binds to the 3′ termini of transcripts, especially to tRNAs that are synthesized by RNA polymerase III, and regulates their termination. Interestingly, the La protein induces an autoimmune reaction in patients with lupus erythematosus and Sjögren syndrome. Additional IRES-binding components include polypyrimidine tract binding protein, poly (rC)-binding protein 2, poly(A)-binding protein and the Unr factor (upstream of N-ras). In addition to these proteins, also the translation initiation factor eIF-2 and other not yet characterized proteins have been identified as interaction partner of the IRES.

Following the initial translation steps, the whole polyprotein is uniformly synthesized. During translation, it is folded into the individual protein domains. In most picornaviruses (exceptions are hepatitis A virus and parechoviruses), the first proteolytic cleavage reactions occur cotranslationally during the synthesis of the polyprotein, namely immediately after translation of the sequences encoding the 2A protease. This enzyme operates autocatalytically, leading to cleavage of the protomer region at the amino terminus. The 3C and 3CD proteases are involved in processing the other viral components; owing to their carboxy-terminal location within the polyprotein, they are synthesized later. In hepatitis A virus and

parechoviruses, the protomer remains linked with the 2A domain. Owing to proteolytic cleavage, RNA-dependent RNA polymerase ($3D^{pol}$) and Vpg are available as a prerequisite for the replication of the viral genome in the infected cell. In vitro, the synthesis of the polyprotein takes approximately 15 min.

The formation of a primer molecule, Vpg-pUpU-OH, is necessary for replication of the viral genome, which leads to the generation of a complementary negative-sense RNA molecule as an intermediate product. Mediated by the hydrophobic domain of the 3AB protein, the direct precursor of Vpg, primer synthesis occurs in association with intracellular membrane compartments and is catalysed by $3D^{pol}$. The template for the uridylylation reaction is a conserved sequence motif (AAACA), which is located in the loop of a hairpin structure within the *cis*-responsive element. This *cis*-responsive element is situated in different regions of the genome among the various members of the picornaviruses: in enteroviruses, it is located within the 2C-encoding region (poliovirus 1: nucleotides 4,444–4,505); in rhinovirus A, it resides within the coding sequences for the 2A domain; in parechoviruses and rhinovirus B (rhinovirus 14), it is situated within the VP1-encoding sequences, whereas in rhinovirus C it is found within the sequences encoding VP2. In FMD virus, the *cis*-responsive element is located within the 5′ UTR upstream of the IRES sequences, whereas in hepatitis A virus, it is situated within the 3C-encoding region. Mutations in the *cis*-responsive element lead to a replication failure, which results in an inhibition of negative-strand synthesis owing to the absence of uridylylation. The Vpg-pUpU primer associates with the poly(A) tail at the 3′ terminus of the viral genome and forms the initiation structure with a free 3′-OH end as a recognition site for the RNA-dependent RNA polymerase ($3D^{pol}$), which synthesizes the complementary strand (Fig. 14.4). The initiation of negative-sense RNA synthesis is, however, also dependent on the presence of an intact cloverleaf secondary structure at the 5′ terminus of positive strands. This suggests that the positive-sense RNA genomes form a circular structure, at which the negative-strand synthesis is initiated. The circularization is mediated by the interaction of the terminal-binding proteins poly(A)-binding protein and poly(rC)-binding protein 2 as well as by the viral proteins $2C^{ATPase}$, $3CD^{pro}$ and $3C^{pol}$, which bind to the 5′ and 3′ ends of the RNA genome. A transitory intermediate double-stranded RNA molecule is formed during the RNA polymerization. The 3′ end of the newly synthesized negative strand contains two adenosine residues. These constitute a perfect structure for the complementary interaction with Vpg-pUpU primers, and initiate the synthesis of new positive-sense RNAs. The synthesis of a complete RNA molecule takes about 45 s. Besides $3D^{pol}$ and Vpg-pUpU primers, a cellular protein (67 kDa) is essential for the correct course of this process. Between five and eight positive-sense RNA strands can be synthesized from a single intermediate negative-sense RNA molecule. Only 5–10 % of all viral RNA molecules are negative-sense RNA. New viral polyproteins are translated from the newly synthesized positive-sense RNA strands. In this way, the amount of viral components can rapidly multiply within infected cells. The entire process of polyprotein synthesis and genome replication occurs in the cytoplasm in association with intracellular membrane compartments. The polyproteins become embedded in

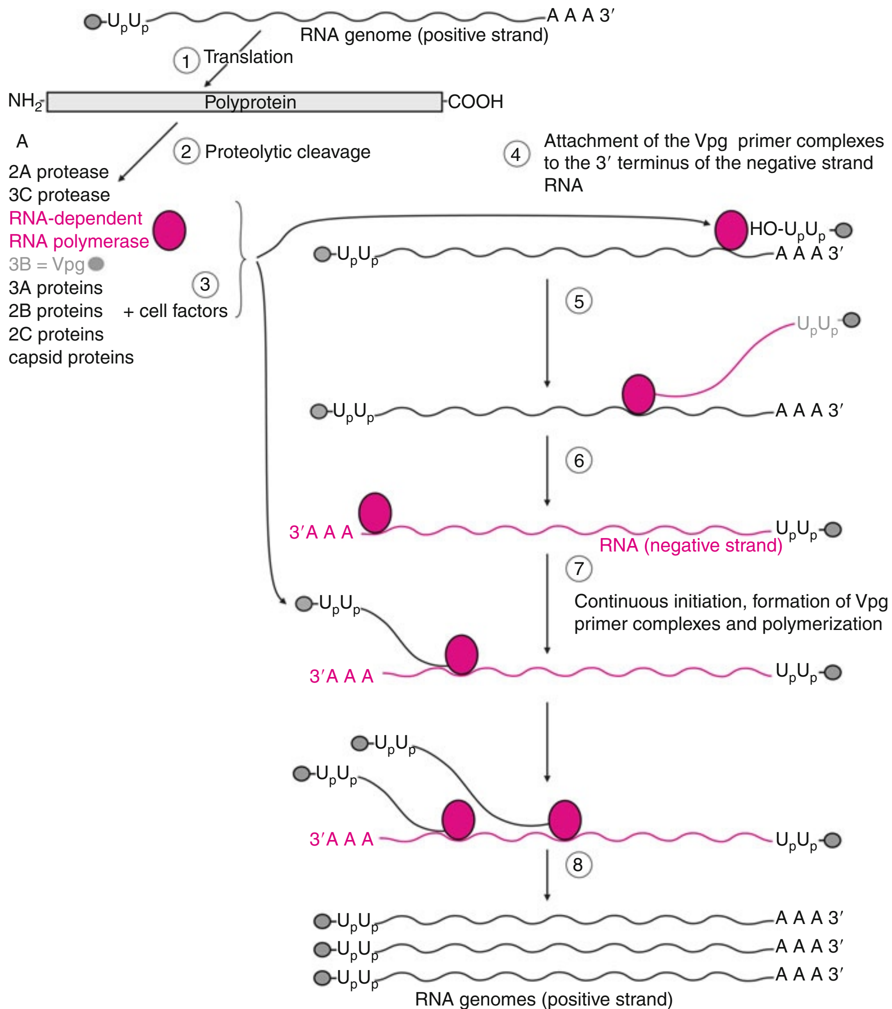


Fig. 14.4 Genome replication of picornaviruses. In a first step, the RNA genome is translated (*1*), leading to the synthesis of a polyprotein, which is processed into the individual protein components by the activity of the proteases 2A and 3C/3CD (*2*). The protein Vpg, which is subsequently uridylylated, arises during this process (*3*). Cellular proteins are also needed for this process. The Vpg-pUpU complex attaches to the 3′ terminus of the RNA genome (*4*), acting as a primer for the synthesis of the negative-sense RNA strand by the viral RNA-dependent RNA polymerase (*5*), which is also generated by proteolytic processing of the polyprotein. New Vpg-pUpU complexes attach to the 3′ terminus of the newly synthesized negative-sense RNA molecules (*6*). They function as primers for the synthesis of more RNA molecules (*7*), which now possess positive-sense polarity (*8*) and serve both as messenger RNA (mRNA) for further protein synthesis and as viral genomes

such compartments by their amino-terminal myristoylation and the hydrophobic 3AB domain.

The cellular metabolism is strongly influenced by viral replication. The large number of newly synthesized viral genomes that are present in the cell shortly after infection and the high affinity of their IRES elements for cellular ribosomes result in the cellular translation machinery being involved exclusively in the synthesis of viral proteins. In addition, the 2A protease of poliovirus and rhinoviruses induces the degradation of p220 (eIF-4G), a member of the cap-binding complex that is essential for initiation of translation of eukaryotic mRNA molecules. This is a second mechanism for inhibition of cellular protein synthesis. The poliovirus 2B and 3A proteins influence both the transport and the secretion of cellular glycoproteins. Furthermore, as described above, proteolytic degradation of TFIIIC by $3C^{pro}$ inhibits RNA polymerase III dependent transcription. Finally, even mRNA synthesis is blocked. In this case, a still unknown viral protein apparently affects the activity of RNA polymerase II. These processes are responsible for virus-host shutoff, i.e. the virus-mediated deactivation of the cellular metabolism.

As soon as sufficient amounts of viral proteins and RNA genomes are produced, the components assemble into infectious virions (self-assembly). In a first step, the myristoylated protomer, which is attached to membrane compartments, is cleaved by the activity of $3C^{pro}$ to form VP0, VP1 and VP3. These proteins remain initially bound as a complex and associate with four additional VP0/VP1/VP3 aggregates to form pentamers, the precursor structures of the vertices of the icosahedron. In hepatitis A virus, the 2A protein domain does not possess proteolytic activity and remains linked to the protomer, forming the protein pX. In this case, the 2A domain facilitates the formation of pentamers and induces the process of morphogenesis. In a further step, 12 pentameric complexes assemble and form capsid precursors. These protein shells surround the RNA genome. However, is not completely understood how the RNA genome arrives at the interior of the capsids. Sequences of the IRES region are also important for this process: they enhance the encapsulation of the RNA genome. Furthermore, considerable rearrangements of the capsid protein structure are associated with this process, which is expressed in an altered sedimentation behaviour of the particles. It is conceivable that the RNA genome reaches the interior of the so-called procapsids through an orifice, or by preliminarily binding to the outside of the particle precursors with subsequent translocation into the interior by an ample refolding process. The last step in virion formation involves the activation of the protease within the VP2 domain of VP0, which is induced by interaction between VP0 and the viral RNA. Once activated, this protease performs the maturation cleavage that leads to the mature capsid proteins VP2 and VP4. In hepatitis A virus, a still unknown cellular protease catalyses the cleavage of pX to form VP1 and 2A in this late step. The final release of the virus is triggered by alteration of membrane permeability due to infection-induced cell death. The complete replication cycle, beginning from viral attachment to the release of progeny viruses, takes approximately 8 h in poliovirus.

The intracellular processes during viral replication and apoptosis lead to morphologic alterations of the cell structure, which can be microscopically observed as a cytopathic effect: the chromatin structure is disintegrated and nucleic acid/protein complexes accumulate at the inner side of the nuclear membrane. The cytoskeleton also undergoes alterations by structural rearrangements in proteins that are associated with the microtubules. The cells take on a rounded morphology. Numerous vesicles are formed in the cytoplasm during viral replication. Finally, the cell membranes change their permeability and become more permeable because of the incorporation of increased amounts of phosphocholine.

Translation Initiation by IRES Sequences

So far, IRES sequences have been identified in the genomes of picornaviruses and pestiviruses and hepatitis C virus, which belong to the family *Flaviviridae* (Sect. 14.5). This sequence motif facilitates the translation of eukaryotic mRNA independently of both the 5′-cap structure and the cap-binding complex. Nevertheless, also a cellular gene was found to contain an IRES within the 5′ UTR of its mRNA: it encodes the binding immunoglobulin protein (BiP; immunoglobulin heavy chain binding protein; an alternative designation is 78-kDa glucose-regulated protein, GRP-78). This protein is a protein-folding enzyme (chaperone) which is active in the ER and in the Golgi apparatus. It is involved in the interaction between light and heavy chains of immunoglobulins that leads to functional antibodies.

Function of the 5′-Cap Structure

In eukaryotic protein translation, the cap-binding complex attaches to the cap region, a methylated guanosine nucleotide that is linked to the 5′ terminus of the messenger RNA via a 5′–5′ ester bond. The cap-binding complex is composed of the cap-binding protein, which interacts with the 5′ terminus, factor eIF-3 and proteins eI-4A and eI-4B, which bind to the region located up to 100 nucleotides directly upstream of the start codon, maintaining it in a single-stranded extended configuration. The small ribosomal subunit along with $tRNA^{Met}$, initiation factor eIF-2 and GTP interact with this 5′ terminally associated complex to initiate protein translation. The genome of picornaviruses functions as mRNA, but does not possess a 5′-cap structure. Therefore, it cannot perform the necessary interaction with the components of the cap-binding complex to interact with the ribosomes.

14.1.5 Human Pathogenic Picornaviruses

Some members of the picornaviruses account for various severe diseases in humans. These include, in particular, poliomyelitis and hepatitis A. However, cases with

severe clinical courses are increasingly found in connection with infections with other human enteroviruses.

14.1.5.1 Poliovirus

Epidemiology and Transmission

Poliomyelitis (infantile paralysis) was known as long ago as 1,500 BC. Major epidemics appeared especially when there were large numbers of susceptible individuals within a population, e.g. in the years between 1940 and 1950. Many of the infected children died, whereas in other cases the consequences were lifelong paralysis symptoms. Some children survived the poliovirus infection only by temporary placement in an iron lung, which allowed breathing for a limited period of time. Franklin D. Roosevelt, President of the USA from 1933 to 1945, was infected with poliovirus as an adult, and had lifelong muscular dysfunctions.

Originally, the three different types of poliovirus were distributed worldwide. They exhibit differences especially in the amino acid sequences of the variable loop regions of VP1, VP2 and VP3 and can be distinguished serologically: type 1 (Mahoney, also known as Brunhilde) has a relatively high incidence and causes severe diseases, whereas type 2 (Lansing) induced rather mild illnesses; infections with poliovirus type 3 (Leon) also have a severe clinical course. More than 150,000 cases of poliomyelitis were registered in the 1992. By virtue of the immunization programme of the World Health Organization (WHO), poliomyelitis is considered as eradicated, except for in about ten nations: as a result, Europe was declared polio-free by the WHO in June 2002. Nevertheless, serious recurrences have temporarily appeared owing to transregional dissemination: in the nations concerned (e.g. Nigeria), the immunization programme was stopped for political reasons. In 2008, infections with polioviruses 1 and 3 were reported only in a few developing countries, 98 % of them in Nigeria, India and Pakistan. Nonetheless, starting from such countries, poliovirus infections have also been imported to some regions which had already been declared as polio-free. However, the WHO has achieved successful control of such disseminations by implementing massive outbreak-control immunization measures. For this reason, acute poliovirus infections were not reported in India in 2011.

Under natural conditions, polioviruses infect only humans; however, they can be transmitted to various monkey species, and can cause epidemics in chimpanzee populations, as described by Jane Goodall. Further, poliovirus 2 could be adapted to mice as well. Polioviruses preferentially proliferate in the lymphatic tissue of the intestine, the Peyer plaques, and are excreted in the faeces of infected individuals for about 5 weeks. In the initial stage of the disease, an infection of the pharyngeal mucosa and the tonsils can frequently be observed, so the virus is present in the sputum and in the pharyngeal lavage. Polioviruses are transmitted by aerosols, via the faecal–oral route, via contact with dirt or smears and by contaminated drinking water.

Infantile Paralysis: It Became an Adult Paralysis

During past centuries, poliovirus infections were widespread and occurred mostly during the first 6 months of life under the protection of the maternal IgG antibodies, which are transferred through the placenta. Children did not become ill if they were infected in this early stage of life, but they developed an active immune protection. Later contacts with the poliovirus led to diseases only in children who had not experienced such an infection in that early period of life, because of the now lacking maternal immune protection. All in all, less than 1 % of all seronegative children became ill with poliomyelitis after contact with the virus. These data indicate that poliovirus is not highly neuroinvasive. Indeed, non-paralytic poliovirus infections were the most frequent reason for summer flu. With the increase of living standards, the first contact with poliovirus has been postponed to an advanced age; thus, the infantile paralysis became an adult paralysis.

Clinical Features

The vast majority of poliovirus infections are asymptomatic. After an incubation period of 1–2 weeks, only a few cases of poliovirus infection lead to stomach and intestinal disorders, followed by fever and flu-like symptoms, which typically resemble summer influenza. Most patients recover completely from this type of poliovirus infection (abortive polio). Approximately 1–2 % of patients subsequently develop a non-paralytic poliovirus infection; in such cases the virus infects the central nervous system, causing aseptic meningitis accompanied by muscle cramps and back pain, which may persist for about 2–10 days. In up to 2 % of patients, this illness is subsequently accompanied by symptoms of flaccid paralysis because the anterior horn cells are damaged (paralytic poliovirus infection). Approximately 10 % of these patients have a lethal outcome, e.g. due to paralysis of the respiratory muscles; about a further 10 % recover without lasting health impairments; however, in around 80 % of cases, paralyses of different magnitudes remain as permanent injury.

In recent years, the phenomenon of the so-called post-polio syndrome has been observed. The symptoms emerge 15–40 years after the occurrence of the original polio disease. It is a recurrent progressive myasthenia in association with muscle pain, joint aches and severe fatigue. It is believed that originally hardly damaged or also undamaged neural cells die owing to permanent strain, thus leading to the syndrome; many cases of such late complications are expected in the USA.

Furthermore, there were cases of vaccine-induced polio after polio immunizations with the live oral vaccine (Sabin). The attenuated vaccine virus is transiently excreted with the faeces of vaccinated patients, and can be transmitted to other members of the family; in such cases, immunosuppressed patients are particularly endangered.

Pathogenesis

After being transmitted, polioviruses infect the lymphatic tissue of the gastrointestinal tract and proliferate in the lymphatic tissue of the nasopharyngeal region. They are transported through the stomach into the intestine, where they preferentially infect the Peyer plaques, which represent the lymphatic tissue of the small intestine, and the mesenteric lymph nodes. The viruses are released directly into the bloodstream or the intestine through the lymphatic vessels. The virus infects activated monocytes expressing CD155 (the poliovirus receptor), and proliferates within them. The consequence is a mild viraemia, which elicits an initial fever attack (preliminary disease, abortive polio). Subsequently, the viruses spread throughout the organism and proliferate in the reticulohistiocytic system as well as in endothelial cells; this process is associated with a second viraemia, in which neurons can be infected, leading to a retrograde progression of the infection penetrating further into the spinal cord and brain. Especially, the large anterior horn cells and the motor neurons are infected, resulting in damage to and destruction of these cells. Poliomyelitis arises by infection of the cell-rich grey matter of the brain (*polios* is Greek for "grey"). Muscle cells and brain regions that were formerly innervated by the infected nerves are no longer innervated. The consequences are flaccid paralyses. If they are lasting, then the musculature atrophies because of inactivity. Subsequently, destroyed neurons are removed by macrophages (neuronophagy). The motor neurons remain atrophic: even years after the acute disease, generation of new glial cells (gliosis) as well as mild inflammations can be observed. Patients with paralysis symptoms of the intercostal musculature can survive the acute phase only with the aid of an iron lung. Extirpation of the tonsils, which is usually performed at the time of epidemic poliomyelitis, and irritations of the musculature (by movement, injections, etc.) frequently lead to respiratory paralysis; in such cases, the virus reaches the medulla oblongata along the damaged nerve fibres, destroying there the respective motor neurons.

Immune Response and Diagnosis

In the course of a poliovirus infection, IgM, IgA and IgG antibodies are produced against both capsid and non-structural proteins. Cross-reactivity between the three poliovirus types can only be observed by using heat-denaturated viruses. Reinfections lead to a new increase of antibody concentrations. Immunoglobulins against specific epitopes of structural proteins are neutralizing, and are measured in three discretely performed neutralization tests (polioviruses 1–3). Polioviruses can easily be cultivated in cell cultures (e.g. in primary monkey kidney cells, HeLa cells or Vero cells). The typing of isolates from faeces, pharyngeal lavage or cerebrospinal fluid is performed by neutralization tests using type-specific sera. This is the only way to reliably determine prevalence rates or increasing titres. In appropriate cases, the polymerase chain reaction (PCR) can alternatively be applied for viral genome detection and for identification of mutants. For such purposes, a pan-enterovirus PCR analysis is performed as a preliminary screening test and facilitates the identification of all human enteroviruses.

Therapy and Prophylaxis

The poliovirus vaccine (oral polio vaccine) that was developed by Albert Sabin is an attenuated live vaccine. Only 0.4–1.0 cases of reactions per million vaccinations have been observed. This vaccine induces the generation of virus-neutralizing IgG and IgA antibodies. Its efficiency has been demonstrated, for example, in the control of ongoing polio epidemics (outbreak-control immunization). It is hoped that poliovirus infections can be eradicated in the coming years by the consequent and worldwide application of this vaccine, particularly in developing countries. However, because of the possibility that polio infection symptoms can appear (vaccination-induced polio), this live vaccine must not be used in people with humoral immunodeficiencies (e.g. agammaglobulinaemia), or in patients who are immunocompromised owing to medicament usage, infections or for hereditary reasons. Since the vaccine virus is temporarily excreted in the faeces of vaccinated individuals, it can be transmitted to other members of the family; immunosuppressed individuals are particularly endangered.

The attenuated virus of the live vaccine against poliovirus 1 differs from the wild-type virus by 57 mutated nucleotides, which lead to 21 alterations in the amino acid sequence. In comparison with the wild type, the vaccine virus of poliovirus 3 exhibits ten mutations. However, the mutations that lead to the attenuation of poliovirus 2 are still unknown because the source virus for the vaccine strain has not been identified so far. Especially mutations in the nucleotide sequence of the IRES region seem to be responsible for the attenuation effect (positions 480, 481 and 472 in vaccine strains Sabin 1, 2 and 3, respectively). They influence the stability of the secondary structure of the IRES element, and consequently the association with the ribosomes, thereby affecting the effectiveness of translation. In addition, the attenuated vaccine strains exhibit mutations in genome sequences that encode for the capsid protein VP1. In this respect, vaccine polioviruses 1 and 3 exhibit 12 and two amino acid substitutions, respectively, which contribute to attenuation. In countries where poliovirus infections no longer occur, the live vaccine has not been used since 1999. The risk of acquiring a vaccine-induced poliomyelitis from the attenuated virus in such countries is higher than the risk of contracting the disease by a natural polio infection. A vaccine based on inactivated polioviruses was developed by Jonas Salk a few years before the live vaccine was developed. An improved variant of this vaccine with an enhanced antigen content is available and is now applied for basic immunizations. This inactivated vaccine is also able to induce the production of IgG antibodies. Booster vaccinations are recommended especially when travelling to developing countries (in Asia and Africa).

14.1.5.2 Human Enteroviruses and Parechoviruses

Epidemiology and Transmission

Today, human enteroviruses are subdivided into four species (A–D) and represent, together with the two serotypes of parechoviruses (originally known as echoviruses 22 and 23), a large number of infectious pathogens. Genetic recombination events between members of the same viral species are frequent and contribute to a broad range of virus variants. The viruses are distributed worldwide and their

epidemiology resembles that of polioviruses as they are primarily transmitted via the faecal–oral route and less frequently by aerosol or airborne particles. Infections with human enteroviruses occur principally in the warm months (summer flu), but in tropical regions they occur throughout the year. Seven percent of examined individuals excrete such viruses in the USA, but in tropical regions a level of up to 50 % has been reported. Therefore, recurrent epidemics are reported particularly in tropical countries of Southeast Asia, where outbreaks of enterovirus 71 are frequent. This virus type is considered among the so-called non-polio enteroviruses as the virus whose infections most frequently lead to diseases. Coxsackieviruses cause diseases only in humans; however, after experimental infections of chimpanzees, the viruses induce a clinically inapparent infection. The original classification of coxsackieviruses into subgroup A (serotypes 1–22, 24) and subgroup B (six serotypes) was based on differences concerning the histopathological lesions that they cause in experimentally infected newborn mice. Today the cultivation of coxsackieviruses is also possible in vitro (monkey kidney cells, human HeLa cells or human A549 lung carcinoma cells).

Clinical Features

The incubation period of human enterovirus infections lasts up to 2 weeks until the onset of the disease, and in rare cases more than 4 weeks. The viruses are excreted through the throat and the intestine for several weeks. The infections are characterized by a broad spectrum of different clinical manifestations. It is not possible to make unambiguous correlations between individual virus types and a particular clinical manifestation: different virus types can cause the same symptoms, whereas the same virus type can frequently be associated with many different symptoms. Patients are often simultaneously infected with several virus types; therefore, it is difficult to associate individual virus types with specific clinical pictures or symptoms. Human enteroviruses generally cause mild, cold-like illnesses, which can be associated with diarrhoea ("summer flu"); many infections are asymptomatic. Severe courses with neurological symptoms, meningitis, gastroenteritis, hand, foot and mouth disease, acute haemorrhagic conjunctivitis, myalgia, myocarditis, pleurodynia (Bornholm disease) or uveitis are only rarely observed. Patients with inherited or acquired immunological deficiencies frequently develop persistent infections, which can be associated with chronic enteritis, arthritis or meningoencephalitis. Meningitis is the predominant clinical picture in severe infections with human enteroviruses. In addition to herpesviruses, enteroviruses are considered the most frequent viral pathogens for meningitis and encephalitis. Coxsackie B viruses, particularly type B3, are believed to be the pathogen of viral myocarditis (perimyocarditis). Infantile myocarditis is frequently lethal in newborn babies. It occurs predominantly by infections acquired during delivery, when the mother has not produced antibodies against the coxsackievirus. Besides such acute forms, chronic disease types (dilatative cardiomyopathy) are also seen, especially in adults.

Some human enteroviruses (parechoviruses 1 and 2, coxsackievirus B4) are also discussed as a trigger of type 1 diabetes, which is often observed in patients with

a specific HLA type (HLA-DR and HLA-DQ) after infections with coxsackievirus B4. However, whether these infections have a causal connection to the autoimmune disorder is still controversial.

Pathogenesis

The uptake of human enteroviruses and their spread throughout the organism are similar to those for polioviruses. After initial proliferation in (the) intestinal and pharyngeal lymphatic tissues, the viruses are transported by the blood system as free virus particles or by infected lymphocytes during the second viraemic phase into the different target organs; these include the musculature, skin, meninges, myocardium, intestinal epithelia, central nervous system and respiratory system, where they settle, depending on the virus type. The molecular mechanisms that contribute to the development of the different symptoms are largely unknown. Besides hyperaemia, the following have been found in the conjunctiva of patients infected with enterovirus 70: dot-shaped bleedings (petechiae) and haemorrhages as well as infiltrations with mononuclear cells with diffuse distributed lymphocytes, which develop into striking large and swollen lymph follicles. In such cases, the cornea can be affected by epithelial opacity. Flaccid paralysis symptoms, which rarely occur during infections with enterovirus 70, are associated with degeneration of motor neurons, with haemorrhages and with neuroglial proliferation. The presence of viral proteins in microglia and neurons was demonstrated by immunofluorescence.

In the case of coxsackievirus infections, there is a primary infiltration of granulocytes, and this is followed by infiltration of mononuclear cells. The infected muscles exhibit focally necroses and the cells are disintegrated in a clod-like pattern. Necrotic areas can also be observed in neurons and glial cells of the central nervous system. After in vitro infection with coxsackievirus B3, monocytes release the cytokines TNF-α, IL-1β and IL-6. These are probably involved in the inflammation reaction. The chronic persistent myocarditis associated with coxsackievirus infections is characterized by significant lower quantities of infiltrating cells. Only single muscle cells disintegrate. Coxsackievirus RNA can be detected in heart muscle. Apparently, in this case the ratio between positive-sense and negative-sense RNA molecules is shifted to 2:1, whereas usually a larger excess of genomic RNA molecules is present in infected cells. In such cases, only a few viral genomes are synthesized and also the number of infectious coxsackieviruses is clearly lower in chronically infected regions. Type 1 diabetes mellitus, which is often diagnosed after infections with coxsackievirus B4, is probably triggered by autoimmune processes. There is evidence that similarities between viral proteins and cellular glutamate decarboxylase may be involved.

Immune Response and Diagnosis

During the course of the infection, virus-type-specific IgM, IgG and IgA antibodies are produced, and have, in part, neutralizing properties. The IgG-mediated immune reaction is possibly boosted by cross-reacting epitopes during reinfections with other virus types. In individual cases, a protective effect of IgA antibodies on the

gastrointestinal tract has been found. The IgM response plays only a minor role. Little is known about the influence of the cellular immune response on elimination of viruses; however, cytotoxic T cells seem to be responsible for the elimination of coxsackievirus B4 from the myocardium.

Human enterovirus infections are diagnosed by isolation of the virus and subsequent in vitro neutralization assays or by detection of viral RNA by PCR in faeces, urine, pharyngeal lavage, cerebrospinal fluid or eye secretions. Antibodies can be detected by virus-specific neutralization tests. Some viruses possess haemagglutination properties, which can be used for diagnosis. Because of the high prevalence, cross-reactions are frequent; therefore, ELISAs are only applicable for primary infections and often do not provide information about the virus type.

Therapy and Prophylaxis

There are neither vaccines nor therapeutic agents for prevention or treatment of human enteroviruses infections. Pleconaril, a drug that inhibits the uncoating process by hindering the release of the viral genomes, is also effective against enteroviruses. Although its efficacy against enteroviral meningitis and encephalitis has been demonstrated in clinical studies, it has not received marketing approval.

Ljungan Virus is Suspected of Causing Intrauterine Fetal Death

Human parechoviruses have a global distribution and cause, like human enteroviruses, disorders of the respiratory system and the gastrointestinal tract; severe diseases are only rarely observed. A species closely related to human parechoviruses is Ljungan virus, which is endemic in bank voles (*Clethrionomys glareolus*), especially in northern Sweden. These rodents appear in intervals of 3–4 years in large quantities and trek into barns and houses in autumn. The viruses are excreted in faeces and urine and can be transmitted to humans. Zoonotic transmissions to pregnant women are suspected of causing intrauterine fetal death in the late phase of gestation. In a Swedish study, viral genomes were detected in the placenta and in the brain of deceased fetuses in nearly 50 % of all cases examined.

14.1.5.3 Hepatitis A Virus

Epidemiology and Transmission

Hepatitis A virus was depicted by Stephen Feinstone by means of electron microscopy in 1973. It was independently isolated by Philip Provost and Gert Frösner and their respective co-workers in 1979. It has a worldwide distribution, but infections are mainly observed in tropical and subtropical regions as well as in developing countries. Infections generally occur during infancy. Six genotypes have been identified worldwide: genotypes I, II and III were isolated from infected patients, genotypes IV and VI were isolated from macaques (*Macaca fasicularis*) and genotype V was isolated from a vervet monkey (African green

monkey, *Cercopthecus aethiops*). Several antigen variants have been identified among the human genotypes, but there is only one serotype of hepatitis A virus. Whereas in central Europe and North America the seroprevalence among people older than 50 years was very high before the introduction of vaccination, acute infections with hepatitis A viruses are now relatively rare and appear in adults rather as travelling illnesses: approximately 1,000 cases are reported yearly in Germany. Hepatitis A viruses can be cultivated in primary and continuous kidney cell cultures of African green monkeys without a cytopathic effect; however, the viral reproduction cycle is very slow.

The virus is very stable against environmental influences and is excreted in large amounts in the faeces of infected individuals during the incubation period of 3–6 weeks. The virus is primarily transmitted via the faecal–oral route, by contact with dirt or smears as well as by contaminated food and drinking water. In rare cases, the infection can be transmitted by blood and saliva of ill people during viraemia. The virus is frequently transmitted among residents of asylums and retirement homes, among drug-addicted individuals, but also in kindergartens, vacation camps and in population groups with low socio-economic status.

Accumulation of Hepatitis A Virus in Mussels

The transmission of hepatitis A virus by contaminated mussels is known. In some regions of the world, untreated domestic sewage is released into rivers, lakes and seas. Since in such countries infections with hepatitis A viruses are frequent, this stable virus can reach the natural environment in this way. Mussels that grow in seas containing contaminated sewage near large towns are effectively able to filtrate the viruses from the surrounding water, thus leading to accumulation and concentration of the virus in the mussels. If such mussels are insufficiently heated during the cooking process, the virus is transmitted to the gastrointestinal tract after consumption of infected mussels.

Clinical Features

The incubation period until the appearance of the first symptoms lasts 3–6 weeks. The principal symptom of a hepatitis A virus infection is liver inflammation with jaundice (icterus), which arises by the transfer of bile salts (bilirubin) into the blood (hyperbilirubinaemia) and their elimination in the urine. The disease is, especially in children, usually inapparent, i.e. without symptoms. Jaundice symptoms begin suddenly, and are accompanied by nausea, fever and a general state of sickness that can continue for several weeks; particularly in older adults, occasionally there are also fulminant forms that can have a lethal outcome owing to cirrhosis and severe hepatic failure. The hepatitis A virus genome can be detected in the faeces by PCR even several weeks after remission of symptoms. In immunosuppressed patients, it is detectable for a considerably longer time. Persistent infections have not been observed.

Pathogenesis

Hepatitis A virus reaches the gastrointestinal tract generally by contaminated food. There is evidence that the virus infects the crypt cells of the small intestine, before it spreads via the blood system and reaches its principal target organ, the liver, and replicates there in hepatocytes. The proteins TIM-1 and TIM-3 have been identified as cellular receptors; an alternative entry pathway seems to exist for virus particles complexed with IgA molecules. These immunocomplexed viruses can bind to IgA receptors on the surface of hepatocytes. Replication of hepatitis A virus occurs in the liver 8–10 days before the onset of symptoms. The viruses spread to the intestine through the bile duct, and are excreted in the faeces. During this phase, viruses are also present in the blood, reaching concentrations of up to 10^5 virus particles per millilitre. The peak of virus release has already passed at the onset of the disease. Liver cells are destroyed by infection-induced mechanisms, viral proteins are found in the cytoplasm and even virus particles can be detected by electron microscopy. The very massive damage of liver cells leads to the release of bilirubin and transaminases into the blood. In contrast to other picornaviruses, in hepatitis A virus, functions responsible for the virus-host shutoff effect are not or are only weakly developed. Liver cells are not primarily destroyed by the viral infection. Instead, the cellular immune response is principally responsible for this process, as cytotoxic $CD8^+$ T cells have been detected in the liver of patients with acute hepatitis A. These cells release interferon-γ and give rise to the active immigration of other immunologically active cells into the liver. Infiltrating mononuclear cells are mostly located near the liver portal regions. Besides liver cells, also macrophages of the spleen and Kupffer's star cells (also known as stellate cells) contain viral proteins. They are derived from viruses. In a later infection stage, immigration of $CD4^+$ lymphocytes can be observed. In rare cases, a hepatitis A virus infection can cause a transient granulocytopenia and damage of bone marrow cells.

Initially, hepatitis A virus proliferates very slowly in infected patients; the incubation period lasts several weeks until the appearance of the first symptoms, which indicate liver cell destruction. Infected liver cells synthesize only marginal amounts of interferon-α (IFN-α) and IFN-β, with the consequence of a delayed activation of the host immune response. Phosphorylation and activation of IRF-3 is inhibited. IRF-3 activation is usually achieved by the signal transduction cascades induced either by activation of Toll-like receptor 3 (TLR3) or the RIG helicase by double-stranded RNA molecules (see ▶ Chaps. 7 and ▶ 8) which arise during the replication cycle of hepatitis A viruses. The viral non-structural protein 2B seems to block this process. Therefore, the synthesis of IFN-β is inhibited in infected cells, and the virus is able to proliferate largely uninfluenced by the immune system during the early infection.

Immune Response and Diagnosis

During hepatitis A virus infection, IgM antibodies are already present in serum at the onset of the clinical disease. IgG antibodies against capsid proteins, and to a lesser extent also against non-structural proteins, are subsequently produced, and persist lifelong. Both IgM and IgG antibodies are capable of neutralizing the virus; they account for the control of viral proliferation and for the rapid decrease of the

viral titre in blood and faeces. Neutralizing antibodies target specific domains on the capsid surface of hepatitis A virus particles. They form an immunodominant region, which involves amino acids 102–114 of VP1 and the amino acid at position 70 of VP3. Another VP1-specific epitope has been characterized around residue 221. Diagnosis of acute infection is performed by detecting viral RNA genomes in blood and stool by PCR as well as by detecting specific IgM antibodies in the serum. The presence of IgG indicates a past infection. As usual for each type of hepatitis, the transaminase and bilirubin levels are additionally determined.

Therapy and Prophylaxis

Vaccines based on in vitro cultivated and formalin-inactivated hepatitis A viruses confer very good protection after two immunizations. Whether the vaccine can last lifelong or whether subsequent booster immunizations are necessary cannot yet be definitely assessed. In fact, the vaccine against hepatitis A is considered as very effective. Current studies based on 10–12 years of vaccination experience have revealed that protecting antibodies have been detected in more than 95 % of all vaccinated individuals. Mathematical calculations predict a persistence of more than 25 years for vaccine-induced antibodies in more than 95 % of all vaccinated individuals.

By administrating virus-specific immunoglobulins, one can achieve passive immune protection lasting for 4–6 weeks. For example, such immunizations are performed as immunoprophylaxis when the time period for production of active antibodies (10–14 days) is too short before the start of the journey. There is no specific antiviral therapy.

Mini Epidemics of Hepatitis A

Because of imported single infections with hepatitis A virus which have been acquired abroad, small epidemics repeatedly emerge also in Europe; occasionally larger mini epidemics can also be observed, and these can affect more than 100 people. For example, a German butcher was infected with hepatitis A virus during his holydays in the Canary Islands. Thereafter, he infected members of his family and at least five co-workers in the butcher's shop in Germany. Apparently, there was contamination of sausage products with this very stable virus. In this way, more people were infected by foodstuff. Further hepatitis A virus infections emerged in small hospitals that were supplied by the contaminated butcher's shop. After identification of the infection chain, public health authorities initiated appropriate measures to control the mini epidemics: This was followed by simultaneous active and passive vaccinations of the workers in the butcher's shop, the exposed customers and the physicians involved. Furthermore, even effective chlorination of public swimming pools was performed. Additionally, relevant blood donor services were informed because hepatitis A viruses can also be transmitted by blood during the viraemic phase.

14.1.5.4 Rhinoviruses

Epidemiology and Transmission

Human rhinoviruses infect only humans. However, they can be transmitted to some ape species and ferrets. There are more than 100 serotypes, and these are classified into groups A and B. Another classification is based on receptor binding: 90 members of the rhinoviruses (major group) bind to ICAM-1 as a cellular receptor, whereas the rest (minor group) bind to the LDL receptor. Rhinoviruses 54 and 89 use heparan sulphate for attachment to host cells. Several serotypes exist simultaneously in a single virus population.

Rhinovirus infections appear especially in both the spring and the autumn. Presumably, they cause 40 % of all acute infections of the respiratory tract. Every person experiences one to three rhinovirus infections per year, the number decreasing with increasing age. Rhinovirus infections cause high morbidity and have great economic importance because of the associated loss of working hours. Transmission generally occurs indirectly by contact with contaminated hands or door handles and only seldom by aerosols or droplets. Rhinoviruses spread rapidly within families, kindergartens and schools.

Clinical Features

The incubation period lasts 1–3 days. Nearly 50 % of rhinovirus infections are asymptomatic; the disease begins with sneezing, coughing and a scratchy throat (flu-like infection). Fever, swelling of lymph nodes and general malaise are absent. The main syndromes are catarrh and nasal congestion. The secretion is initially aqueous and later viscous and yellowish. The symptoms last for a maximum of 1 week. If the symptoms do not subside, this is a sign that a bacterial infection of the paranasal sinuses or an inflammation of the middle ear has possibly occurred.

Pathogenesis

Rhinoviruses invade the body through the mucous membranes of the upper respiratory tract, where they bind to their cellular receptors by the canyon structures. Rhinovirus replication has adapted to the temperature of the mucosae (32–33 °C) and proceeds very fast. Progeny viruses are released by cell lysis 8–10 h after infection of epithelial cells. Maximum viral titres are found after 2–3 days. Release of the virus decreases approximately 4 days after infection. With the scanning electron microscope one can see that large quantities of cells are shed from the ciliated epithelium. This is directly caused by the cell-damaging effect of the virus, and is an ideal basis for bacterial superinfections.

Pathohistologically, hyperaemia and oedema as well as intensified production of mucous secretion with threefold to fivefold increased protein content can be observed at the onset of the disease. The secretion contains a number of inflammatory cytokines, such as IL-1β, TNF-α, IL-6 und IL-11 as well as chemokines such as IL-8, CCL5 (RANTES) and monocyte chemoattractant protein 1; particularly IL-8 induces the migration of neutrophils, monocytes and dendritic cells to the infected regions. Bradykinin, lysylbradykinin and other vasoactive substances

which increase vessel permeability are produced and secreted intensively like vascular endothelial growth factor. Altogether, the viral infection stimulates the local production of many inflammation mediators, and thus causes the typical symptoms.

Analogously to hepatitis A virus, rhinoviruses also inhibit synthesis of IFN-β. However, rhinoviruses do not inhibit phosphorylation and activation of IRF-3, but they prevent the formation of the functionally active IRF-3 dimers, which are formed in the nucleus after transport of the phosphorylated IRF-3.

Immune Response and Diagnosis

Five to 10 days after the infection, IgM, IgG und IgA antibodies against viral proteins can be found in secretions and blood. Whereas IgG is detectable for several years, IgA can be detected for only a few months; particularly IgA mediates protection against reinfections, but only against the same virus type. Most people produce $CD4^{+}$ T cells, which exhibit cross-reactivity with epitopes of various rhinovirus types. Nothing is known about the occurrence of cytotoxic T cells. Diagnosis is performed only clinically. Antibody determination and virus detection are not done in routine diagnostics.

Therapy and Prophylaxis

Vaccines against rhinovirus infections are not available. Because of the large number of different virus types, the development of vaccines is very difficult. It is possible to avoid a rhinovirus infection by application of interferon. However, a long-term interferon therapy should not be performed owing to the associated injuries to mucous membranes. Pleconaril, which embeds within the canyon structures of virus particles, can decrease the infection, but it also induces the fast formation of resistant viruses, so administration of this substance seems to be futile.

14.1.6 Animal Pathogenic Picornaviruses

A large number of picornaviruses have been described in various animal species and they induce a broad spectrum of diseases. FMD virus is of eminent economic importance, and can infect all ungulates (ruminants and swine). For the differential diagnosis of FMD, swine vesicular disease virus plays an important role in pigs. This virus is a member of porcine enterovirus B and causes a disease in swine that is clinically indistinguishable from FMD. Swine vesicular disease virus is acid-resistant, and is predominantly transmitted by meat products from infected swine. It remains infectious for several months in non-heated meat products such as raw sausages (e.g. salami). Diagnosis is performed by cultivation of the virus or PCR, as well as by antibody detection.

Other picornaviruses (various types of enterovirus and encephalomyocarditis virus) can induce fertility disorders or general diseases in swine, but these are relatively rare today. A formerly very important viral disease in swine is induced by porcine teschoviruses. They cause, in rare cases, a polioencephalitis in swine which largely

resembles the poliovirus-induced poliomyelitis in humans. An antigenetically very similar strain of porcine teschoviruses (Talfan virus) has a substantially lower virulence. Generally, its infection is clinically inapparent, but it induces a resilient, protective cross-immunity against the virulent techovirus. Talfan virus is spread worldwide in swine populations. Therefore, clinical cases of Teschen disease are only rarely observed owing to the high infection rate of Talfan virus.

FMD in Europe

Most countries in Europe and all countries of the European Union were free from FMD for many years. This status was essentially achieved by stringent measures in cases of epidemic outbreaks and by the extensive annual vaccination of all cattle older than 6 months from the reference date. In 1991, vaccination was stopped because the virus was eradicated within the countries of the European Union and the control of FMD was uniformly implemented throughout the European Union. From then, only sporadic outbreaks were registered until 2001, e.g. in Italy in 1993 and 1994 and in Greece in 1996. However, a massive FMD outbreak occurred in the UK in 2001. The virus (serotype 01) was probably introduced by infected pork from Asia which was used as animal food. After the initial porcine infection cycle, the virus was mainly spread by infected sheep, a species which develops only mild symptoms. Nearly 10,000 farmsteads were affected in the UK by January 2002. More than four million cloven-hoofed animals (cattle, sheep, pigs, goats and deer) were killed in the course of veterinary measures. From England, the virus was only sporadically transmitted to other countries, and the outbreaks (25 in the Netherlands, two in France and one in Ireland) were rapidly controlled by culling, and partially also by ring vaccinations. From 21 January 2002, Europe was considered to be free from FMD once more. However, on 2 August 2007, new cases of FMD emerged in southern England, and further cases were also diagnosed in the surrounding cattle farms a little later. During this outbreak, the agent was FMD virus strain BFS 1860 O1 1967 (British Field Strain 1860, serotype O, subtype 1, isolated in 1967), which was cultivated in the immediate vicinity of the Pirbright Laboratory of the Institute for Animal Health for the purpose of vaccination. Most probably, a laboratory virus escaped through old and leaky sewage systems into the environment. Owing to the rapid action of the relevant authorities, the setting up of security zones and the culling of all infected livestock, the virus was contained rapidly.

Owing to financial losses for the affected farmers as well as the ban on transport of live animals and their meat, the outbreak in England had severe financial consequences for husbandry in the UK, which had already been shocked by the BSE crisis. The outbreak in the UK has shown that there is great danger from the FMD virus and that faster diagnostics, an inexorable culling of infected animals and the consequent surveillance of animal transport are still necessary.

14.1.6.1 FMD Virus

Epidemiology and Transmission

FMD virus is one of the most important and economically most relevant animal pathogenic viruses. Although it has not played an important role in Europe for a few decades, and the countries of the European Union are considered to be FMD-free owing to systematic disease control, the FMD epidemic in the UK in 2001 has shown that infiltration of the virus is still possible at any time, and that it can induce great epidemics. Although vaccinations against FMD are no longer performed in the countries of the European Union, a reserve vaccine stock is still retained for control of potential epidemics with ring and emergency vaccinations. The measures required to prevent viral infiltration as well as production, checking and storage of the serotype-specific vaccines require huge financial expense. In Europe, FMD is a notifiable disease; veterinarians and animal owners are obliged to notify the relevant veterinary authority about every suspicion of disease. After notification, the official order to kill single animals follows, if necessary, for diagnostic purposes. If FMD is officially ascertained in the animals, killing of all cloven-hoofed animals of the livestock is ordered, and extensive epidemiological examinations are initiated to control possible dissemination of the virus. These measures concern not only FMD, but also diseases induced by viruses that cause FMD-like symptoms and which can be therefore clarified by differential diagnostics. These viruses include swine vesicular disease virus, vesicular stomatitis virus (▶ Sect. 15.1) and the now eradicated vesicular exanthema of swine virus (Sect. 14.6). FMD virus is widespread worldwide and is endemic in vast regions of Africa, South America and Asia, and also in the Asiatic part of Turkey (Anatolia) as serotypes O, A and Asia 1. From such regions, the virus is occasionally imported into the European part of Turkey and rarely also into other European countries, such as Bulgaria and the Balkan countries in 1996 (serotype O) and Greece in 1996 and 2000 (serotype Asia1).

FMD virus is considered as a prototype of the genus *Aphthovirus*. It is acid-sensitive and infects all cloven-hoofed animals; that means in Europe it infects cattle, small ruminants such as sheep and goats, including endemic and exotic ruminants (deer and also antelopes and wild cattle in zoos), and domestic swine and boars.

Epidemiologically important is the differential expression of clinical symptoms: whereas in cattle and swine FMD is a febrile disease that is accompanied by the typical formation of aphthae in the planum nasolabiale of the muzzle and the planum rostrale of the snout as well as in the coronary band of the hoofs, in sheep the infection is less apparent and can easily be overlooked. However, sheep are extremely susceptible to the highly contagious virus. Since infected sheep excrete high amounts of infectious virus, an FMD-infected sheep flock can be the origin of a fast-spreading, devastating epidemic. Epidemiologically significant is also the fact that the viruses are excreted in large amounts by swine, but also by cattle. In an epidemic wave and its spread, infection of these animal species can lead to a drastic increase in the circulating virus quantities in the animal populations.

Of further epidemiological importance is the fact that there are different serotypes of FMD virus, and against which the infected animals do not develop cross-immunity – similarly to what is found in human infections with the three different poliovirus serotypes. Besides the seven serotypes – O (Oise), A (Allemagne), C, Asia 1 and SAT (South African Territories) 1–3 – there are numerous subtypes, whose infections induce partially only a slight cross-immunity. This antigen diversity plays an important role in the control of FMD.

The virus is transmitted by droplet infections. It is relatively stable and contagious, so under favourable climatic conditions infections can be spread through the air even over long distances. This was demonstrated during an FMD outbreak on the Isle of Wight in 1981. Starting from the French mainland, the viruses were transmitted to the island over the English Channel. The distance is 250 km. Inasmuch as the virus is also excreted in milk, transmission to swine is also possible by feeding with milk products from infected cattle. The virus is acid-labile; therefore, it is inactivated in the meat of slaughtered animals after sufficiently long meat maturation. However, it remains infectious over extended periods of time in insufficiently salted and smocked raw sausages. Hence, it can be transmitted by such products that are prepared from infected animals.

Clinical Features

Infected animals excrete the viruses before the appearance of the first clinical symptoms during an incubation period of a few days. The typical aphthae appear in the planum nasolabiale of cattle as well as in the planum rostrale and in the tongue of swine, in the coronary band of the hoofs and in distinct mucosal regions of the gastrointestinal tract, e.g. in the rumina of cattle. The aphthae contain large quantities of viruses. Whereas alterations in the planum nasolabiale and in the mucous membranes of the gastrointestinal tract are the main symptoms in cattle, inflammation of the coronary band (coronitis) is usually the prevalent symptom in swine. The morbidity is high, but the mortality is low. The virus can persist in infected cattle or sheep for several weeks.

Human infections with FMD virus are extremely rare; however, single cases have occasionally been reported. These are subclinical, but they can also be associated with fever and aphthae formation in mucous membranes, like in animals.

Pathogenesis

The infection is oronasal. Initially, viruses proliferate in the mucosa of the muzzle and tongue and first small aphthae emerge (primary aphthae). Thereafter, the viruses spread through the blood system and reach all inner organs, where they replicate and cause the classic symptoms such as blistering in epithelia (accompanied by fever, drop in performance, salivation, lameness) and the typical, nearly pathognomonic (pathological) alterations such as extensive epithelial colliquative necroses and fibrosis. Infections in young cattle and swine can also affect the heart muscle by inflammation, degeneration and fibrosis. Because of its fibrillar structure, the heart muscle appears “streaked”: this condition is called tiger heart. The virus

reaches the mucosa of the muzzle via the bloodstream, Here the typical aphthae (secondary aphthae) are produced as characteristic symptoms of the disease.

It is important that the virus can persist in cattle. The virus can be isolated from throat epithelium of experimentally infected cattle (probang sample). However, it has been shown in various independent studies that these animals do not excrete viruses, and contact animals are not infected.

Immune Response and Diagnosis

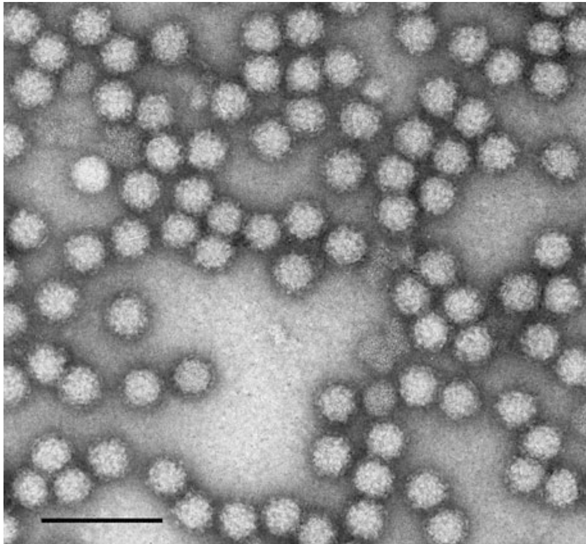
The infection confers a serotype-specific immunity. It is believed that it does not last lifelong. The diagnosis is performed by propagation of the virus in cell culture, preferentially in baby hamster kidney cells or in diverse primary and permanent bovine cell lines. Subsequently, the virus serotype can be determined by using specific antibodies in ELISAs or by means of the complement fixation reaction. Alternatively, the serotype analysis is performed by PCR followed by sequencing of the amplified fragments.

Control and Prophylaxis

FMD is a notifiable disease in many countries. In these countries each suspicion of disease has to be notified to the respective veterinary authorities. After diagnosis, the control includes killing and complete elimination of all ungulates of the livestock concerned as well as all cloven-hoofed animals in neighbouring livestock that are suspected of being infected. Quarantine areas are established in the region and extensive disinfection measures are performed. According to estimates by authorities, an FMD outbreak in a medium-sized country (e.g. Germany) would result in direct and indirect costs of several billion euros.

Recently, the non-vaccination policy regarding highly infectious epidemics such as FMD has been critically discussed within the European Union, and regulations are increasingly being relaxed. The European Commission can, in principle, allow protective vaccinations against FMD (ring vaccination) to protect not yet infected livestock, or suppressive vaccinations to reduce dissemination of the virus in already infected animal stocks or regions; in the latter case, the animals are killed later. The main problem that arises as a consequence of vaccination is the identification and distinction of vaccinated from infected animals. The development of ELISA systems that can detect antibodies against non-structural proteins, which are produced during an infection but not by vaccination, has significantly improved the acceptance of vaccination measures. The principle of distinction is as follows. During FMD virus replication, viral structural and non-structural proteins are synthesized in an animal, and are immunologically recognized; consequently, infected animals produce antibodies against both protein groups. Since vaccines are based on purified, inactivated virus particles, they solely consist of structural proteins and do not contain non-structural proteins. Therefore, vaccinated animals have only antibodies against structural proteins. Commercial ELISA systems which contain antigens of non-structural proteins 3A, 3B and 3C (also known as "3ABC ELISA") and which can detect the presence of antibodies against such proteins have proven to be sensitive and reliable enough to distinguish between vaccinated and infected animals.

14.2 Astroviruses



Little is known about the molecular biology of the family *Astroviridae*. Like picornaviruses, caliciviruses and hepeviruses, astroviruses possess a non-enveloped capsid and a positive-sense RNA genome. During the replication cycle, they synthesize a subgenomic mRNA species which is necessary for the production of structural proteins. The name is derived from Greek *astron* (*cσtpov*), which means "star", alluding to the star-like shape of a part of the astrovirus particle (Fig. 14.5).

14.2.1 Classification and Characteristic Prototypes

The family *Astroviridae* is currently subdivided into two genera, the members of which differ in regard to their host specificity (Table 14.5). The viruses of the genus *Mamastrovirus* infect humans and several mammals (swine, ovines, bovines, felines). The genus *Avastrovirus* includes viruses that infect birds (chickens, ducks, turkeys). Most astroviruses can easily be cultivated in vitro.

14.2.2 Structure

14.2.2.1 Virus Particle

Members of the astrovirus family have non-enveloped, spherical and icosahedral capsids with a diameter of about 28–30 nm (Fig. 14.5). At the capsid surface, approximately 10 % of the virions are characterized by structures which resemble a five- or six-pointed star. The particles are formed from a precursor protein, pV87,

Fig. 14.5 Structure of an astrovirus particle. *ssRNA:* single-stranded RNA

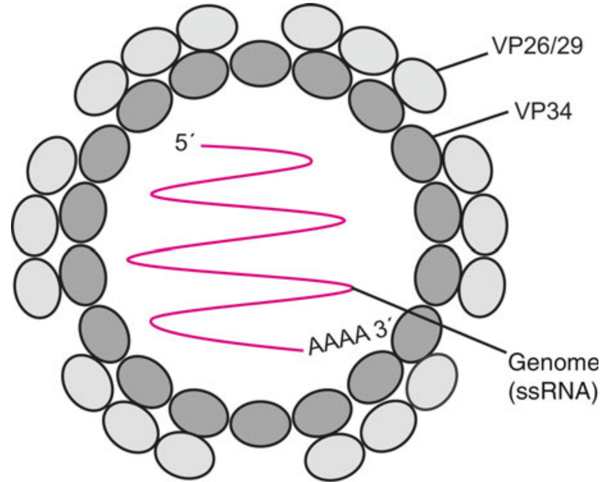


Table 14.5 Characteristic representatives of astroviruses

Genus	Human virus	Animal virus
Mamastrovirus	Human astrovirus types 1-8	Feline astrovirus Bovine astrovirus types 1 and 2 Ovine astrovirus Porcine astrovirus
Avastrovirus		Avastrovirus of ducks Avastrovirus of turkeys Avastrovirus of chicken

i.e. proteolytically cleaved, thereby generating capsid proteins VP34 and VP26/VP29. The processing is necessary for astrovirus infectivity. When cultivated in CaCo-2 cells (human colonic carcinoma cell line), newly synthesized virus particles must be treated with trypsin in order to gain infectivity.

14.2.2.2 Genome Organization and Structure

The astrovirus genome consists of single-stranded, positive-sense RNA; it is polyadenylated at its 3′ terminus and has a length of about 6,800 nucleotides (6,771 and 6,813 nucleotides in human astroviruses 1 and 3, respectively). Sequence analyses revealed three open reading frames with overlapping ends (Fig. 14.6). ORF1a and ORF1b encode non-structural proteins NSP1a and NSP1a/1b. A sequence of 20 nucleotides in the region overlapping ORF1a and ORF1b is highly conserved; it forms a hairpin structure that leads to a partial translational frameshift during translation of the genomic mRNA. Thereby, the codons encoded by ORF1a are fused in frame with those of ORF1b, resulting in the

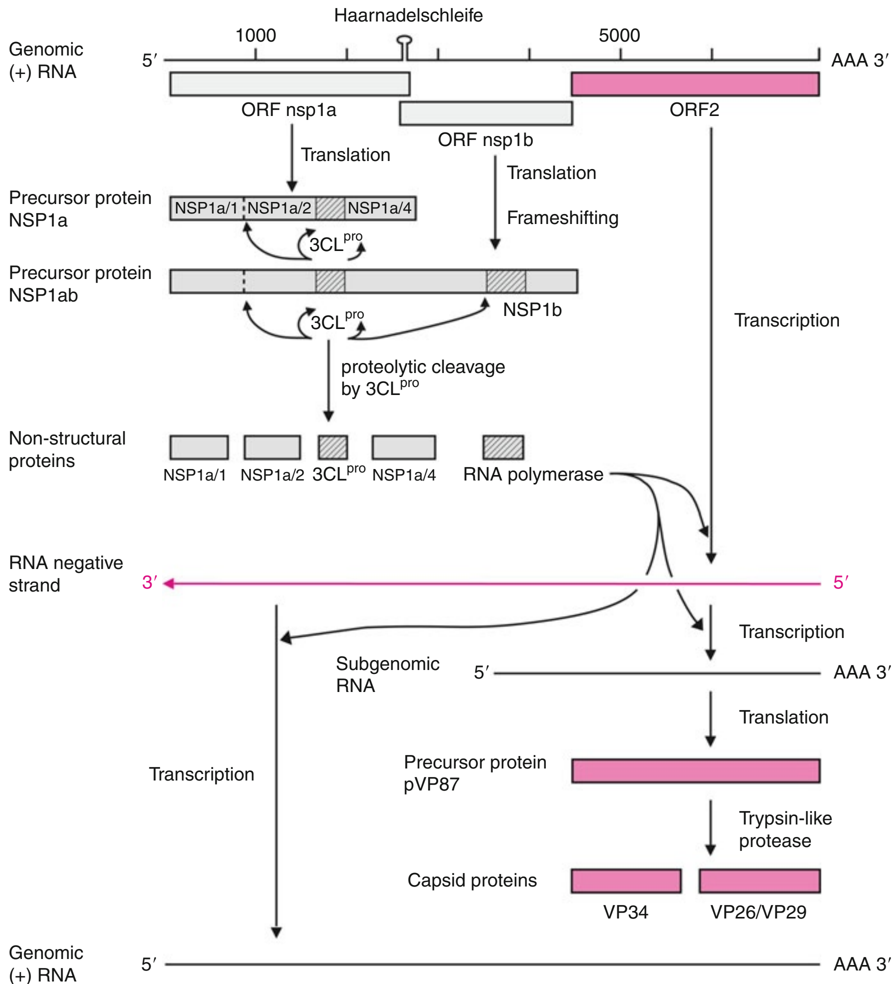


Fig. 14.6 Genome organization of astroviruses. The genome of astroviruses contains three open reading frames. The 3′ and 5′ ends of ORF1a and ORF1b overlap. A hairpin structure induces a translational frameshift leading to an in-frame fusion of ORF1a and ORF1b. The precursor protein NSP1ab contains, in addition to a protease ($3CL^{pro}$), also an RNA-dependent RNA polymerase. This enzyme is responsible for the synthesis of the negative-sense RNA molecules, from which the positive-sense RNA genomes of progeny viruses are transcribed. ORF2 encodes the precursor protein of structural proteins. It is cleaved by the activity of a trypsin-like protease as a part of the clearage product NSP1b

production of a joint precursor protein, NSP1ab. ORF2 encodes the precursor protein for the capsid proteins. At the 5′ terminus of the genomic RNA, there is a short untranslated sequence upstream of the start codon of ORF1a which comprises 10–20 and 45–85 nucleotides in avastroviruses and mamastroviruses,

respectively. Also at the 3′ end of the genomic untranslated sequences are present (130–135 nucleotides in avastroviruses and 59–85 nucleotides in mamastroviruses).

14.2.3 Viral Proteins

14.2.3.1 Non-Structural Proteins

Two precursor proteins are produced: NSP1a (103 kDa) comprises 920 amino acids and is encoded by ORF1a; NSP1ab has a molecular mass of 160 kDa and is a precursor fusion protein that arises from a hairpin-mediated translational frame-shift between ORF1a and ORF1b. After translation, $3CL^{pro}$ (3C-like protease) mediates cleavage of the precursor protein NSP1a into probably four proteins (NSP1a/1, NSP1a/2, NSP1a/3 and NSP1a/4); $3CL^{pro}$ resembles the 3C protease of picornaviruses and is located in the central domain NSP1a/3 of the precursor protein. The RNA-dependent RNA polymerase is encoded by ORF1b. Few data are available concerning the function of the other cleavage products (Table 14.6).

14.2.3.2 Structural Proteins

The precursor protein (pVP87) for the production of the capsid proteins is encoded by ORF2 and comprises on average 780 amino acids; depending on the virus type, the molecular mass ranges between 70 and 90 kDa. The precursor protein

Table 14.6 Functions and properties of astrovirus proteins

Protein	Molecular mass (kDa)	Reading frame	Function/properties
NSP1a/1	20	ORF1a	????
NSP1a/2	23	ORF1a	Hydrophobic, contains putative transmembrane domains
NSP1a/3	27	ORF1a	3C-like protease $3CL^{pro}$, cleavage of precursor protein(s)
NSP1a/4	26–35	ORF1a	Contains nuclear transport signal, located in perinuclear region, associates with ER
NSP1b	≈57	ORF1b	RNA-dependent RNA polymerase, transcription, replication
pVP87	70–87	ORF2	Precursor protein of capsid proteins; different size in different virus types; cleaved by cellular typsin-like proteases
VP34	34	ORF2	Capsid protein, amino-terminal cleavage product of pVP87, conserved
VP26, VP29	26–29	ORF2	Capsid proteins, carboxy-terminal cleavage product of pVP87, variable; proteins are generated by alternative use of cleavage sites within pVP87, induction of neutralizing antibodies, substrate of cellular caspases

The protein succession order in the table corresponds to the real order of the reading frames in the genome and in the precursor proteins
ORF open reading frame, *ER* endoplasmic reticulum

aggregates to particular structures, and is subsequently cleaved by trypsin-like cellular proteases into a conserved amino-terminal domain (VP34) and a carboxy-terminal domain (VP26, VP29); the size of the latter can vary as additional trypsin cleavage sites are located within the neighbouring amino acid sequences and may be alternatively recognized and used by the enzymes. In the carboxy-terminal regions of both precursor protein pVP87 and its cleavage products VP26/VP29, a series of acidic amino acids have been described which are cleaved by cellular caspases. Both these proteolytic cleavage reactions by trypsin-like enzymes and caspases are necessary for the production of infectious virus particles.

14.2.4 Replication

The replication cycle of astroviruses has hardly been investigated. The cellular receptor to which the viruses bind on their target cells is unknown. Also, the processes that lead to penetration of virus particles and to the release of the viral genome are not well understood. The infection provokes, by still unknown mechanisms, the activation of the extracellular-signal-regulated kinase (ERK)-mediated signal pathway. This mitogen-activated signalling pathway is induced by extracellular stress signals and results in phosphorylation of ERK1/2. Thereby activated, ERK1/2 reach the cell nucleus and induce the expression of various cellular genes involved in regulating cell division and differentiation. The finding that activation of ERK-mediated signalling is important for viral replication provides evidence that astrovirus replication depends on dividing cells.

The initial step of the reproduction cycle is the translation of non-structural precursor proteins NSP1a and NSP1ab. How protein synthesis is initiated is unclear. No covalently linked protein has been found at the 5′ terminus, as observed in picornaviruses and caliciviruses; the UTR does not seem to form an IRES (see Sects. 14.1 and 14.3). Whether the 5′ end is capped is also not clear. In some translation events, a shift of the reading frame occurs in the carboxy-terminal region of NSP1a, resulting in an in-frame fusion of NSP1a and NSP1b. This process resembles the synthesis of the non-structural proteins of togaviruses (Sect. 14.6). After synthesis, the NSP1a and NSP1ab precursors are both autocatalytically cleaved by $3CL^{pro}$, whereby the functions of the diverse non-structural proteins, including RNA-dependent RNA polymerase, become available. The synthesis of complementary negative-sense RNA molecules is achieved by the activity of RNA polymerase. The negative RNA strands serve as templates for the synthesis of (1) new genomic mRNA molecules and (2) subgenomic mRNA species of about 2,000 nucleotides. The latter contain the ORF2 coding sequences and serve for translation of precursor protein pVP87 (Fig. 14.6). Apoptosis is induced in infected cells during viral replication, resulting in cell death and release of progeny viruses. However, it is unknown which of the viral proteins are responsible for this process. Caspase activities that are induced during apoptosis cleave capsid proteins VP26 and VP29, thus increasing the infectivity of released progeny viruses.

14.2.5 Human Pathogenic Astroviruses

14.2.5.1 Epidemiology and Transmission

Human astrovirus infections were first described during an outbreak of infectious gastroenteritis in a maternity clinic in the UK in 1975. Afterwards, it became evident that astroviruses are distributed worldwide and that they are, after caliciviruses, the second most frequent cause of non-bacterial diarrhoea (see Sect. 14.3). Eight serotypes have been found in human astroviruses to date, but serotype 1 is predominant. Astrovirus infections are preferentially found in children aged less than 2 years, but are also found in older people and in immunosuppressed patients. Excretion of the virus in the faeces persists for 1–2 weeks, but in immunosuppressed individuals also for considerably longer. Transmission occurs via the faecal–oral route.

14.2.5.2 Clinical Features

The incubation period is short and usually lasts 2–3 days. In some cases, astrovirus infections are asymptomatic; in general, they induce gastroenteritis accompanied by diarrhoea and occasionally also by vomiting; muscle and joint pains are rarely observed. The disease is mild and self-limiting and usually lasts 3–4 days. In immunosuppressed patients, astrovirus infections are severer and may persist for longer.

14.2.5.3 Pathogenesis

Astroviruses infect enterocytes in the small intestine and replicate in these cells. Only few signs of cell destruction and inflammation can be found histologically. The relatively low inflammatory activity can be associated with the property of capsid proteins binding to the C1q complement component, thus inhibiting the activation of the complement system as a defence reaction of the unspecific immune system (see ▶ Chap. 7). Furthermore, capsid proteins seem to exert an additional function that determines the pathogenesis of astrovirus infection: they cause an enhancement of the permeability of the epithelium of the small intestine. Because of this, the interaction of occludin, a tight-junction protein, with the cellular actin skeleton is impaired. This process is independent of active viral replication, and it may contribute to the diarrhoea symptoms.

14.2.5.4 Immune Response and Diagnosis

Astrovirus infections can be diagnosed by detection of viral proteins via antigen-capture ELISA, by electron-microscopic representation of virus particles from samples of faeces or by amplification of viral RNA by PCR. During infection, IgM antibodies and later IgG and IgA antibodies against viral structural proteins and are detectable by ELISA. The antibody concentration declines rapidly after the disease. Neither cultivation of the virus in cell cultures nor serological methods play a role in diagnostics. Nothing is known about cellular immunity.

14.2.5.5 Prophylaxis and Therapy

The best measures to avoid infections with human astroviruses are good hygiene and disinfection. Chemotherapy or vaccines are not available. In several countries, astrovirus infections have to be notified to the respective health authorities.

Mammalian Astroviruses are Veterinarily Irrelevant

Mammalian astroviruses have been isolated from swine, cattle, sheep, red deer, cats and mink; all can be propagated in vitro. They differ genetically by up to 40 %, and viruses that infect individual species form their own clusters in phylogenetic analyses. This suggests a high degree of host specificity. An exception is feline astrovirus. It is relatively closely related to human astroviruses and belongs to this cluster. Like for human astroviruses, in vitro cultivation of feline astrovirus requires the addition of trypsin to the culture medium to facilitate infection. In all cases, only mild or subclinical infections have been described; hence, the virus is without clinical relevance for these animals.

14.2.6 Animal Pathogenic Astroviruses

14.2.6.1 Epidemiology and Transmission

Astrovirus infections are frequent in poultry; there are different distinguishable serotypes. Avian nephritis virus is widespread in chickens. It causes minor growth disorders, which can become evident at the herd level. The virus is excreted and transmitted through the faeces of infected animals.

14.2.6.2 Clinical Features

Like human and mammalian astroviruses, avian astroviruses cause subclinical infections and mild diseases. Both serotypes of turkey astrovirus provoke gastroenteritis and are associated with the poultry enteritis mortality syndrome. These viruses replicate in epithelial cells of the intestine as well as in various other tissues, including thymus, bursa of Fabricius, spleen and kidney. Avian nephritis virus infects kidney epithelial cells, and is capable of inducing an interstitial nephritis. Duck astrovirus is associated with hepatitis; a mortality rate of more than 50 % has been observed in experimental transmissions.

14.2.6.3 Pathogenesis

The pathogenesis of avian astrovirus infections has barely been investigated. In contrast to infections in mammals and humans, avian astroviruses do not proliferate exclusively in epithelial cells of the intestine, they also proliferate in several other tissues; therefore, a wider range of different diseases have been observed.

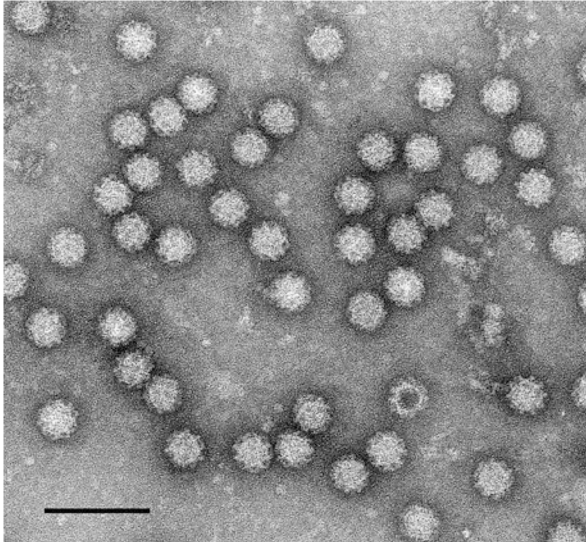
14.2.6.4 Immune Response and Diagnosis

Avian astroviruses can be propagated in cell culture. Diagnostics can be performed by detecting viral RNA genomes by PCR.

14.2.6.5 Prophylaxis and Therapy

Neither antiviral therapy nor immunoprophylaxis is available.

14.3 Caliciviruses



There is limited knowledge with regard to the molecular biology of the family *Caliciviridae*. The name is derived from the Greek word *kalyx* (meaning “goblet” or “chalice”) and alludes to the cup-shaped deepened structures in the lateral surfaces of the icosahedron which can be observed in electron microscope images. Like picornaviruses, caliciviruses possess non-enveloped capsids and single-stranded, positive-sense RNA genomes. In contrast to picornaviruses, caliciviruses synthesize a subgenomic RNA species during their replication cycle. By this property, they exhibit similarities to hepeviruses and togaviruses.

14.3.1 Classification and Characteristic Prototypes

The family *Caliciviridae* is subdivided into five genera, which differ especially in the structure of their genome (Table 14.7). The genera *Vesivirus* and *Lagovirus* comprise only animal pathogens. Feline calicivirus, which belongs to the vesiviruses, is a widespread cat flu pathogen. Rabbit haemorrhagic disease virus is most prominent among the genus *Lagovirus* because it was used to combat the rabbit plague in Australia some decades ago. Human pathogenic viruses that cause diarrhoea are classified into the genera *Norovirus* and *Sapovirus*. Their members are characterized by considerable sequence differences in their genomes, and are

Table 14.7 Characteristic representatives of caliciviruses

Genus	Human virus	Animal virus
Vesivirus		Vesicular exanthema of swine virus San Miguel sea lion virus Feline calicivirus
Lagovirus		Rabbit haemorrhagic disease virus European brown hare syndrome virus
Norovirus	Norovirus; genogroups I, II, IV Norwalk virus Southhampton virus Mexico virus Desert Shield virus	Norovirus, genogroup III (bovines) Norovirus, genogroup V (mice) Porcine enteric calicivirus Jena virus Newbury agent Canine norovirus
Sapovirus	Sapovirus, genogroups I, II, IV, V Sapporo virus Parkville virus Manchester human calicivirus	Sapovirus, genogroup III (swine)
Nebovirus		Newbury-1 virus
Valovirus[a]		St-Valérien virus

[a]Proposed as a new genus

subdivided into five groups (GI–GV), which in turn contain a large number of different genotypes. They have denominations that commonly are derived from the place where they were isolated. Norovirus types GI, GII and GIV are human pathogenic viruses, whereas genogroups GIII and GV infect cattle and mice. Sapoviruses infect predominantly humans; only members of genogroup III have been isolated from swine. Until now, only one species, Newbury-1 virus, has been assigned to the genus *Nebovirus*: it infects cattle and causes diarrhoea in calves. Recently, new members of caliciviruses have also been isolated from pigs in Canada. These St-Valérien viruses cannot be assigned to any genera today; therefore, the new genus *Valovirus* has been proposed. Only a few caliciviruses can be cultivated in cell culture: feline calicivirus and San Miguel sea lion virus can be propagated largely without problems, whereas the propagation of rabbit haemorrhagic disease virus is only possible in primary hepatocytes. No cell culture system has been described for noroviruses and sapoviruses.

14.3.2 Structure

14.3.2.1 Virus Particle

Members of the family *Caliciviridae* have non-enveloped, spherical and icosahedral capsids with a diameter of about 34–39 nm and depressions in the lateral faces (Fig. 14.7). They consist of 90 units of the dimeric VP1 (60 kDa). The virus particles contain one or two molecules of VP2 (23 kDa in noroviruses), which is associated with the RNA genome, and Vpg, which is covalently linked to the 5′ terminus of the genome.

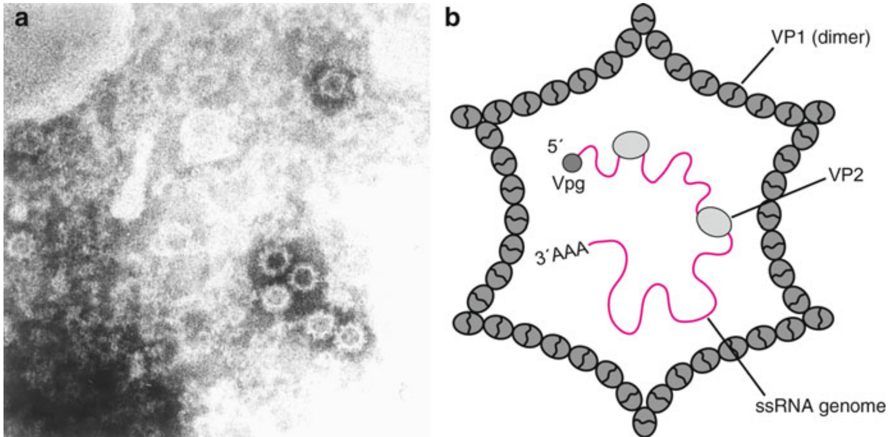


Fig. 14.7 (**a**) Electron microscope image of a calicivirus using recombinantly produced empty capsids of feline calicivirus. (**b**) The structure of a calicivirus particle

14.3.2.2 Genome Organization and Structure

The genome of caliciviruses is constituted of single-stranded, positive-sense RNA, which is polyadenylated at the 3′ terminus and has a length ranging from 7,000 to 8,000 nucleotides (7,338–7,708 nucleotides in noroviruses, 7,437 nucleotides in rabbit haemorrhagic disease virus, 7,690 nucleotides in feline calicivirus and 7,431–7,490 nucleotides in sapoviruses). A viral protein (15 kDa) is covalently linked to the 5′ terminus; it is equivalent to Vpg of picornaviruses (Sect. 14.1). Short untranslated sequences of 10–14 nucleotides in length are located in the 5′ region of the genome upstream of the start codon of ORF1. Genome sequence analyses revealed two open reading frames in sapoviruses and lagoviruses and three open reading frames in noroviruses and vesiviruses; their ends partially overlap (Fig. 14.8). ORF1 is located in the 5′ half of the genome and encodes the precursor of the non-structural proteins. ORF2 is located in the 3′ half of the genome and encodes capsid protein VP1; in sapoviruses, the VP1 open reading frame is directly interconnected with ORF1. The additional reading frame ORF3 (ORF2 in sapoviruses) is responsible for the synthesis of VP2.

14.3.3 Viral Proteins

14.3.3.1 Non-Structural Proteins

The non-structural proteins of caliciviruses are synthesized as a precursor protein of about 180–190 kDa. It is post-translationally cleaved into single components by $3CL^{pro}$, which resembles the 3C protease of picornaviruses (see Sect. 14.1). The arrangement of the individual components is similar in all caliciviruses: the

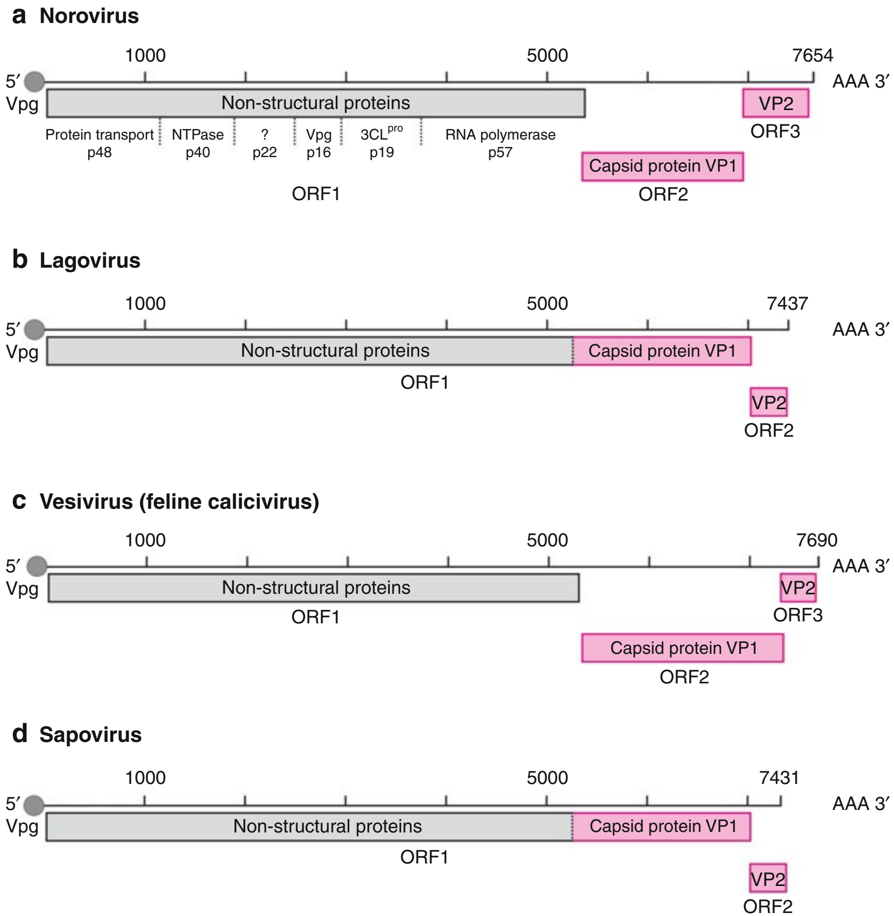


Fig. 14.8 Genome organization of caliciviruses. (**a**) Norovirus (Norwalk virus). (**b**) Lagovirus (rabbit haemorrhagic disease virus). (**c**) Vesivirus (feline calicivirus). (**d**) Sapovirus (Sapporo virus). The mRNA genome of caliciviruses is modified by a covalently linked protein (Vpg) at the 5′ end, and is polyadenylated at the 3′ terminus. It is translated in the cytoplasm. The organization of the open reading frames differs among the different genera: in noroviruses and vesiviruses there are three open reading frames. ORF1 is separated from ORF2 and is responsible for the synthesis of the precursor for the non-structural proteins. It is autocatalytically cleaved by the serine protease 3CL^{pro} into Vpg, an NTPase (helicase), RNA-dependent RNA polymerase and two additional non-structural proteins (p48 and p22 in noroviruses). The non-structural protein precursors of lagoviruses, sapoviruses and vesiviruses contain the respective protein functions, but they have been scarcely examined. The RNA polymerase is responsible for the synthesis of the negative-sense RNA molecules, from which the RNA genomes of progeny viruses are synthesized during the replication cycle. ORF1 encodes capsid protein VP1 and ORF3 contains the genetic information for VP2, a protein associated with the RNA genome. In lagoviruses and sapoviruses, the reading frame encoding the non-structural protein precursor is fused in frame to the open reading frame of capsid protein VP1. The small ORF2 encodes VP2

Table 14.8 Functions and properties of astrovirus proteins

Protein	Norovirus	Sapovirus	Feline calicivirus	RHDV	Function
Non-structural protein	37–48 kDa	32 kDa	32 kDa	? kDa	Associates with Golgi vesicles, influences intracellular protein transport
Non-structural protein	40–41 kDa	35 kDa	36–38 kDa	37 kDa	NTPase, helicase? essential for transcription and replication
Non-structural protein	20–22 kDa	32 kDa	30 kDa	29 kDa	Function unknown; membrane associated?
Vpg	16 kDa	14 kDa	15.5 kDa	13 kDa	Covalently linked with the 5′ terminus of RNA, translation initiation
Protease, 3CL^{pro}	19 kDa	14 kDa	30 kDa	15 kDa	3C-like protease, cleavage of precursor proteins
Polymerase	57 kDa	57 kDa	50 kDa	58 kDa	RNA-dependent RNA polymerase, transcription, replication
Capsid protein VP1	68 kDa (ORF2)	60–62 kDa (ORF1)	60–65 kDa (ORF2)	60 kDa (ORF1)	Structural protein, dimer, major capsid protein
Structural protein VP2	23–29 kDa (ORF3)	23–29 kDa (ORF2)	12 kDa (ORF3)	10 kDa (ORF2)	Structural protein, RNA binding

The protein succession order in the table corresponds to the real order of the reading frames in the genome and in the precursor proteins
RHDV rabbit haemorrhagic disease virus

amino-terminal sequences constitute a non-structural protein (p48 in noroviruses) of variable length that is characterized by a transmembrane domain and that is associated with Golgi vesicles. It interacts with cellular vesicle-associated membrane-protein-associated protein A (VAP-A), which regulates the SNARE-mediated fusion of intracellular vesicles; the function of this viral protein is not completely resolved. The directly adjacent sequences represent an NTPase with similarity to the picornaviral 2C protein and which possibly functions as a helicase. The domain of Vpg, which is covalently linked to the 5′ terminus of the genome, is separated from the NTPase by an amino acid region of unknown function; it contains many hydrophobic amino acids and possibly induces the interaction of the precursor protein with intracellular membrane compartments, such as the 3A protein in picornaviruses. The adjoining sequences represent the domains of 3CL^{pro} and RNA-dependent RNA polymerase (Table 14.8).

14.3.3.2 Structural Proteins

In lagoviruses and sapoviruses, the sequences of capsid protein VP1 are fused to the carboxy-terminal end of the RNA-dependent RNA polymerase, and the separation into individual proteins is performed by the proteolytic activity of 3CL^{pro}.

In noroviruses and vesiviruses, VP1 is encoded by separate reading frames. Vesiviruses exhibit the peculiarity that VP1 is initially synthesized as a precursor protein (73–78 kDa), which is processed by $3CL^{pro}$ into the VP1 that is part of mature virus particles. In this case, a leader sequence of 128 amino acids is removed from the amino-terminal end; its function is still unknown. If VP1 proteins are expressed in eukaryotic systems by genetic engineering methods, they can autonomously associate to form virus-like particles by a self-assembly process. The structural protein VP2 is encoded by a separate open reading frame (ORF2 in lagoviruses and sapoviruses; ORF3 in noroviruses and vesiviruses). It contains many basic amino acids and is associated in low copy numbers with the RNA genome.

14.3.4 Replication

Noroviruses and sapoviruses use the carbohydrate structures of the ABH and Lewis blood group antigens as cellular receptors, whereby distinct virus types bind to different versions of these histo-blood group antigens. Histo-blood group antigens are complex sugar structures which are present on the cell surface of erythrocytes and epithelial cells of the mucosa of the respiratory tract and the intestine. The cellular receptors of feline calicivirus are α(2,6)-linked sialic acid molecules, which are modifications of junction adhesion molecule A, a member of the immunoglobulin superfamily. After adsorption, virus particles are probably incorporated by receptor-mediated endocytosis. Nothing is known concerning the processes that lead to the release of the viral genome from capsids and endosomes.

The first step during viral replication is the translation of non-structural proteins in noroviruses and vesiviruses, as well as the translation of the fusion products of the non-structural and capsid proteins in lagoviruses and sapoviruses using the positive-sense RNA genome as mRNA. The viral genome is characterized by neither a 5′-cap structure nor an IRES element at its 5′ terminus, which otherwise mediate binding of ribosomes for cellular or picornaviral translation, respectively (see Sect. 14.1). Instead, caliciviruses have developed an alternative mechanism for translation initiation that is mediated by Vpg linked to the 5′ terminus of the RNA genome. Vpg interacts with the cellular translation initiation factor eIF-3, a component of the 40S ribosomal subunit; binding of Vpg to eIF-3 mediates attachment of the small ribosomal subunit to the 5′ end of the viral genome. This probably induces binding of the large ribosomal subunit and the translation of ORF1 from the adjacent start codon. The synthesized precursor proteins are autocatalytically cleaved by $3CL^{pro}$, whereby the functions of the different non-structural proteins, including RNA-dependent RNA polymerase, become available.

RNA-dependent RNA polymerase catalyses the synthesis of complementary negative-sense RNA molecules. The negative-sense RNA molecules are used as a template for the synthesis of new Vpg-primed genomic and subgenomic RNAs. These have a length of 2,400–2,700 nucleotides and encode the structural proteins.

This process is similar to the replication process in astroviruses (Sect. 14.2). The subgenomic RNAs are bicistronic and are responsible for the synthesis of VP1 and VP2 in lagoviruses and noroviruses. Translation initiation of VP2 occurs by an unusual mechanism. After termination of VP2 translation, the ribosomes remain bound to the mRNA. Next, translation is reinitiated, resulting in the synthesis of VP2. VP1 capsid proteins aggregate to precursor virus particles, into which the viral RNA genomes are deposited. Interestingly, subgenomic RNAs are also packaged in some virions; this has been shown in rabbit haemorrhagic disease virus, but not in feline calicivirus.

Animal Noroviruses and the Risk of Zoonotic Transmissions

Diverse animal species are infected by noroviruses; there are isolates especially from cattle and swine. Recently, norovirus infections have been observed also in dogs. In general, noroviruses cause no or only mild diseases in animals. Noroviruses of genogroup II can be isolated from swine and humans, whereas viruses of genogroup III have only been found in bovines. The direct transmission from animals to humans was hitherto considered as improbable because animal noroviruses differ substantially from human isolates. However, the isolation of a human genotype II/4 virus from swine and pork demonstrated that this assumption may not be generalized. Besides direct transmission from swine to humans, even genetic recombinations between animal and human noroviruses are conceivable in principal. However, although this has not been directly demonstrated, such a scenario does not appear impossible. It is based on the findings that human noroviruses can replicate in swine and cattle, and that genetic recombination between different genogroups occurs.

14.3.5 Human Pathogenic Caliciviruses: Noroviruses and Sapoviruses

14.3.5.1 Epidemiology and Transmission

Infections with noroviruses and sapoviruses have a global distribution. Both virus types contain several genogroups with variants that are pathogenic in humans and which can be subdivided into different genotypes. This suggests that caliciviruses have a very high mutation rate because of the high error rate of RNA-dependent RNA polymerase, which essentially contributes to the high norovirus and sapovirus variability. Infections cause gastroenteritis that is associated with severe diarrhoea and vomiting. The introduction of obligatory notification has revealed that noroviruses and sapoviruses are responsible for most intestinal infections in the countries of central Europe. The viruses are transmitted by infected individuals via stool and vomitus. Excretion of the virus lasts some days longer than the symptoms; therefore, infected individuals should not return to work not until 2 days after cessation of diarrhoea.

Faeces of immunosuppressed individuals can remain infectious for weeks or months. This causes considerable logistic problems in hospitals because patients cannot be transferred unless viruses are undetectable in stool. The non-enveloped calicivirus particles possess a high environmental resistance, which has to be considered when choosing appropriate disinfectants. Fewer than 100 virus particles are sufficient to induce an infection. Therefore, these pathogens can easily be transmitted by the faecal–oral route, but also by contaminated food and drinking water. They induce epidemics in collective facilities such as schools and homes or on cruise ships, where many people have to cohabit in a relatively confined space. Particularly affected are retirement homes and hospitals, where there is a high turnover of patients, nursing staff and visitors. In such outbreaks, the infection is also frequently transmitted by infected personnel. Whereas sapoviruses have principally been observed in gastrointestinal diseases of children, and until now have not been detected in food, noroviruses also cause food-borne infections (food poisoning); therefore, a strict code of conduct is prescribed for kitchen personnel. More recently, noroviruses have also been detected in the faeces of calves and swine. This suggests a zoonotic potential for this virus group, but this has not been demonstrated so far, however.

14.3.5.2 Clinical Features

The incubation period is very short and usually lasts 2–3 days. Noroviruses and sapoviruses cause short-lasting (2–3 days) but severe gastroenteritis with vomiting and diarrhoea. Severe general symptoms are not observed. The disease is self-limiting and does not cause long-lasting damage. Protracted courses are observed in immunocompromised patients who excrete infectious viruses for considerably longer periods. Threatening situations can emerge in older patients owing to massive fluid loss.

14.3.5.3 Pathogenesis

Noroviruses and sapoviruses infect and destroy mature intestinal enterocytes. As cellular receptors, noroviruses and sapoviruses use carbohydrate structures which are present in ABH and Lewis blood group antigens as well as on the surface of enterocytes. These saccharide structures are present not only in cell-bound form, but also in soluble form, e.g. in breast milk. Individuals belonging to the secretor-negative type are resistant because of the lack of genetic information for the enzyme α(1,2)-fucosyltransferase. Breast-fed infants of secretor-positive mothers ingest the sugar molecules via breast milk and are temporarily protected because the saccharide molecules can complex with the viruses in the intestine of children, thereby preventing infection of enterocytes.

14.3.5.4 Immune Response and Diagnosis

Norovirus and sapovirus infections can be diagnosed by detecting virus particles via antigen ELISA or by detecting viral RNA by reverse transcription PCR (RT-PCR) from stool samples. Evidence of virus particles in stool samples by means of

electron microscopy does not play a role in diagnosis. Serological analyses to detect virus-specific antibodies do not have diagnostic significance. Nothing is known concerning cellular immunity.

14.3.5.5 Prophylaxis and Therapy

The best measure to avoid infections with human caliciviruses is systematic compliance with both personal and general hygiene measures. This also includes disinfection by using appropriate disinfectants, the strict isolation and non-transport of infected patients within the hospital, and cleaning of plants for food production and water supply. In extreme situations, entire hospital units and sections and even whole hospitals have to be closed to admission of patients. Cruise liners are temporarily quarantined. Chemotherapy or vaccines do not exist.

Genetic Differences Within Blood Group Antigens Mediate Resistance Against Viral Infections

Blood groups are characterized by the individual composition of glycolipids or proteins on the surface of erythrocytes and other somatic cells. In humans, there are a myriad of different blood group systems; the so-called histo-blood group antigens can be assigned to three families, namely the Lewis, secretor and ABO families. For example, the common feature of all groups of the ABO system is the presence of *N*-acetylglucosamine as the central sugar molecule of carbohydrate structures on cell surfaces. They are linked to galactose molecules that carry fucose residues. Together they compose the basic structure of all blood groups representing blood group 0. If one or two additional *N*-galactosamine molecules are linked to the galactose, these carbohydrate structures represent blood groups A and B, respectively. The biosynthesis of blood group antigens depends on a number of enzymes that are subject to genetic variation. If viruses use carbohydrate structures of blood group systems as receptors, the susceptibility of the respective infection is genetically determined. Therefore, people with genetic defects in the enzyme α(1,2)-fucosyltransferase are not able to synthesize the carbohydrate structures that are necessary for binding of noroviruses and sapoviruses to the cell surfaces. Hence, these "non-secretor" phenotypes cannot be infected by certain types of noroviruses and sapoviruses: they are resistant. Inasmuch as noroviruses frequently mutate, and as a result also change their receptor preferences, it must be assumed that such resistance does not apply for all virus types. Similar genetically determined resistances are also known from parvovirus B19 infections: this virus binds to blood group antigen P, a glycosphingolipid. People who do not possess the genetic information for the enzymes which are necessary for the synthesis of the corresponding carbohydrate structures cannot be infected by these types of parvoviruses (see ▶ Sect. 20.1)

14.3.6 Animal Pathogenic Caliciviruses

The clinical pictures caused by caliciviruses that are pathogenic in animals are clearly different from those of human pathogenic caliciviruses. Vesicular exanthema of swine virus is the prototype of the genus *Vesivirus*. Retrospective genetic analyses revealed that vesicular exanthema of swine virus is closely related to San Miguel sea lion virus, which displays different serotypes; it may therefore be of marine origin. Vesicular exanthema virus of swine exhibits a broad host spectrum, which includes various mammals, reptiles, amphibians and even fishes and nematodes. It can also be cultivated in human cell lines in vitro. Between 1930 and 1950, vesicular exanthema of swine virus caused diseases in swine in the USA which could not be distinguished by differential diagnosis from FMD. In mammals, blistering of the skin can be observed, particularly in the extremities (coronary band as well as fins), and in the mucosa of the muzzle. The last outbreak was detected in the USA in 1952; since then the virus has been deemed to be eradicated.

14.3.6.1 Feline Calicivirus

Epidemiology and Transmission

Feline calicivirus is a very important animal pathogen; it is the causative agent of cat flu. It is transmitted by direct contact and by secretions from the respiratory tract. Many infected cats develop the status of persistent infected virus carriers, which excrete the virus for weeks and months. Feline caliciviruses are antigenetically not uniform, and although distinct serotypes cannot formally be distinguished, there are many isolates whose infections induce no or a non-protecting and incomplete cross-immunity. The variability within the capsid proteins is primarily limited to a little domain of about 100 amino acids, the hypervariable E region. The nucleotide sequences of different isolates can differ in this region by up to 70 %.

Recently, feline caliciviruses have been isolated from cats with a new clinical picture. These cats show oedemas in the head and neck and ulcers in the nose, ears and paws, as well as sporadic icterus. Such isolates have been denominated as virulent systemic feline caliciviruses. This is an unfortunate designation as every feline calicivirus infection is systemic and the haemorrhagic component in the clinical picture is rare; in particular, these new isolates do not show any resemblance to rabbit haemorrhagic disease virus. Interestingly, these highly virulent isolates originate from vaccinated and mainly old cats. Genetically, they cannot be grouped together; they exhibit a similar heterogeneity like other feline caliciviruses. A genetic marker has not yet been found for these highly virulent strains.

Clinical Features

The virus induces an acute respiratory disease in cats, and it is associated with rhinitis and ulcers of the oral mucosa; polyarthritis is rarely found.

Particularly, kittens become ill within the first few months of life. Feline calicivirus can frequently be detected in old cats that suffer from chronic inflammation of the oral mucosa (stomatitis). However, an aetiological significance of the virus in this clinical picture has not been proven.

Pathogenesis

Viraemia arises during the course of infection, which also leads to the infection of synovial cells in the joints. The tonsils are a site for persistence of the virus. This virus replicates in feline cell cultures; hence, it is well investigated.

Immune Response and Diagnosis

Infected cats develop an IgG response against capsid proteins, which can be detected by ELISA and Western blotting. The diagnosis of the acute infection is done by cultivating the virus in cell culture or by detecting viral RNA by PCR.

Control and Prophylaxis

Vaccines against cat flu are available on the basis of both attenuated and inactivated viruses, and these are routinely used to vaccinate domestic cats. However, these vaccines have only limited effectiveness owing to high antigenic variability.

14.3.6.2 Rabbit Haemorrhagic Disease Virus

Epidemiology and Transmission

Around 1985, a seemingly new severe epidemic disease appeared in rabbits (*Oryctolagus cuniculus*) in China. The illness, named rabbit haemorrhagic disease, spread rapidly worldwide. It is characterized by severe haemorrhagic symptoms and fulminant hepatitis. Parvoviruses were primarily assumed as the aetiologic agent; however, calicivirus was rapidly described as the true pathogen. The origin of rabbit haemorrhagic disease virus in China has not yet been finally determined. Retrospective serological analyses have revealed that the virus was disseminated in the UK as long ago as 1950 without having shown any epidemic signs at that time.

Meanwhile, all rabbit populations are contaminated, but the initially high mortality has strongly decreased. Apparently, fast attenuation of the virulence seems to occur in rabbits, as shown by the “field study” in Australia and New Zealand. Therefore, animal health measures have been lifted. Besides direct contact between animals and coprophagy (eating faeces), the passive transmission of the virus by arthropods plays an additional role. The virus is deemed to be host-specific; it cannot be propagated in cell culture. European brown hare syndrome virus, a similar but different virus type, causes disease in the European brown hare (*Lepus europaeus*). This virus is not contagious for domestic rabbits.

Clinical Features

The virus causes a severe disease in rabbits older than 8 weeks. Irrespective of whether maternal antibodies are present, the kittens cannot be infected before that period. The disease is usually fulminant, and the animals die within hours of infection. In protracted cases, the animals bleed from all mucous membranes. Recovery is exceptional.

Pathogenesis

The virus has a tropism for hepatocytes and causes a lytic infection leading to massive liver necroses.

Immune Response and Diagnosis

Because of the fulminant course of the infection, an immune response cannot be established in the animals; the serological diagnosis is generally irrelevant. Subclinically infected animals can be identified by detecting specific antibodies in ELISA. The viral genome can be detected by RT-PCR in samples from liver, blood and faeces. Electron-microscopic verification of virus particles in the liver is also possible and is commonly used. Since the virus does not replicate in cell cultures, isolation of the virus is not possible.

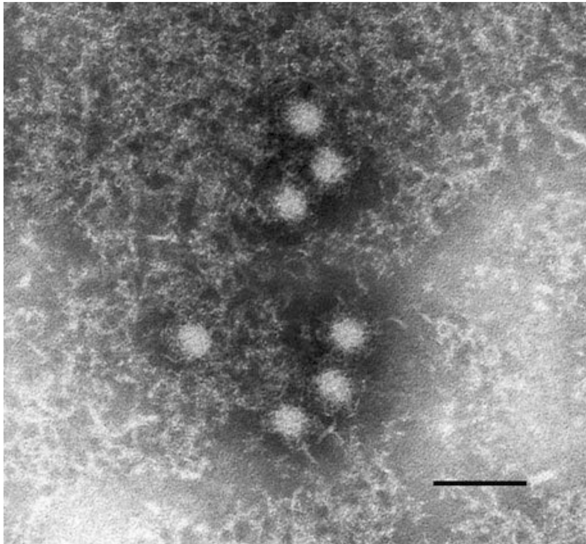
Control and Prophylaxis

For rabbits in livestock husbandry, a vaccine based on inactivated whole virus is available, and this is extracted from the liver of infected rabbits.

Rabbit Haemorrhagic Disease

Rabbit haemorrhagic disease virus gained dubious popularity when it was released for biological control of rabbit populations under the name “rabbit calicivirus” in Australia in 1996. It has been and is being controversially discussed whether other species of the unique Australian fauna may be endangered in addition to rabbits. On the other hand, the tremendous rabbit plague in Australia had considerable consequences for the flora and consequently also for the fauna. The application of the virus led to a significant reduction of the rabbit population (60 % or more) in some regions of Australia; however, in other regions it had no influence. Farmers disseminated the virus over long distances within the continent, and a lively trade in infected rabbits flourished. Furthermore, the virus was illegally introduced to and spread in New Zealand. As expected, healthy rabbits carrying infectious viruses were found in Australia and New Zealand after a short time. This is an infallible sign of natural attenuation of the virus in its host.

14.4 Hepeviruses



Hepatitis E viruses were long considered to belong to a group of unclassified viruses, the so-called non-A, non-B hepatitis viruses. Later they were classified into the family *Caliciviridae* because of electron-microscopic analyses which revealed some structural similarities. However, further investigations regarding the replication cycle and genome structure have revealed notable differences; therefore, hepatitis E viruses are now classified into their own family, the *Hepeviridae*. The family name is derived from the denomination of the hepatitis E virus.

14.4.1 Classification and Characteristic Prototypes

Hepatitis E viruses are classified into the genus *Hepevirus* (Table 14.9). They are serotypically homogenous, but are subdivided into four genotypes: genotypes 1 and 2 have only been found in humans, whereas genotypes 3 and 4 represent porcine hepatitis E viruses and are closely related to genotypes 1 and 2 and can also infect humans. Human hepatitis E virus infection is a classic zoonosis in which the pathogens are transmitted from swine to humans. Furthermore, there is also a hepatitis E virus variant which can only reproduce in chickens and other birds.

Table 14.9 Characteristic representatives of hepeviruses

Genus	Human virus	Animal virus
Hepevirus	Hepatitis E virus	Porcine hepatitis E virus Avian hepatitis E virus

14.4.2 Structure

14.4.2.1 Virus Particle

Hepatitis E viruses have non-enveloped icosahedral capsids with a diameter of 34–39 nm. In electron-microscopic images they resemble caliciviruses (Fig. 14.9) and are composed of 180 units of a capsid protein with a molecular mass of 76 kDa. The particles additionally contain various amounts of a small soluble protein of 14.5 kDa, the origin of which is still uncertain. It is possibly a cleavage product of the capsid protein.

14.4.2.2 Genome Organization and Structure

The genome of hepatitis E viruses consists of single-stranded, positive-sense RNA with a length between 7,194 and 7,232 nucleotides, depending on the different genotypes. The 5′ terminus contains a cap structure and the 3′ terminus is polyadenylated. Short non-coding sequences are located at the 5′ and 3′ ends of the genomic RNA (Fig. 14.10). Sequence analyses revealed three open reading frames with partially overlapping ends. ORF1 is situated in the 5′-oriented part of the genome half and encodes the precursor of the non-structural proteins. The second open reading frame (ORF2) directs the synthesis of the capsid protein. A further open reading frame (ORF3) encodes the small protein pORF3, which comprises 112–132 amino acids and is important for the infectivity of the virus. This open reading frame overlaps with ORF1 in most isolates. Exceptions are the Chinese isolates of hepatitis E viruses (genotype 4), in which the 3′ end of ORF1 encoding the non-structural protein precursor is separated by 28 nucleotides from the translation initiation codon of ORF3.

14.4.3 Viral Proteins

The non-structural protein precursor (186 kDa) comprises 1,692–1,709 amino acids and contains the domains of a methyltransferase/guanylyltransferase at the amino-terminal region (Fig. 14.10). It is necessary for modification of genomic and subgenomic mRNAs with a cap structure. The other non-structural proteins are a protease, which cleaves the precursor protein into the individual functional components, an NTPase/helicase and an RNA-dependent RNA polymerase (Table 14.10). The protease resembles cysteine proteases of the papain type. Whether this protease is responsible for all cleavage reactions and at which sites has not been finally elucidated. In hepatitis E viruses, the sequences encoding the capsid protein are located in a separate reading frame (ORF2) that exhibits the highest degree of conservation. The capsid protein generally has a length of 660 amino acids (about 72–76 kDa) and forms homodimers. It is characterized by a signal-peptide-like sequence followed by a signalase cleavage site at the amino terminus. Glycosylated and unglycosylated forms may be found in the cytoplasm of infected cells. Probably the capsid protein is synthesized in association with the ER membrane and translocated into the ER lumen, whereby the signal peptide is excised and the

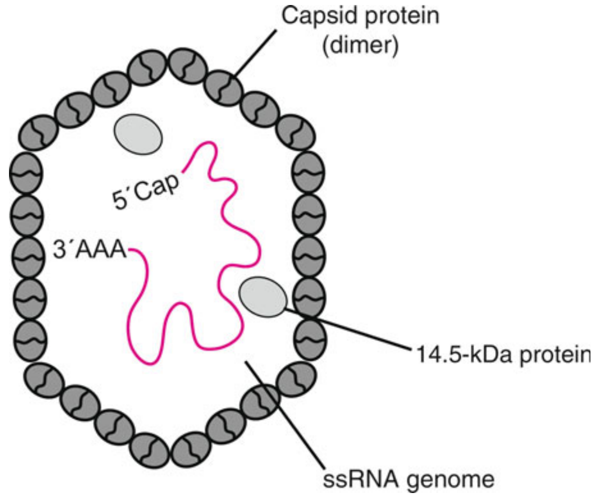


Fig. 14.9 Particle structure of a hepatitis E virus

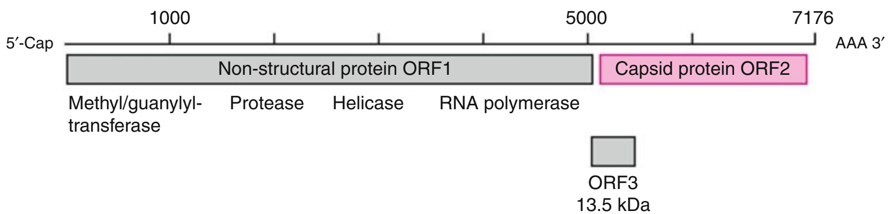


Fig. 14.10 Genome organization of hepatitis E viruses. The mRNA genome contains a cap structure at the 5′ terminus, and is polyadenylated at its 3′ end. It comprises three open reading frames: ORF1 encodes a non-structural protein containing the domains for a methyltransferase, a protease, a helicase and an RNA-dependent RNA polymerase. The protease cleaves the precursor protein into the different functional domains. The RNA-dependent RNA polymerase is responsible for the synthesis of both the antigenomes and new viral RNA genomes. ORF2 encodes the capsid protein and ORF3 encodes a non-structural protein of 13.5 kDa

amino acid chain is modified with sugar groups. Subsequently, retransport of the capsid proteins into the cytoplasm seems to occur. ORF3 encodes a small phosphoprotein, pORF3. Its function has not been conclusively resolved. Nevertheless, two-hybrid analyses have shown that it interacts with the capsid proteins; phosphorylation of the serine residue at position 80 of pORF3 is necessary for interaction. Moreover, binding to bikunin, a Kunitz-type protease inhibitor of serine proteases, and binding to haemopexin, a glycoprotein present in blood plasma that belongs to the acute phase proteins (see ▶ Chap. 7), have also been observed. To what extent these in vitro results are relevant for in vivo replication of hepatitis E viruses is not clear. New data suggest an interaction of pORF3 with dynein of microtubules. It has further been shown that pORF3 is localized in endosomes, where it interferes with protein

Table 14.10 Function and properties of hepatitis E virus proteins

Protein	Size (kDa)	Properties	Function
Capsid protein	76	Dimer; signal peptide is cleaved, partially glycosylated	Capsid protein
Capsid protein	14.5	?	Soluble protein in virions, cleavage product of the 76-kDa capsid protein?
Methyltransferase/ guanylyltransferase	110	?	Capping of genomic and subgenomic RNA molecules
Protease	?	Homology to cysteine proteases	Cleaves the precursor of non-structural proteins
RNA helicase	?	NTP-binding	Resolution of RNA secondary structures
RNA-dependent RNA polymerase	?	?	Replication Synthesis of subgenomic RNA
pORF3	13.5	Phosphorylated, homodimer	Associated with cytoskeleton

transport into the compartments of late endosomes, e.g. the transport of the epidermal growth factor receptor. Since this process is a prerequisite for the transport of phosphorylated STAT3 proteins into the nucleus and thus for initiating the non-specific immune responses (see ▶ Chaps. 7 and ▶ 8), these cannot be properly developed. By inhibiting the early immune defence, the virus may become capable of replicating without impairment.

14.4.4 Replication

The first step of hepatitis E virus reproduction is the translation of non-structural proteins using the positive-sense RNA genome as mRNA. The non-structural protein precursor is probably autocatalytically cleaved by the protease in analogy with other positive-sense RNA viruses. As a result, the functional activities of the following proteins become available in addition to the viral protease: RNA-dependent RNA polymerase, helicase and methyltransferase/guanylyltransferase. They are involved in the production of negative-sense RNA molecules, which are synthesized using the viral genome as a template. This process occurs in association with the ER membrane. The negative-sense RNA molecules are used as a template for the synthesis of new viral genomes. In addition, two subgenomic mRNAs with lengths of 2.0 and 3.7 kb are transcribed from negative-sense RNAs. The bicistronic subgenomic 2.9-kb RNA spans the region of the 3′-oriented ORF2 and ORF3. This mRNA is used for translating the capsid proteins and pORF3 (Fig. 14.10). Whether the 3.7-kb subgenomic RNA has a similar function is uncertain. The capsid proteins interact to form precursor virus particles in which the viral RNA is embedded. New viruses are released from the surface of infected cells.

14.4.5 Human and Animal Pathogenic Prototypes of Hepeviruses

14.4.5.1 Hepatitis E Virus

Epidemiology and Transmission

A new hepatitis epidemic emerged in New Delhi in 1955, and was transmitted via the faecal–oral route by contaminated drinking water; it infected 29,000 people. Further outbreaks were observed in Central America, Africa, India, China and the south of the former USSR. Occasionally, patients in non-endemic regions developed similar forms of infectious liver inflammation, e.g. also in central European countries. Owing to the mode of transmission, it was initially thought that hepatitis A viruses had provoked these diseases. They could only be identified retrospectively as hepatitis E virus epidemics. After the first isolation of the virus by Mikhail S. Balayan, whereby he inoculated himself with pathogenic material, the molecular characterization of hepatitis E viruses was performed by Daniel W. Bradley and co-workers in 1988. They were able to isolate virus particles from stool samples; these showed a positive reaction with sera from patients, and caused hepatitis in macaques after feeding. Later, it was demonstrated that the virus can be transmitted to other primates, and even to animal species such as rats, sheep and cattle. Hepatitis E viruses were isolated from swine for the first time in 1997. These viruses, as shown in subsequent investigations, are spread worldwide in both wild boar and domestic pig populations. It is assumed that adolescent animals, which are no longer suckling, are mainly infected via the faecal–oral route. At least 50 % of all animals older than 6 weeks have antibodies against the virus.

Hepatitis E viruses seem to have been disseminated in human populations for a long time. Almost 90 % of adults in Egypt have antibodies specific for hepatitis E viruses. The seroprevalence is about 40 % in India, it ranges from 4 % in southern and eastern coastal states of the USA to 30 % in the Midwest, it is approximately 4 % in Germany and it is around 16 % in southwestern France. The various genotypes are distributed in different geographical regions. Genotype 1 viruses are found in Asia and North Africa. Genotype 2 has been found in hepatitis E epidemics from Central America and Central Africa. Genotype 3 is prevalent in various European countries, in North America, South America and Japan. Genotype 4 viruses have especially been detected in China, Taiwan, Japan and Vietnam. Whereas genotypes 1 and 2 of hepatitis E virus have only been found in humans so far, genotypes 3 and 4 can infect both humans and swine; therefore, their regional distribution is coincident. Hepatitis E virus genotype 3 has been detected in 5 % of all blood samples from wild boars. In the Netherlands, hepatitis E viruses of genotype 3 have also been found in commercially available swine liver. Inoculation of swine with such viruses led to acute infections, strongly suggesting that those liver samples contained infectious hepatitis E viruses. Efficient in vitro cultivation of hepatitis E viruses is not possible today. The virus particles do not display the high particle stability of hepatitis A viruses (Sect. 14.1). Besides the usually observed faecal–oral transmission (via contaminated water) and the suspected zoonotic transmission, hepatitis E viruses are also transmitted directly from person to person by droplet and smear infections.

Clinical Features

Hepatitis E virus infections apparently do not induce clinical symptoms in swine. In humans, the severity of the disease seems to correlate with the quantity of hepatitis E virus particles transmitted upon contact. In connection with very low amounts of the virus, infections are asymptomatic, especially in children. After an average incubation period of 6–7 weeks, influenza-like symptoms appear, such as sickness, nausea, fever, itching, joint pain and headaches, which are accompanied by a steep increase in the levels of liver enzymes. The intrahepatic cholestatic jaundice which develops during the disease and is caused by disintegration of liver cells and congestion of bile can last for several weeks (light stool, dark urine and yellow eyes). The mortality rate is 1–4 %, which is considerably higher than that of infections with hepatitis A virus (Sect. 14.1). Particularly striking is the high death rate among acutely infected pregnant women, which is observed mainly in India; it is around 20 %. Apart from the mother, the unborn child may also be affected. Chronic, persistent infections can be established in immunocompromised patients; recipients of organ transplants (liver, kidney, pancreas) have been reported to develop chronic hepatitis and liver cirrhosis, which apparently are linked to persistent hepatitis E virus infections.

Pathogenesis

The hepatitis E virus enters the body predominantly through contaminated food and settles in the liver cells. How it arrives there is unknown, as is the nature of its cellular receptor. From the liver the virus is shed into the blood and through bile ducts into the intestine. Similarly to hepatitis A, both excretion of infectious hepatitis E viruses and viraemia reach their peaks before the onset of symptoms. Hepatitis E is pathohistologically characterized by cell necroses and degenerations in the liver; they can also be observed in the otherwise asymptomatic infection in swine. Immigrating granulocytes can be detected in intratubular infiltrates, whereas there are more lymphocytes than granulocytes in the portal region.

Immune Response and Diagnosis

During infection, IgM and then IgG antibodies against the viral capsid protein are initially produced, and can be detected by ELISA or Western blotting. The diagnosis of acute infections is performed by detecting IgM antibodies against capsid proteins and viral RNA in serum or stool by PCR.

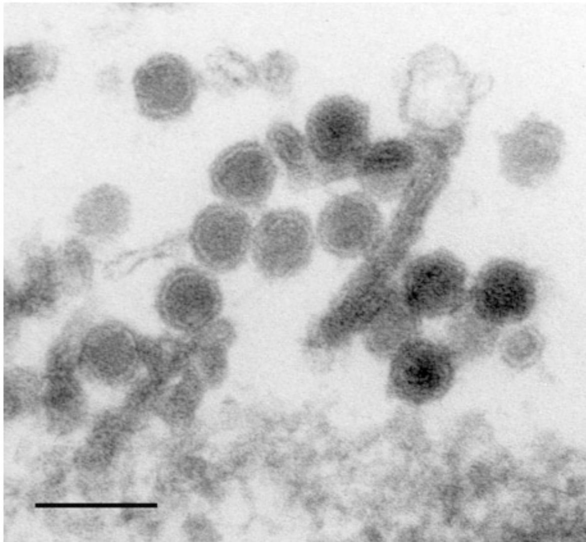
Therapy and Prophylaxis

In endemic areas, the best measure to prevent infections is maintenance of the cleanliness of water supply facilities. A vaccine based on capsid proteins produced in insect cell cultures has shown good protective results and hardly any side effects in clinical studies; however, this vaccine is not yet commercially available. An antiviral chemotherapy does not exist.

Avian Hepatitis E Virus

Avian hepatitis E virus was originally detected in the USA in tissues of a chicken which had hepatitis–splenomegaly syndrome. It was shown that this virus infects only chickens, and is spread worldwide in poultry. A high sero-prevalence of about 30 % can be found in poultry. Hepatitis–splenomegaly syndrome is characterized by an increased mortality in flocks; the affected animals have an enlarged liver and spleen, as well as ovaratrophies. Histologically, bleeding and widespread or multifocal necroses can be detected in the affected livers. There is no evidence that avian hepatitis E virus can be transmitted to humans. The genome organization is identical to that of human and porcine isolates; the sequence homology is, however, relatively low (about 50–60 %). Similar to the human and porcine hepeviruses, avian hepatitis E virus cannot be propagated in cell culture Comparative analyses of genome sequences from different isolates of avian hepatitis E viruses show a significant genetic heterogeneity: the sequence identity between some isolates is only 70 %.

14.5 Flaviviruses



Flaviviruses are characterized by a single-stranded mRNA as the genome and, similarly to picornaviruses, translate it into one precursor polypeptide comprising both structural and non-structural proteins; like picornaviruses, they do not produce subgenomic mRNAs. However, flaviviruses differ from picornaviruses, astroviruses, caliciviruses and hepeviruses by an envelope which surrounds the capsids and contains viral surface proteins.

14.5.1 Classification and Characteristic Prototypes

The family *Flaviviridae* encompasses more than 70 different flavivirus types, which are classified into three genera (Table 14.11): The genus *Flavivirus* includes yellow fever virus; the jaundice that is caused by the virus was eponymous for the denomination of both the family and the genus (*flavus*, Latin for "yellow"). It was the first virus for which an insect-associated mode of transmission had been demonstrated (*Aedes* spp.). The genus *Flavivirus* includes a number of other viruses which are also transmitted by arthropods (insects and arachnids) and are pathogenic in humans. Dengue virus, which is also transmitted by *Aedes* spp., and Japanese encephalitis virus, St. Louis encephalitis virus and West Nile virus (transmitted by *Culex* mosquitoes) have been associated with febrile, haemorrhagic or neurological disorders and encephalitis, especially in tropical countries. In central Europe, the pathogen of meningoencephalitis is spread endemically in certain regions. This disease is a typical prototype of tick-borne encephalitis (TBE), which is transmitted by tick bites. On the other hand, some other flaviviruses infect only mammals; they are presumably transmitted from animal to animal (Rio Bravo virus infects only bats and Jutiapa virus infects solely rodents).

The second genus includes pestiviruses, which cause severe animal diseases such as classical swine fever (hog cholera). These viruses are not transmitted by arthropods.

Table 14.11 Characteristic prototypes of flaviviruses

Genus	Vector/carrier	Human virus	Animal virus
Flavivirus	Mosquitoes	Yellow fever virus Dengue virus types 1–4 West Nile virus Japanese encephalitis virus St. Louis encephalitis virus	Yellow fever virus (monkeys) Wesselsbron virus (ovines, bovines) West Nile virus
	Ticks	Tick-borne encephalitis virus Kyasanur forest disease virus Omsk haemorrhagic fever virus	Tick-borne encephalitis virus Kyasanur Forest disease virus Omsk haemorrhagic fever virus Louping iII virus Jutiapa virus (cotton rats) Rio Bravo virus (bats)
Pestivirus	–		Classical swine fever virus Bovine viral diarrhoea virus (mucosal disease) Ovine border disease virus
Hepacivirus	–	Hepatitis C virus	GB Virus B (New World monkeys)
Pegivirus	–	T Human pegivirus (Hepatitis G virus (GB virus C))	Theiler's disease associated virus of horses Simian pegivirus (GB virus A) bat pegivirus (GB virus D)

Because of its molecular characteristics, hepatitis C virus has been classified as a separate genus (*Hepacivirus*) within the family *Flaviviridae*. It is primarily transmitted through contaminated blood, and usually provokes a chronic infection with hepatitis in humans. It causes liver cirrhosis and primary hepatocellular carcinoma as late complications. Hepatitis C virus is similar to human pegivirus (formerly known as hepatitis G virus or GB virus C), which was isolated from a patient with liver inflammation. The virus is widespread, but contrary to initial speculations, human pegivirus infections do not cause hepatitis. Since the amino acid sequence of its precursor polyprotein exhibits only about 28 % and 20 % homology to the sequences of hepatitis C virus and yellow fever virus, respectively, it has been classified in a separate, new genus in the family *Flaviviridae*. So far, there are only very few data on the molecular biology and pathogenesis of this virus. Similar viruses have been isolted from tamavirus (GB virus A or similar pegivirus) and bat (GB virus D or bat pegivirus). Recently, Theiler's disease associated virus has been characterized to cause hepatitis in horses. In contrast, GB virus B seems to represent an additional species in the genus hepacivirus, it causes hepatitis in New World monkeys.

Arboviruses

Viruses that are transmitted by insects or arachnids are denominated arboviruses (arthropod-borne viruses). They exist mainly in tropical and subtropical climates. A prerequisite for transmission of the viruses by arthropods is that the viruses must be able to infect specific organs of the vectors, such as the epithelial cells of the gut and salivary glands. The mere uptake of virus-containing blood by mosquitoes or ticks is not enough: the viruses must be able to perform a productive infection cycle in both arthropod and mammalian cells.

14.5.2 Structure

14.5.2.1 Virus Particle

Infectious flaviviruses have a diameter of 40–50 nm. The spherical capsids consist of only one type of viral protein (C protein), and are surrounded by an envelope, in which two viral surface proteins are embedded. They are designated by the letters M and E in flaviviruses (Fig. 14.11). M protein is relatively small and has a molecular mass of 7–8 kDa; 90 dimers of the virus-type-specific E proteins are found per particle. M protein is absent in hepatitis C virus and pestiviruses; instead a smaller glycosylated surface protein, E1 (gp33), can be found in addition to the main glycoprotein, E2 (gp70). The RNA genome is contained inside the capsids and interacts strongly with the highly basic C protein.

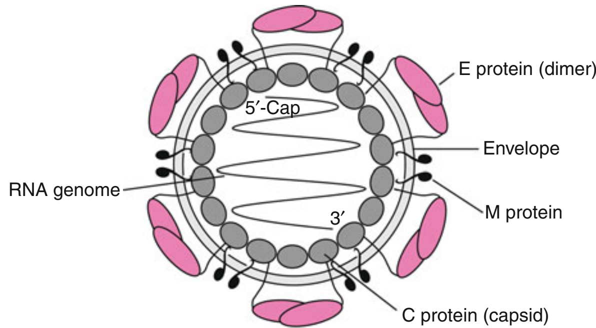


Fig. 14.11 Structure of a flavivirus particle (tick-borne encephalitis, TBE, virus). The icosahedral capsid consists of C proteins. The RNA genome is associated with the protein domains on the inner side of the capsid. The capsid is surrounded by an envelope, which is intercalated with homodimeric E and M proteins

Non-A, Non-B Hepatitis Viruses

Hepatitis C virus was assigned to the so-called non-A, non-B hepatitis viruses until its molecular identification in 1989. Initially all pathogens of liver inflammation which could not be diagnostically identified as hepatitis A virus or hepatitis B virus were classified into the group of hepatitis non-A, non-B viruses. Even after the characterization of hepatitis C virus and hepatitis E virus (the latter was characterized and classified into the family *Hepeviridae* in 1988; see Sect. 14.4), some other, hypothetical viruses are still assigned to this group. For example, there is only indirect evidence for the existence of hepatitis F virus. It has long been unclear whether hepatitis C virus should be incorporated into the family *Flaviviridae* or the family *Togaviridae*. Detailed knowledge of the molecular-biological characteristics allowed hepatitis C virus to be included as a separate genus, *Hepacivirus*, within the family *Flaviviridae*.

14.5.2.2 Genome Organization and Structure

The genome consists of single-stranded RNA and has a length of about 9,100–12,000 nucleotides: 10,862 in yellow fever virus, vaccine virus 17D; 10,664–10,723 in dengue virus; 11,141 in TBE virus (TBEV), strain Neudörfl; 9,340–9,589 in hepatitis C virus, 9,143–9,493 in human pegivirus (GB virus C), 12,308–12,573 in bovine viral diarrhoea virus (BVDV); 12,297 in classical swine fever virus. The RNA is present with positive-sense polarity and spans a large reading frame, which has a length of 10,233 nucleotides in yellow fever virus (Fig. 14.11). Like in picornaviruses, it directs the synthesis of a single common precursor protein that is cleaved into the individual components during infection. The RNA genome of flaviviruses has a cap structure at the 5′ terminus. In the case of yellow fever virus,

the reading frame is flanked at the 5′ and 3′ termini by untranslated sequences, which are 118 and 511 nucleotides in length, respectively. The 3′ terminus is not polyadenylated. However, adenosine-rich sequences of variable length are found in this region.

In contrast to members of the genus *Flavivirus*, the genomes of pestiviruses, hepaciviruses and pegiviruses do not have a cap structure at the 5′ end of the genome, but they possess – like picornaviruses – IRES elements. The IRES structure mediates binding of the ribosomal subunits and initiation of translation of the polyprotein (Sect. 14.1.4). Therefore, the 5′ UTR of hepatitis C virus is significantly longer than that of flaviviruses, it comprises about 340 nucleotides. A short sequence of uridine and adenosine residues is found at the 3′ end of the hepatitis C virus genome. Similarly to the untranslated sequences in the 3′ region of other flaviviruses, they have important functions in initiating negative-sense RNA synthesis during genome replication.

14.5.3 Viral Proteins

14.5.3.1 Polyprotein

The yellow fever virus polyprotein contains 3,411 amino acids (3,412 in TBEV). The sequences of the structural proteins are located in the amino-terminal third in the following order: capsid protein, viral envelope proteins PrM (as a precursor product of the M protein) and E (Fig. 14.11, Table 14.12), followed by the sequences of non-structural proteins NS1–NS5.

The protein arrangement is different in hepatitis C virus: the polyprotein, which comprises on average 3,000 amino acids, does not contain the sequences of the PrM protein. The E1 protein, a glycosylated envelope protein with a molecular mass of about 33 kDa, is localized after the capsid protein. Thereafter follow the sequences of a second glycoprotein (E2, gp68–72) and the small protein p7. In hepatitis C virus, NS1 is present neither as a gene nor as a protein (Fig. 14.11). The protein succession C–E1–E2–p7 is followed by non-structural proteins NS2 to NS5B. Table 14.12 provides a comparative summary of the properties of the proteins.

Like the polyproteins of hepaciviruses, the polyproteins of pestiviruses also do not contain the sequences of NS1 protein; pestiviruses also have two glycosylated envelope proteins, E1 and E2, followed by the sequences of p7 protein. Furthermore, the polyproteins of pestiviruses have some additional features: differing from flaviviruses and hepaciviruses, their genomes encode the non-structural protein N^{Pro} at the 5′ terminus of the viral genome; in the polyprotein it is located before the domains of the structural proteins (Fig. 14.11). After the sequences encoding the capsid protein C, the genetic information for an RNase (E^{rns} protein, for "envelope protein, RNase secreted") follows; this RNase is part of the virus particle and is also secreted by infected cells.

Processing of the structural protein moiety of the precursor product into the individual, functionally active components of the C, PrM and E proteins (flaviviruses), the C, E1, E2 and p7 proteins (hepaciviruses) or the C, E^{rns}, E1, E2

Table 14.12 Comparison and functions of flavivirus proteins

Protein	Yellow fever virus	TBEV	Hepatitis C virus	BVDV	Function
N^{pro}	–	–	–	23 kDa	Protease, autocatalytically cleaved from precursor protein; causes ubiquitylation and degradation of IRF-3
C	12–14 kDa	13–16 kDa	22 kDa	14 kDa	Capsid protein, interaction with RNA genome
PrM/ M	18–19 kDa 7–9 kDa	24–27 kDa 7–8 kDa	–	–	Membrane protein; cleavage by the protease furin
E	51–59 kDa	50–60 kDa	–	–	Glycosylated membrane protein, neutralizing antibodies, haemagglutinin, attachment
E^{rns}	–	–	–	44–48 kDa	RNase, secreted, glycosylated
E1	–	–	31–35 kDa	25–33 kDa	Membrane protein, glycosylated
E2	–	–	70–72 kDa	53–55 kDa	Membrane protein, glycosylated
p7	–	–	7 kDa	7 kDa	Ion channel, hydrophobic
NS1	19–25 kDa	39–41 kDa	–	–	Highly conserved, glycosylated, secreted, cell-membrane-associated, not a component of virus particles
NS2A	20–24 kDa	20 kDa	–	–	ER-membrane-associated, morphogenesis
NS2B	14 kDa	14 kDa	–	–	Zn^{2+} metalloproteinase, associates with NS3 protease (TBEV and similar viruses)
NS2	–	–	21–23 kDa	38–54 kDa	Zn^{2+}-binding
NS2/3	–	–	90–95 kDa	120–125 kDa	Zn^{2+}-binding, autocatalytic protease, it performs the cleavage into NS2 and NS3 in hepatitis C virus and in pathogenic BVDV strains
NS3	68–70 kDa	70 kDa	70 kDa	75–80 kDa	Serine protease, cleaves the non-structural proteins from the polyprotein; dsRNA helicase

(*continued*)

Table 14.12 (continued)

Protein	Yellow fever virus	TBEV	Hepatitis C virus	BVDV	Function
NS4A	16 kDa	16 kDa	8–10 kDa	7–10 kDa	Hydrophobic, associates with ER membrane, it inhibits INF-α/INF-β-mediated signalling (flaviviruses), it forms heterodimers with NS3 protease in hepatitis C virus and BVDV
NS4B	26 kDa	27 kDa	27 kDa	30 kDa	Hydrophobic, associates with ER membrane
NS5	103–104 kDs	100 kD*	–	–	Methyltransferase, RNA-dependent RNA polymerase
NS5A	–	–	56–58 kDa	58–70 kDa	Phosphorylated, membrane anchored; virus morphogenesis
NS5B	–	–	68–70 kDa	75–78 kDa	RNA-dependent RNA polymerase

The protein succession order in the table corresponds to the real order in the precursor polyprotein
BVDV bovine viral diarrhoea virus, *IRF-3* interferon regulatory factor 3, *dsRNA* doubled-stranded RNA, *INF* interferon, *TBE* tick-borne encephalitis virus

and p7 proteins (pestiviruses) is done by the cellular signalase, which is associated with the ER membrane. In cellular metabolism, this protease removes the signal peptides from the amino-terminal ends of proteins that are translated at the ER. The pestivirus N^{pro} protein is an autocatalytically active protease which cleaves itself cotranslationally from the nascent precursor protein. For all further processing reactions, viral proteases are primarily responsible: the cleavage between the NS2 and NS3 moieties is performed by the proteolytic activity of the NS2B protein; in hepatitis C virus and in non-cytopathogenic strains of BVDV, a proteolytic activity that is located in the amino-terminal domain of the NS3 protein is responsible for such reactions, but this activity is only exhibited in a fusion product of the NS2 and NS3 proteins. The NS3 protein acts as a serine protease and performs all other cleavage reactions; in hepatitis C virus, the NS4A protein is required as a cofactor for this purpose.

14.5.3.2 Structural Proteins

The capsid is constituted of C proteins. It contains a large number of basic amino acids which interact with the RNA genome, thereby forming the nucleocapsid. The carboxy terminus of the C protein is highly hydrophobic. Together with further hydrophobic domains, it mediates the interaction of the polyprotein with the ER membrane. Cleavage reactions are induced by the signalase between the following

domains: C and PrM (flaviviruses), C and E1 (hepaciviruses) and C and E^{rns} (pestiviruses). A peculiarity has been found in BVDV: mutants have been described completely lacking the sequences that encode the C protein, but which, nevertheless, exhibit the morphology of flaviviruses. Accordingly, the C protein is not essential for the formation of infectious particles. In this case, the functions of the C protein are assumed by the NS3 protein, which interacts with the RNA genome and E proteins.

The PrM protein, which is glycosylated at asparagine residues, is the precursor of the very small, non-glycosylated M protein that is anchored in the viral envelope. Exhibiting a molecular mass of approximately 19 kDa in yellow fever virus and 24–27 kDa in tick-borne flaviviruses, the PrM protein is significantly larger than the M protein in infectious particles. The amino-terminal part of the PrM protein is cleaved by the cellular protease furin at a late stage of viral morphogenesis during the passage of the immature virus through the Golgi apparatus. This cleavage reaction is essential for the infectivity of virus particles; it induces the fusogenic properties of the E protein to fuse endosomes with the viral envelope after the virus has entered the cell.

In addition, the viral envelope contains 90 dimers of glycosylated E proteins. The structure of the E protein of TBEV was elucidated by Félix A. Rey and colleagues by means of X-ray diffraction in 1995. In comparison with other structurally known viral surface proteins (▶ Sect. 16.3; haemagglutinin of influenza viruses), the E protein has an unusual structure: it is anchored in the membrane by a hydrophobic amino acid sequence in the carboxy-terminal region, it lies flat on the membrane and determines the size of the particle because of a bending effect caused by protein folding (Fig. 14.12). The E protein mediates attachment of the virus to target cells, and induces fusion of the viral and endosomal membranes by low pH after entry of the virus via receptor-mediated endocytosis. It is also responsible for the haemagglutination properties of flaviviruses. Virus-neutralizing antibodies directed against the E protein are induced during infection. They protect against reinfections with the same virus type.

The glycosylated E1 and E2 proteins of hepatitis C virus share sequence homologies with the corresponding proteins of pestiviruses. E1 proteins are not covalently associated with E2 proteins; they are anchored in the membrane by hydrophobic, carboxy-terminal amino acid sequences. There is a highly variable region in the C-terminal region of the E2 protein, and this differs among the different serotypes of hepatitis C virus and also among individual virus isolates.

In pestiviruses, another envelope protein has been characterized: E^{rns} protein. It is a virion component, has double-stranded RNase activity, is secreted by infected cells and induces the formation of neutralizing antibodies. The function of this protein during viral replication has not been fully clarified. In BVDV, the E^{rns} protein counteracts the immunological responses that are triggered by double-stranded RNA by its ability to degrade double-stranded RNA. These include, among others, the activation of TLR3, whereby the production of IFN-α and IFN-β is induced (see ▶ Chaps. 7 and ▶ 8) (Fig. 14.13).

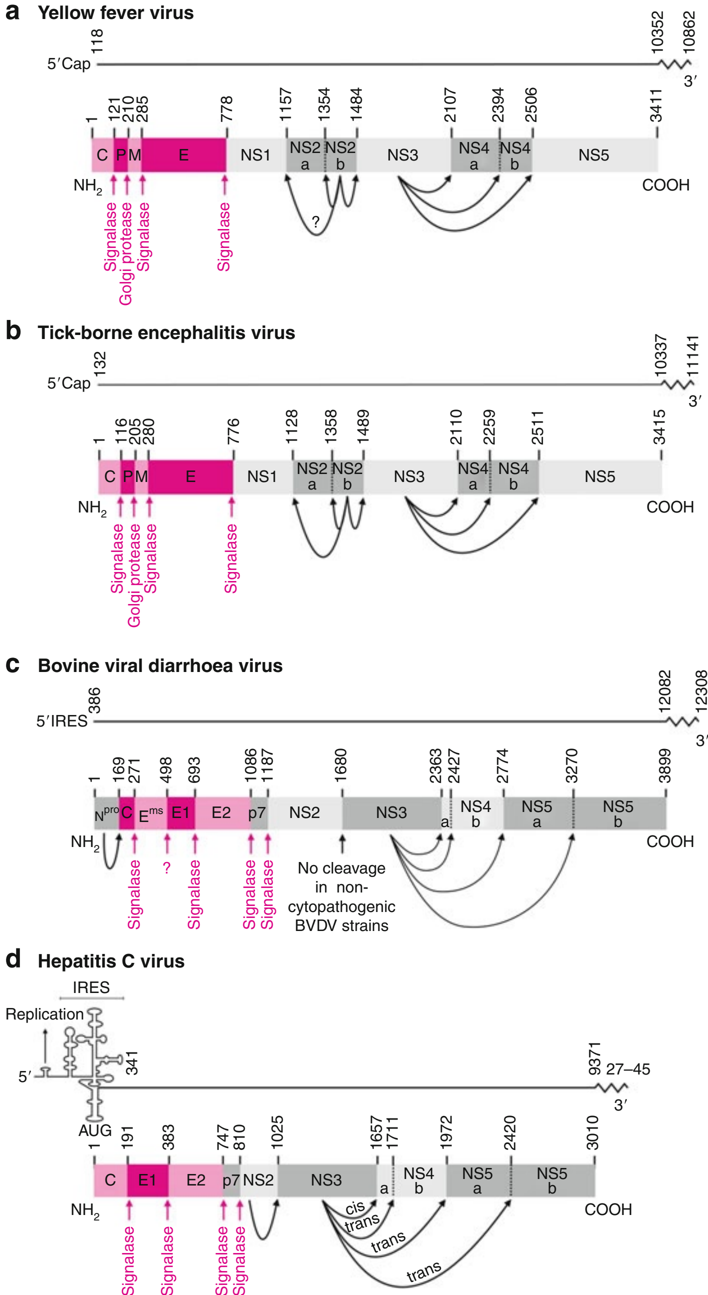


Fig. 14.12 Genome organization of flaviviruses. (**a**) Yellow fever virus, vaccine strain 17D. (**b**) TBE virus, strain Neudörfl. (**c**) Hepatitis C virus. (**d**) Bovine viral diarrhoea virus (*BVDV*), strain SD-1 (non-cytopathogenic). In hepatitis C virus and pestiviruses (BVDV), an IRES is

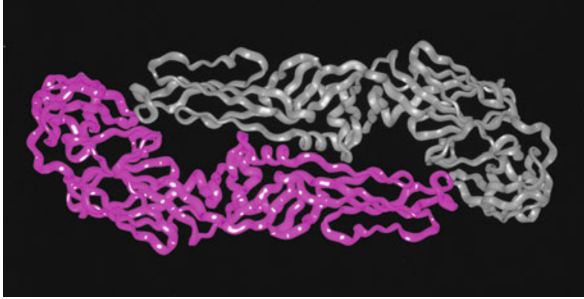


Fig. 14.13 Structure of the TBE virus E protein, represented in a ribbon model. A plan view of the homodimeric protein complex (the protein is located here on the surface of the virus) is shown. The carboxy-terminal domain, which contains the transmembrane region, was removed by proteolytic digestion (Courtesy of Franz X. Heinz, University of Vienna)

Numerous Viruses Are Able to Agglutinate Erythrocytes

Many viruses can cause haemagglutination by the respective activities of their surface proteins. That means the virus-induced agglutination and aggregation of red blood cells. Before the development of highly specific ELISA and PCR tests for detection of viral infections, the haemagglutination and haemagglutination-inhibition tests were very important diagnostic methods (Chap. 13). In rare cases, they are still used today.

14.5.3.3 Non-Structural Proteins

The flavivirus NS1 protein is associated with the cell membrane. In infections of mammalian cells, but not insect cells, a soluble variant of the NS1 protein which is secreted by the cells can be observed. There is evidence that the membrane-associated NS1 protein exists as a dimer; in contrast, the secreted form is present as a hexamer. In some flaviviruses, NS1-specific antibodies seem to induce the antibody-mediated lysis of infected cells, thus exerting a protective effect. The function of the protein during the infection cycle is unknown. It is possibly involved in the replication of viral genomes, as well as in intracellular transport of viral structural proteins and in release of the virus. An immunomodulatory function has recently been found for the NS1 protein of West Nile virus: it blocks the signal transduction pathway that is mediated by TLR3 by impeding the transport of IRF-3

Fig. 14.12 (continued) located in the 5′ UTR. In members of the genus *Flavivirus* (yellow fever virus and TBE virus), the 5′ end of the genome is modified by a cap group. The genomes of flaviviruses have a continuous open reading frame. It encodes a polyprotein that is proteolytically cleaved into the different protein components (structural proteins in *colour*). Responsible for this process are enzymes which are a constitutive part of the polyprotein and autocatalytically activate themselves, as well as cellular proteases and signalases. The *numbers* refer to the amino acid positions in the polyprotein at which the cleavages occur

and nuclear factor κB into the nucleus. As a result, the production of IFN-β and proinflammatory cytokines such as IL-6 is inhibited. Furthermore, both the soluble and the membrane-associated variant of the West Nile virus NS1 protein bind to protein factor H, a regulator of complement activation. This leads to decreased accumulation of both the C3 component and the membrane attack complex (C5B to C9; see ▶ Chap. 7).

The 7-kDa protein of hepatitis C virus and pestiviruses is a small hydrophobic protein; it is believed to be present as a membrane-anchored protein, which possibly functions as an ion channel protein.

In flaviviruses, NS2 protein is cleaved into proteins NS2A and NS2B. There are few data regarding the function of NS2A. It is associated with the ER membrane and plays an important role during flavivirus morphogenesis. It also seems to inhibit the interferon-mediated antiviral immune response. The NS2B protein of flaviviruses is an essential cofactor of NS3 protease. In hepatitis C virus, the NS2 moiety fused with NS3 forms the catalytic domain of a Zn^{2+}-dependent protease which catalyses the cleavage between NS2 and NS3.

The NS3 protein of all flaviviruses is bifunctional: in the amino-terminal region, a serine protease has been localized that is responsible for all cleavage reactions in the regions of the polyprotein that follow the NS3 domain. NTP-binding sites and a helicase activity are found in the carboxy-terminal region. The latter belongs to the helicase DExH/D box superfamily, and is necessary for unwinding the highly structured double-stranded RNA intermediates that are formed during genome replication and during translation of the polyprotein. The NS3 protease of flaviviruses is a heterodimer consisting of NS2B and NS3. In hepatitis C virus, NS3 interacts with NS4A by its amino-terminal region, which contains the active centre of the serine protease, thereby forming a heterodimer that is anchored in the ER membrane by the hydrophobic domains of NS4A.

The NS4A protein of hepatitis C virus primarily has the function of interacting with the NS3 protease. Thereby the NS3/NS4A protein complex associates with the ER membranes and remains as part of the replication complex. The NS4A protein of flaviviruses is also associated with the ER membrane, but does not interact with the NS3 protein. It contributes both to the rearrangement of ER membranes and to inhibition of the signal transduction pathways that are mediated by IFN-α/IFN-β, thereby inhibiting phosphorylation of STAT1 and STAT2 proteins.

Little is known concerning the function of the membrane-anchored NS4B protein. In hepatitis C virus, it induces the formation of specific intracellular membrane compartments, where replication of the viral genomes occurs. In the case of flaviviruses, there are indications that the NS4B protein, like NS4A, impedes the interferon-mediated immune response; however, these data are controversial.

The NS5 protein of flaviviruses is a multifunctional enzyme: the amino-terminal domain possesses a methyltransferase activity that is required for capping the 5′ terminus of the genome. Since viral replication and proliferation occur in the cytoplasm of infected cells, viruses cannot rely on and use respective cellular enzymes since these are localized in the nucleus. In addition, the

amino-terminal NS5 domain also functions as an antagonist of interferon: it blocks the interferon-stimulated Jak/STAT signal transduction pathways and prevents the expression of interferon-stimulated genes. The carboxy-terminal domain of the NS5 protein has RNA-dependent RNA polymerase activity. The NS5 protein of hepatitis C virus and pestiviruses is cleaved by the NS3 protease into the moieties NS5A and NS5B. The NS5B protein constitutes the RNA-dependent RNA polymerase, which is essential for replication of the RNA genome. The membrane-anchored NS5A protein is phosphorylated and binds to RNA. The carboxy-terminal domain of NS5A has important functions in viral morphogenesis: it induces the accumulation of C proteins in intracellular membrane compartments. Deletions of the carboxy-terminal domain of NS5A suppress the formation of infectious progeny viruses.

The non-structural proteins of pestiviruses have some peculiarities: in BVDV, they are responsible for the formation of different biotypes and play an important role in the pathogenesis of mucosal disease (Sect. 14.5.6). Unique is the already mentioned N^{pro} protein, which acts as a protease and constitutes the first domain of the polyprotein. It is capable of autocatalytic cleavage from the precursor protein. Additionally, N^{pro} has another function that is important for the pathogenesis of pestivirus infections. Both in classical swine fever virus and in BVDV, it is apparent that N^{pro} binds to IRF-3 and that this interaction induces the ubiquitination and degradation of IRF-3 via proteasomes. Thus, infected cells have a significantly reduced expression of IFN-β (see also ► Chaps. 7 and ► 8).

14.5.4 Replication

The cellular receptors of many flaviviruses are known. In hepatitis C virus, receptor binding is a very complex and multifaceted process. The initial attachment to the target cells requires glycosaminoglycan and LDL receptors. Thereafter follow interactions of the viral E2 proteins with at least three entry factors, namely scavenger receptor class B type 1 (SR-B1), tetraspanin 28 (CD81) and the tight-junction protein claudin 1. The E2 protein binds to CD81 (25 kDa), a member of the tetraspanin superfamily, which spans the cytoplasmic membrane with four transmembrane regions, thereby forming two extracellular domains. CD81 has functional activities in cell adhesion, cell activation and cell motility, as well as in signal transduction pathways; it is found on the surface of many cell types, such as hepatocytes and B lymphocytes, which can be infected by hepatitis C viruses. The E2 proteins also bind to SR-B1, an 87-kDa protein found on the surface of many different cell types that are involved in cellular lipid metabolism. Serum proteins such as high-density lipoproteins are ligands for the SR-B1 receptor and enhance the infectivity of hepatitis C virus, possibly because they form complexes with the E1/E2 proteins on the surface of the virus and mediate the interaction with the SR-B1 receptor. A similar binding mechanism has also been proposed for LDLs: these serum proteins can also bind to virus particles, thereby mediating the interaction with the LDL receptor.

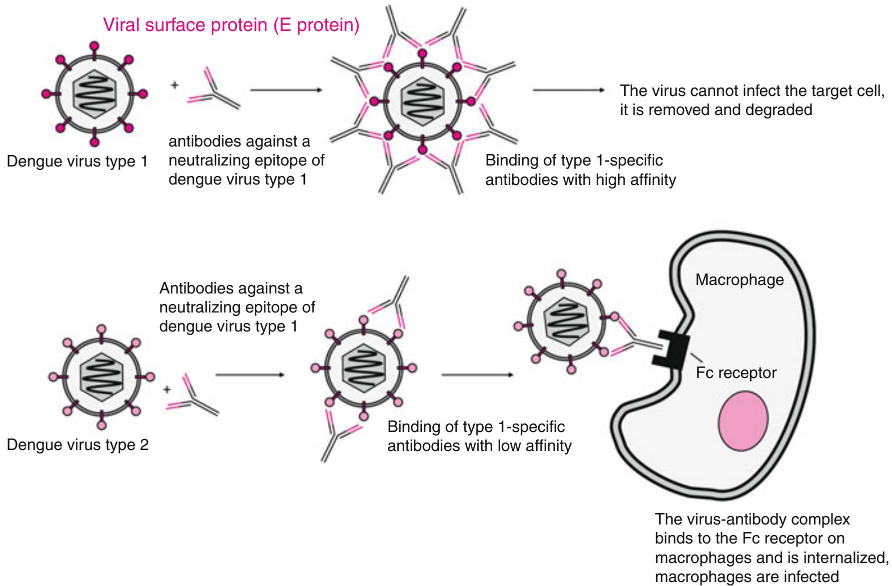


Fig. 14.14 Mode of operation of infection-enhancing antibodies. After an infection with a particular subtype of dengue virus, e.g. dengue virus type 1, subtype-specific antibodies are developed in the organism. In the case of reinfections with the same virus subtype, they can bind to the virus particles, neutralizing the virus. However, if a subsequent infection with another dengue virus subtype occurs, e.g. dengue virus type 2, the antibodies will also bind to the surface of dengue virus type 2 capsids since the amino acid sequences of both virus types are similar. Type 1-specific antibodies are not able to neutralize dengue virus type 2, however. Via the Fc region of immunoglobulins, virus–antibody complexes bind to Fc receptors present in the membrane of macrophages. This results in internalization of the complex, leading to infection of macrophages

Dengue virus and TBEV bind with low affinity to heparan sulphate, whereas integrins ($\alpha_v\beta_3$) have been described as interaction partners for West Nile virus. Besides the direct interaction of the viral envelope proteins with defined cell surface components, a second way of binding and penetration has been described for dengue virus: it depends on the presence of subneutralizing concentrations of virus-type-specific antibodies or cross-reacting immunoglobulins, which can recognize various types of related viruses. The latter is valid for dengue virus, which comprises four different serotypes. The virus-type-specific antibodies can only bind to epitopes that are specific for the respective serotype. However, there are additional domains especially in the E protein of dengue virus which are commonly shared by all serotypes. These virus-type-overlapping and cross-reacting antibodies are usually not neutralizing. If viruses are mixed in vitro with such cross-reacting antibodies or with low concentrations of type-specific antibodies that can bind to the particle surface, entry of the virus and viral infection can be induced via the Fc portion of immunoglobulins by mediating the interaction with Fc receptors on monocytes and macrophages (Fig. 14.14). This phenomenon is known as immune enhancement.

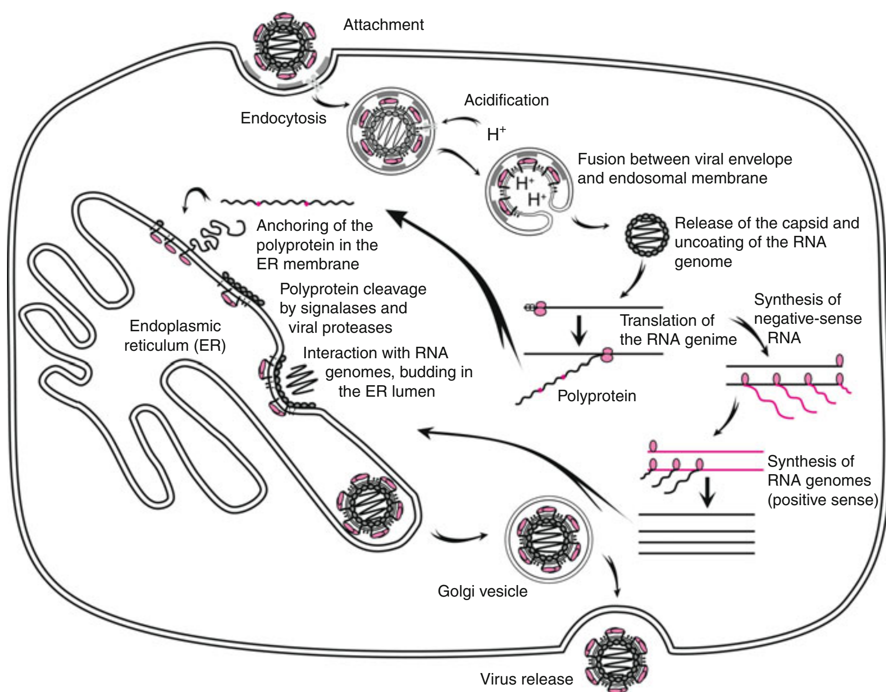


Fig. 14.15 TBE virus life cycle (the nucleus is not shown for clarity). The virus attaches to a still unknown receptor on the cytoplasmic membrane, and penetrates the cell by endocytosis. Endosomal acidification induces the fusion of the endosomal membrane with the viral envelope, whereby the capsid is released into the cytoplasm. The viral genome is used as mRNA and is translated into a polyprotein. The polyprotein is embedded in the endoplasmic reticulum (*ER*) membrane by signal-peptide-like protein domains. All subsequent steps of the infection cycle occur in close proximity to this cell compartment. The signalase, which is associated with the ER, cleaves the precursor protein in the region of structural proteins C, PrM and E. All other cleavage reactions in the region of non-structural proteins are performed by the NS3 protein, which together with the NS2B protein acts as a protease. These processing reactions give rise to the NS5 protein, which carries the RNA-dependent RNA polymerase activity and transcribes the positive-sense genome into negative-sense RNA molecules. In turn, these serve as templates for the synthesis of new viral genomes. Newly synthesized viral genomes accumulate in regions of the ER membrane that contain high concentrations of viral structural proteins. This leads to budding of virus particles into the ER lumen. In the further course, these are transported via Golgi vesicles to the cell surface and released

After attachment to the cell surface, the virus enters the cell by endocytosis (Fig. 14.15). Afterwards, it is present within the endosome in the cytoplasm and must be released from this membrane vesicle. For this purpose, the interior of the endosomes is acidified via an ATP-dependent proton pump, which is a component of the vesicular membrane. The endosomal membrane fuses with the viral envelope. The E and E1 proteins of flaviviruses and hepaciviruses/pestiviruses,

respectively, are actively involved in this membrane fusion process. During this process, the dimeric E proteins change their structure and form trimeric intermediates, in which the fusogenic domain is exposed, developing its activity. In this way, the viral capsid enters the cytoplasm; the mechanism that leads to the release of the viral nucleic acid is poorly understood.

In the next steps, the 5′ end of the genome interacts with cellular ribosomal subunits. In flaviviruses, the 5′ cap structure is responsible for this process; it binds to the components of the cap-binding complex and mediates the interaction with the ribosomes. In the case of hepatitis C virus and pestiviruses, the IRES sequence, which is located in the UTR at the 5′ terminus, is responsible for binding of ribosomes. If the C protein is present at the amino-terminal region of the polyprotein after initiation of translation, the elongation of the amino acid chain stops temporarily: the hydrophobic domain in the carboxy-terminal region of the C protein acts as a signal peptide. It interacts with the signal recognition particle, a complex of cellular polypeptides and the 5S RNA, which induces the transport of the translation complex to the ER membrane. There, the nascent amino acid chain is translocated through the ER membrane, whereby the transmembrane domains of PrM and E proteins cotranslationally anchor the polyprotein in the lipid layer (Fig. 14.15). Signalases perform the processing reactions between the C, PrM, E and NS1 moieties. Further cleavage reactions of the polyprotein are performed by NS2B/NS3 (flaviviruses) and NS3/NS4A (hepatitis C and pestiviruses) proteases (Fig. 14.12). Most of the nonstructural proteins have hydrophobic domains that contribute to their anchorage in the ER membrane. This ensures that the synthesis of the polyprotein and the subsequent steps of replication can be executed in association with this intracellular membrane compartment.

As soon as the RNA-dependent RNA polymerase and the NS5 and NS5B proteins are available in the cytoplasm, they catalyse the transcription of the positive-sense RNA genome into complementary negative-sense RNA molecules, which in turn serve as templates for the synthesis of new positive-sense RNA genomes; in hepatitis C virus, the hairpin structure in the UTR at the 5′ end of the RNA genome is essential for this process (Fig. 14.12d). It seems to have a function similar to that of the cloverleaf structure of the picornavirus genome (see Sect. 14.1). The newly synthesized positive strands are used as both genomic RNAs and mRNAs for the synthesis of further polyproteins. The details of these processes are largely unknown. In general, however, the mechanism is similar to that of picornavirus replication. Since viral replication occurs exclusively in the cytoplasm, where cellular capping enzymes are not accessible, flaviviruses have evolved their own capping enzymes. The NS5 methyltransferase is responsible for the synthesis of 5′-cap structures of positive RNA strands.

In flaviviruses, morphogenesis and formation of infectious particles is performed at the ER membrane. Analogous processes are presumed in hepatitis C virus and pestiviruses. The C, PrM and E components as well as the E1 and E2 polypeptides are inserted into the lipid layer during translation, where they accumulate and form regions containing high concentrations of viral proteins. The membrane-associated C proteins interact with both the carboxy-terminal domain of E proteins, probably

also with that of NS2A proteins, and the RNA genomes via basic amino acids. The membrane invaginates into the ER lumen, thus forming the initial budding complex, which is finally released. In this phase of viral morphogenesis, the virus particles are not yet infectious: E proteins of flaviviruses are present as a heterodimer complex with PrM polypeptides. During the following transport through the Golgi apparatus, the envelope proteins are glycosylated and PrM is processed into the M protein by the protease furin. As a consequence, the complex between E and PrM proteins is resolved and the E proteins form homodimers. Thereby the immature, non-infectious virus particles become mature virus particles. Finally, the Golgi vesicles fuse with the cytoplasmic membrane, releasing the infectious virus particles into the surrounding environment. The replication cycle of flaviviruses is illustrated schematically in Fig. 14.15.

14.5.5 Human Pathogenic Flaviviruses

The members of the genus *Flavivirus* that are pathogenic for humans are transmitted to humans by mosquito or tick bites. The tick-borne viruses are found in Europe and Asia, where they preferentially infect rodents as natural hosts; in central Europe, TBEV belongs to this group. Viruses that are transmitted by mosquito bites can be divided into two groups. Mosquitoes of the genus *Aedes* preferably bite mammals. Viruses that are transmitted by these mosquitoes, such as the pathogens of yellow fever and dengue fever or dengue shock syndrome, may cause febrile illnesses in humans, and these can be associated with haemorrhage. *Culex* mosquitoes prefer birds as hosts and transmit viruses such as the pathogens of West Nile fever, St. Louis encephalitis and Japanese encephalitis that cause neurological diseases such as meningitis and encephalitis in humans. The hepatitis C and hepatitis G viruses are primarily transmitted through contaminated blood or other body fluids; an arthropod-mediated transmission is not known for these infectious agents. As no illnesses have been observed in infections with hepatitis G virus so far, they will not be discussed in detail in the next section.

Origin of Yellow Fever Epidemics

Under natural conditions, yellow fever virus can undergo two infection cycles: jungle or savannah yellow fever and urban yellow fever. Jungle or savannah yellow fever is transmitted by mosquitoes of the species *Aedes africanus* and *Aedes haemagogus*. They breed in water accumulations in tree holes, puddles or holes in the ground. Female insects can vertically transmit the virus to their offspring. Monkeys, as intermediate hosts, also maintain the infection chain of jungle or savannah yellow fever. New World monkeys in South America become sick and may die from the infection, whereas Old World monkeys in Africa have apparently adapted well to the virus during evolution and are often infected only subclinically. As side links in the

transmission chain, the virus can be transmitted to people who stay in those regions, causing sporadic diseases. Yellow fever virus can be carried by infected individuals into urban regions where the mosquito species *Aedes aegypti* is ubiquitous; these species become infected with the virus, leading to its epidemic dissemination.

14.5.5.1 Yellow Fever Virus

Epidemiology and Transmission

Yellow fever virus is transmitted by mosquitoes of the genus *Aedes*. The first historically documented cases occurred in Mexico in 1648. Probably, the virus was originally widespread only in Africa. However, the slave trade between Africa and North America and South America by the Spanish and English conquerors led to the introduction of the mosquito/virus combination during the seventeenth and eighteenth centuries, and thereby to the epidemic dissemination of yellow fever in the tropical regions of America. Yellow fever appeared primarily in coastal cities of Africa, America and southern Europe, and it was one of the major diseases of mankind, claiming thousands of victims. There were more than 100,000 deaths from yellow fever infections during the construction of the Panama Canal. It could only be completed after the mosquitoes had been exterminated as carriers of the infection. As early as 1881, the Cuban physician Carlos Finlay presumed that the disease is transmitted by insects. This assumption was finally proved by Walter Reed in 1900. In 1902, yellow fever virus was identified as the aetiologic agent of the epidemic yellow fever; in 1929, it was transferred to monkeys, thus paving the way for further research.

Today, yellow fever is endemic in regions of Africa south of the Sahara – mainly in the tropical forests of West Africa – and in South America. Yellow fever virus can proliferate with differing efficiency in various *Aedes* species, which are well adapted to different environmental conditions in jungles, savannahs and urban centres. In Asian countries, the disease has not emerged so far. It is assumed that Asian *Aedes* species are less susceptible to the virus, and thus constitute bad carrier vectors. On the other hand, it seems conceivable that cross-immunities with dengue viruses, which are widespread in Asian countries, might prevent the occurrence of apparent yellow fever infections. It also seems possible that people in Africa may have developed a wide cross-reactive immunity owing to numerous viral infections by insects. These conditions may have prevented major epidemics of yellow fever in the population; therefore, only non-immune Europeans were affected. However, because of the advancing urbanization in recent decades, the living conditions in Africa have changed greatly, so yellow fever is now frequently observed. In recent years, epidemic outbreaks of yellow fever with more than 100,000 cases have been reported in Nigeria. The annual number of officially reported infections is approximately 2,000 in South America. Presumably, the number of unreported cases may be very high.

Yellow fever virus is genetically very stable, and there is only one serotype. It is present in the blood of infected individuals for several days during the disease.

If they are bitten by a mosquito during this time, the virus is absorbed with the blood. The virus proliferates in the intestinal epithelium as well as in body and salivary gland cells of insects. This process lasts approximately 1 week, and has been named the extrinsic incubation period. Thereafter, the mosquito is able to transmit the yellow fever virus with the salivary secretion by biting.

Clinical Features

Usually, the first symptoms of fever, nausea, headache and muscle pain appear 3–6 days after the mosquito bite; at this stage, infected people are viraemic. After a short-term improvement, in a subset of patients the symptoms may appear more pronounced, accompanied by resurging fever, vomiting of blood (signalizing haemorrhage), dehydration, hypotension, abdominal pain and signs of renal failure. In this stage of the disease, patients develop the signs of jaundice because of the destruction of liver cells and the associated increase of the level of bilirubin. The virus is then no longer present in the blood. Half of patients who enter the second phase die of severe kidney and liver failure, shock and delirium within 7–10 days. Subclinical or abortive forms of infection, in which the symptoms appear in a mitigated form or do not emerge at all, are more frequent than fulminant forms of yellow fever. The overall lethality of yellow fever is 20–50 %.

Pathogenesis

After having been introduced into the bloodstream by a mosquito bite, yellow fever viruses infect endothelial cells, lymphocytes and preferably macrophages and monocytes near the injection site. These cells transport the virus via lymphatic vessels to the lymph nodes and lymphoid tissues, where they encounter other susceptible target cells. During viraemia, high amounts of infectious viruses are produced that infect liver macrophages (Kupffer cells), which die as a result of viral replication. Next, the virus infects and destroys hepatocytes. This leads to a strong increase in the concentration of transaminases in the blood. In rare cases, infected macrophages may transport the yellow fever virus to the brain, where it may cause encephalitis. The haemorrhages, which become obvious by internal bleeding in the kidney, brain and other organs during the symptomatic phase of infection, are ascribed to a reduced production of clotting factors due to infection and subsequent destruction of liver cells.

Immune Response and Diagnosis

Yellow fever virus can be easily cultivated in human cells (HeLa and KB cell lines) and in monkey kidney cells (Vero cells) in vitro. It may also be reproduced in chicken and duck embryonic cells as well as in continuously growing rodent cell lines. In infected individuals, IgM and IgG antibodies against the E and M proteins can be detected about 1–2 weeks after infection (i.e. 5–7 days after the onset of symptoms) by ELISA, immunoblotting and immunofluorescence, haemagglutination-inhibition and virus-neutralization tests. In addition to these serological methods, detection of viral genomes by RT-PCR in blood samples is the method of choice in the early stages of infection. Neutralizing antibodies

persist lifelong, and confer durable protection against reinfections. During the viraemia, NS1-specific antibodies can induce antibody-dependent lysis of infected cells, thus making an important contribution to the control of infection and to the clearance of the virus from the organism. To what extent the cellular immune system is involved in clearance of the virus by induction of cytotoxic T cells is unclear.

Therapy and Prophylaxis

By continuous cultivation of yellow fever virus in embryonated chicken eggs, in 1937 Max Theiler was able to breed an attenuated yellow fever virus (strain 17D) which in humans does not cause any symptoms. In 1951, Theiler was awarded the Nobel Prize in Physiology or Medicine for the first development of a live vaccine. The molecular basis of attenuation is unknown. In comparison with the wild-type genome, strain 17D has a total of 68 nucleotide mutations, which lead to 32 amino acid changes in viral proteins. Most mutations are located in the gene encoding the E protein, so it is suspected that the vaccine virus may bind less efficiently to the receptors on liver cells. As a consequence, less virus is produced, which results in slower infection rates, and hence in a mild or attenuated form of infection. Low virus concentrations are found in the blood of vaccinated individuals about 3–5 days after inoculation; viraemia lasts for 1–2 days. The first immune response is detectable in 95 % of vaccinated individuals 10 days after vaccination. To maintain protection, booster vaccinations are needed in 10-year intervals. Back mutations to the wild type have never been observed; thus, the yellow fever vaccine is regarded as very successful and safe worldwide. Millions of people have been vaccinated to date. Hence, significant control and reduction of yellow fever infections has been achieved in tropical countries; large-scale epidemics are reported only very rarely today. The attenuated yellow fever vaccine is manufactured and distributed worldwide under the control of the WHO. It must be administered only in government-approved vaccination centres. Yellow fever vaccination is obligatory in many countries for travellers to or from endemic yellow fever regions.

In addition to vaccination of the population, the fight against the mosquito species involved in transmission of the virus is another important measure to control the infection, especially in endemic regions. In this relation, insecticides are just as important as the draining of breeding places for mosquito larvae.

The Attenuation of the Vaccine Virus Has Never Been Reproduced

The isolation of vaccine strain 17D of yellow fever virus by Max Theiler was serendipitous, i.e. a fortunate and accidental discovery: this strain was isolated by continuous cultivation of the wild-type virus in embryonated chicken eggs after 89–114 passages. All attempts to reproduce the result have not been successful so far.

14.5.5.2 Dengue Virus

Epidemiology and Transmission

Dengue fever has been known as a human disease for more than 200 years and was formerly called dandy fever or break-bone fever owing to the very strong joint and muscle pain. The first reports of an epidemic occurrence were from Indonesia and Egypt. A dengue fever epidemic also occurred in North America (Philadelphia) in 1780. Further outbreaks were subsequently observed periodically in almost all tropical and subtropical regions. In 1903, Harris Graham, working as a physician in Beirut, isolated a filterable pathogen from the blood of patients. Thomas L. Bancroft, an Australian physician and botanist, demonstrated its transferability by *Aedes aegypti* in 1906. In 1944, Albert Sabin and R. Walter Schlesinger identified dengue viruses as pathogens by transferring blood of infected soldiers into mice. Four different serotypes of dengue virus are known. Similarly to yellow fever, there are urban and rural forms of dengue fever. The latter is spread by *A. albopictus* and *A. scutellaris*; their natural hosts are considered to be non-human primates in tropical forests of Southeast Asia and South America. *A. aegypti* is especially involved in propagation and transmission of the infection in urban centres. Not all strains of *A. aegypti* mosquitoes are able to transmit dengue viruses. Genetic variants of the mosquitoes which do not produce receptor protein R67/R64 (molecular mass 67 kDa) in their intestinal epithelial cells are responsible for this. These mosquito strains (e.g. IBO-11) are not permissive for dengue virus infections; therefore, they cannot transmit the pathogens.

The spread of mosquitoes on the Asian continent particularly during the Second World War and the subsequent urbanization of the population led to a dramatic increase of dengue fever cases in Asia. Inasmuch as tourist traffic also increased at that time, infected mosquitoes were imported by aircraft from the Pacific regions to Central America and South America as well as to the USA. Today, dengue virus infects approximately 50 million people worldwide each year; hence, it is the most common insect-borne viral infection in humans, and is endemic in most urban areas of tropical countries. Epidemics emerge in intervals of 3–5 years. Every year, millions of people become sick from dengue fever and hundreds of thousands become sick from the associated haemorrhagic fever and the dengue shock syndrome. Whether individual dengue virus isolates differ in their virulence, and thus in determining of the severity of the disease, is uncertain. Possibly, such differences may explain the partially epidemic occurrence of dengue haemorrhagic fever, even in people who have been infected with dengue virus for the first time.

Clinical Features

The incubation period until the onset of symptoms of dengue fever lasts 3–7 days. Dengue viruses cause different disease forms. Especially in young children, the disease is a febrile illness without specific symptoms. Older children and adults develop the classic symptoms consisting of fever, skin rash and joint and muscle pain. These symptoms are associated with sensitivity to light and enlarged lymph nodes, petechial haemorrhages in the mucous membranes of the mouth, nose and

gastrointestinal tract, and thrombopenia and lymphopenia. The symptoms last for about 3–7 days, and most patients recover without any subsequent problems.

In addition to the symptoms described above, dengue haemorrhagic fever induces enhanced vascular permeability and increased internal bleeding. Blood plasma extravasates from the vessels into the surrounding tissues, leading to oedema, particularly in the abdomen and around the thoracic area. Dengue shock syndrome appears in patients with dengue haemorrhagic fever in which vascular permeability and haemorrhages are increased. The critical phase occurs when the body temperature suddenly decreases to normal levels or below (hypothermia), resulting in circulatory failure, bleeding in the gastrointestinal tract and neurological disorders. In such cases, shock conditions can occur, and are manifested by escape of blood plasma into the body cavities. Approximately 50 % of patients with dengue shock syndrome die.

The WHO has established strict criteria for the diagnosis of dengue haemorrhagic fever and dengue shock syndrome: these include severe fever, haemorrhagic symptoms, swelling of the liver and circulatory failure. The disease has been classified, depending on the severity, into four stages: stages I and II correspond to dengue haemorrhagic fever, and stages III and IV correspond to dengue shock syndrome.

Pathogenesis

Dengue viruses penetrate into the body through the bite of an infected mosquito, and infect macrophages that are located in the local environment. The infected macrophages carry the virus via lymphatic vessels to the lymph nodes, where the viruses find further target cells, in which they can replicate. After this phase, the patient is viraemic, and 10^8–10^9 infectious particles can be detected per millilitre of blood. On average, the viraemia lasts 4–5 days. In addition to macrophages, endothelial cells and possibly also bone marrow cells are susceptible to infection. Furthermore, the virus has also been detected in other organs, such as liver, lungs, kidneys and the gastrointestinal tract. To what extent it replicates in these tissues is unclear. The pathological alterations in the tissues are similar to those that can be observed in infections with yellow fever virus.

Dengue haemorrhagic fever and dengue shock syndrome are characterized by increased permeability of capillary vessel walls. Immunopathogenetic mechanisms seem to be responsible for the development of this severe disease form. The four dengue virus serotypes display homology of 63–68 % at the amino acid sequence level of their E proteins; contrarily, the homology between different variants of one dengue virus serotype is more than 90 %. Severe diseases occur especially if patients are infected with dengue virus for a second time, but now with a serotype different from that of the initial infection. Because of the primary infection, these patients possess dengue-virus-specific antibodies, which partially cross-react with the other serotypes. They can bind to the E protein on the surface of the virus. Owing to a low affinity determined by differences in the amino acid sequence of the epitopes, they are not neutralizing, but they enable the viruses, which are complexed with antibodies, to interact with Fc receptors on

macrophages, thus preferentially facilitating a more efficient penetration into the cells; therefore, the cross-reacting IgG molecules exert an infection-enhancing effect (Fig. 14.14). They contribute significantly to the development of dengue haemorrhagic fever and dengue shock syndrome.

Binding of both antibody-complexed and free dengue viruses on the surface of macrophages leads to the interaction of viral proteins with CLEC5A protein (C-type lectin domain family 5, member A, also known as myeloid DAP12-associating lectin, MDL-1). This surface protein does not act as a receptor for the interaction with dengue virus, but is involved in this process. The consequence is the induction of a signalling cascade that results in the release of large amounts of proinflammatory cytokines. If the interaction between the virus and CLEC5A is blocked by CLEC5A-specific antibodies in a mouse model, cytokine release does not occur, preventing the increase of vascular permeability.

It is uncertain to what extent the various activities of the non-structural proteins which suppress the IFN-α- and IFN-β-mediated defence strategies in vivo also influence the pathogenesis of the disease. This also applies to the property of dengue viruses to increase the expression of MHC class I antigens in infected cells in vitro. The high concentration of MHC class I proteins together with increased binding to the receptors, which are inhibitory for NK cells, lead to infected cells evading NK-cell-mediated lysis.

Immune Response and Diagnosis

IgM antibodies against viral E proteins can be detected by ELISA, immunoblotting and indirect immunofluorescence tests from the fifth day after the initial infection with dengue virus. They remain detectable for 2–3 months. The production of IgG antibodies ensues, they reach their maximum concentration about 2–3 weeks after infection and persist for a lifetime. A large percentage of antibodies against the E protein which were produced during the primary infection are not neutralizing and cross-react with other serotypes of dengue virus; only a relatively small portion of IgG molecules are type-specific and exert a neutralizing effect. In addition to the humoral immune responses, cytotoxic T cells may also be important for eradication of the virus from the organism. Clones of cytotoxic T lymphocytes which were able to lyse dengue-virus-infected cells have been detected in different people.

In secondary infections with other dengue virus serotypes, the IgM response is only of short duration. However, because there are already IgG antibodies against group-specific epitopes of E proteins, their synthesis is rapidly induced and IgG antibodies achieve more than the tenfold concentration that was detected during the initial infection.

Since dengue-virus-specific antibodies cross-react with other flaviviruses, particularly in countries where many different members of these viruses are endemic, the diagnosis of an acute infection is difficult by means of antibody detection. Therefore, explicit assertions can only be arrived at by a virus-neutralization test, by detecting viral RNA by RT-PCR or by isolation of the virus from blood samples of infected individuals; dengue viruses can be reproduced in vitro in various continuous cell lines (Vero or baby hamster kidney cells).

Therapy and Prophylaxis

So far, there are neither vaccines for prevention nor suitable antiviral drugs against dengue virus infections. The immunopathogenesis of antibody-dependent enhancement (also referred to as immune enhancement) which is related to dengue haemorrhagic fever and dengue shock syndrome renders the development of suitable vaccines very difficult. However, attenuated viruses of all four serotypes have been developed in Thailand with financial support from the Rockefeller Foundation. They are being tested as live vaccines in clinical trials. In addition, the fight against mosquitoes as the carrier of the disease and the combat against their breeding grounds is still of paramount importance.

14.5.5.3 Tick-Borne Encephalitis Virus

Epidemiology and Transmission

According to the geographical distribution, TBEV is classified into two groups: the Eastern subtypes are found particularly in the Asian part of Russia and the countries of the former Soviet Union; the Western subtypes prevail in the countries of central and eastern Europe, especially in Scandinavia and the European regions of Russia. In the literature, they are also known as Russian spring–summer encephalitis virus and central European encephalitis virus. Related virus types also exist in India (Kyasanur forest disease virus). TBEV is the only member of the group of central European encephalitis viruses which is widespread in central Europe. Louping Ill virus, which infects sheep in Great Britain and can cause encephalomyelitis, is closely related to TBEV; human disorders caused by louping Ill virus have been described only in individual cases. TBEV is endemic especially in Austria (Kärnten) and southern Germany (Danube region, Black Forest), in Slovenia, Croatia, Hungary, the Czech Republic, Slovakia, Poland, Lithuania, Latvia, Estonia and Russia. In endemic regions in central Europe the prevalence of TBEV in ticks is between 0.2 % and 0.5 %. TBEV is transmitted by tick bites, especially by *Ixodes ricinus*, the common wood tick, which is found in forests and alluvial areas. Infected ticks transmit the virus directly during sucking because TBEV accumulates in the salivary glands. Usually, people come into contact with ticks from grass and bushes when walking through infested areas. It is a mere delusion that ticks jump down from trees onto their victims. TBEV can be transmitted to humans and rodents during the period when ticks are active, particularly in the months from April to September/October. Within the tick population, TBEV can be transmitted transovarially to offspring. A rare infection route for humans is the transmission of TBEV by fresh milk and non-pasteurized raw milk products, especially from sheep and goats. These animals can be infected by ticks, and excrete the virus in the milk. However, the reservoirs of TBEV are small rodents. Infection of humans is a dead-end road because it interrupts the spread of TBEV.

Clinical Features

Infections with the central European virus type are relatively mild in comparison with infections with the eastern European virus type. About 70–90 % of individuals

infected remain asymptomatic, whereas the other 10–30 % develop a mostly gentle illness without lasting complications. The time between contact with the virus and the onset of the first symptoms is 1–2 weeks. The first signs of disease are flu-like symptoms such as fever, headache, nausea and sensitivity to light. They last about 1 week. During this time, viruses can be isolated from the blood. After this period, the health improves in most cases, but in about 10 % of patients only a transient improvement is observed for about 1 week. The second phase can range from a mild form of meningitis (inflammation of the meninx in the brain or spine; in roughly 55 % of cases) to severe forms of meningoencephalitis (inflammation of the brain; 35 % of cases) with tremor, dizziness, altered perception and paralysis. The involvement of the spinal cord is referred to as a meningomyelitis (5 %) or meningomyeloencephalitis (5 %). The mortality rate is approximately 1 % of patients with severe clinical courses. About 7 % of survivors of the second phase of infection have neurological sequelae such as paralysis, speech problems and epileptic seizures.

Pathogenesis

After inoculation by a tick bite, TBEV infects endothelial cells and macrophages at the site of the bite. The viruses are transported by them to the lymph nodes, where they find appropriate target cells for further reproduction cycles. From the lymphatic system, the viruses reach the blood. They spread in the body, and settle in the cells of the reticulohistiocytic system, where they proliferate. Infected macrophages transport the viruses into the central nervous system. In addition to specificity for infection of lymphocytes, TBEV has a marked neurotropism. As a result of the infection, the brain swells (cerebral oedema), and there is locally limited bleeding. Histopathologically, the following alterations can be recognized: inflammations in the vicinity of blood vessels, neuronal degeneration and necrosis in the brainstem region, in basal ganglia of the spinal cord and in the upper and lower cortex. The anterior horn cells in the cervical spine are particularly sensitive to the infection. This also explains the emergence of paralyses that occur preferably in the upper extremities in myelitic cases.

The TBEV E protein seems to be the key parameter for the virulence of different virus isolates: mutation of one amino acid (tyrosine to histidine at position 384) can significantly alter the virulence of infection. Apart from virus-specific features, intrinsic genetic differences between infected hosts can also affect the expression and the severity of the disease: a study in Lithuania described that serious infections associated with severe encephalitis are statistically commoner in patients with genetic defects in the gene encoding chemokine receptor CCR5 (the receptor of CCL5, also known as RANTES).

Immune Response and Diagnosis

TBEV can be cultivated in embryonated chicken eggs, in chicken embryonic cell cultures and in mammalian cells lines. However, isolation from patients is very difficult. The diagnosis of acute infection is performed by detecting virus-specific IgM antibodies in ELISA from blood and/or cerebrospinal fluid. Detection of viral

genomes by RT-PCR is possible from blood, and especially from cerebrospinal fluid; however, it is usually not successful in later infection stages. IgG antibodies are produced in the course of infection, and are virus-neutralizing and remain detectable lifelong.

Therapy and Prophylaxis

There is an inactivated vaccine made of purified and formalin-inactivated virus particles, which are cultivated, for example, in primary chicken embryonic cells. The vaccine usually contains aluminium hydroxide as an adjuvant. It exhibits a very good seroconversion rate and protective efficacy after primary immunization (three vaccinations), which lasts 3–5 years. Thereafter, booster vaccinations are required at regular intervals. Preferentially, vaccination is usually performed for people in highly endemic regions or in population groups with a high risk of being bitten by ticks because they have to remain in forests and meadows for a long time for job-related reasons. The formerly postinfection passive immunization is no longer recommended because the vaccine exerts a negative influence on the course of infection in children. A postinfection active vaccination within 3–4 days after a tick bite is currently under discussion. Effective antiviral agents for symptomatic TBEV infections are not available.

14.5.5.4 Hepatitis C Virus

Epidemiology and Transmission

Hepatitis C virus has long been classified into the so-called non-A, non-B hepatitis viruses. In 1989, Daniel W. Bradley characterized the genome of these viruses. Today, six genotypes of hepatitis C virus are known from different geographical regions; their nucleic acid sequences differ by 31–34 %. The genotypes are also subdivided into various subtypes. The most common genotype, 1b, is found in Europe, followed by genotypes 2a, 3b, 2c and 3a; however, in North America, genotype 1a is commonest, followed by genotype 1b. Genotypes 4 and 5 have only been found in Africa; on the other hand, genotype 6 prevails in some regions of Asia (China, Korea). The number of people who are chronically infected with hepatitis C virus has been estimated to be 200 million worldwide. In Germany, the prevalence is 0.5–0.6 %.

Hepatitis C virus is found only in humans. Before the introduction of appropriate test procedures, it was transmitted mostly through blood transfusions or blood products. Today, the residual risk of becoming infected by receiving a positive blood transfusion is 1:100,000. Nearly 70 % of all new infections with hepatitis C virus now occur among drug addicts, and are caused by the common use of syringes. Further methods of transmission are sexual intercourse and, in rare cases, household contact with infected patients under poor hygiene conditions. Hospital staff are endangered by injuries with needles. Even so, the source of infection is not known in approximately 30 % of cases. The virus can be transmitted vertically from mother to child during pregnancy or at birth. However, a hepatitis C virus infection of the gestating mother is not considered an indication for caesarean section.

Discovery of Hepatitis C Virus

The identification and characterization of hepatitis C virus was done using molecular-biological methods. The blood of an experimentally infected chimpanzee was the source for the isolation of the viral RNA. Representative complementary DNA clones were synthesized from the viral RNA. The encoded proteins were expressed. It was attempted to identify proteins that react with sera from patients with chronic non-A, non-B hepatitis. The respective clone was sequenced. Then, oligonucleotides were synthesized, and were used to amplify the viral RNA genome from the blood of the chimpanzee by PCR. Finally, the genome was completely sequenced. In the last step, monoclonal antibodies against the viral proteins were produced, which allowed the identification of the virus particles.

Clinical Features

A generally slight liver inflammation appears after an average incubation period of 6–8 weeks. About 75 % of infections are asymptomatic; severe clinical courses are rare. Acute infections with clinical symptoms have a favourable prognosis. Up to 80 % of all infected individuals develop a chronic persistent hepatitis or reactivated chronic hepatitis. Persistent viral RNA can be detected in the blood of such patients by modern ultrasensitive PCR methods. Chronic infections are characterized by elevated transaminase levels, which, however, can fluctuate and can also be temporarily normal: the more active the infection, the higher the values. Only a few patients who have established a chronic infection show spontaneous elimination of the pathogen. This can be observed in 0.5–0.74 % of patients. Ten percent to 20 % of patients with chronic infection develop cirrhosis over the years, and about 4 % of them develop a primary liver cell carcinoma in the course of decades. A simultaneous infection with human immunodeficiency virus promotes the emergence of cirrhosis. Additional complications include periarteritis nodosa, membrane-proliferative glomerulonephritis and idiopathic Sjögren syndrome. This is determined by circulating mixed cryoglobulins, which are considered to be a consequence of the expansion of B-cell clones, which produce pathogenic IgM with rheumatoid factor activity.

Pathogenesis

The virus directly enters the circulatory system, mainly through contaminated blood or blood products, and is transported by infected macrophages into the liver, where it infects hepatocytes. The result is liver inflammation accompanied by cell necrosis. Cellular damage seems to be primarily induced by the immune response during hepatitis C. The virus itself is only weakly cytopathogenic, as was demonstrated by continuous replication of the entire viral genome in different cell types in vitro. IFN-α is produced and secreted by liver cells. Tubular structures have been observed in the cytoplasm of infected liver cells by electron microscopy. Little is known concerning the details of the pathogenesis of the acute infection.

Antigen–antibody complexes are formed in the chronic infection form, and can be deposited in the glomeruli. They seem to be responsible for the membrane-proliferative glomerulonephritis in such patients.

The hepatitis C virus exhibits a high mutation rate and changes in patients in the course of infection. New quasispecies are formed constantly. The mutations arise during replication with a probability of 2×10^{-3}. They are attributed to the fact that the viral RNA-dependent RNA polymerase, unlike cellular DNA polymerases, does not possess an exonucleolytic proofreading mechanism to control and enhance the accuracy of RNA synthesis. The classification of genotypes and subtypes of hepatitis C virus was originally based on the sequence of the NS5 gene. However, variations are found in all regions of the genome. Only the 5′-terminal UTR containing the IRES element is highly conserved. Mutations within viral genes are not uniformly distributed. There are variable and hypervariable as well as relatively conserved sequences. Hypervariable regions are located in the amino-terminal region of the E2 protein between amino acids 1–27 and 90–97. They are recognized by antibodies, and thus are exposed to a strong immune selection pressure. The virus changes both epitopes during a chronic infection, with the result that the antibodies are no longer able to recognize them. In a similar way, mutations also alter the epitopes which are recognized by cytotoxic T cells. Presumably, this immunological selection pressure promotes the development of viral variants, which can cause a chronic infection. In addition, it has been found that the non-structural proteins of flaviviruses possess several activities that allow the pathogens – including hepatitis C virus – to avoid the defence strategies of the non-specific immune response (Sect. 14.5.3). In the case of hepatitis C virus, the NS3/4A protease cleaves the cellular factors Cardif (CARD-adapter-inducing IFN-β) and TRIF (Toll/IL-1-receptor-domain-containing adapter), which activate the interferon regulatory factor and initiate the synthesis of IFN-α and IFN-β. In vitro, the NS5A protein inhibits protein kinase R, which is activated by IFN-α and inhibits translation. However, whether this mechanism also occurs in vivo to counteract the effect of interferon has not been demonstrated; the same also applies to the other examples mentioned. It is also not known whether specific mutations are important for the virulence of the different quasispecies. Some subtypes appear to differ in their sensitivity to IFN-α, whereby genotypes 1 and 4 are particularly resistant.

It has not been conclusively resolved in which way hepatitis C virus promotes carcinogenesis. There is evidence that specific sequences of the C protein interact with cellular Ras proteins and that this interaction induces transformation. The time span between infection and the formation of a primary hepatocellular carcinoma is approximately 20–40 years. The origin is a chronic infection in adolescents and adults. It is thought that persistent inflammation processes are responsible for the development of cancer over the years. Because of the infiltration of immunologically active cells and their secretion of cytokines, liver cells are destroyed. This cell-damaging process can be intensified by certain cofactors, such as alcohol

consumption. In individual cases, mutations can arise in the cellular genome during such processes, which then stimulate liver cells to proliferate continuously, and contribute to the development of hepatocellular carcinoma.

The perinatal transmission of the virus from infected mothers to their newborn children plays only a marginal role in carcinogenesis associated with hepatitis C virus, unlike carcinogenesis associated with hepatitis B virus (▶ Sect. 19.1). Double infections with hepatitis B and hepatitis C viruses are found in Japan in up to 18 % of primary hepatocellular carcinomas. Simultaneous infection with hepatitis B, hepatitis C and hepatitis D viruses cause a shortening of the incubation period until the emergence of the carcinoma.

Immune Response and Diagnosis

Increased transaminase levels may be elucidative for the diagnosis of hepatitis C virus infections, although this does not allow any further assignment of the pathogen. The main approach for diagnosis of hepatitis C virus infections is ELISA, which is used for screening. If the test findings are positive, it can be inferred that a fresh, chronic or past hepatitis C virus infection has occurred. A more exact serological differentiation is not possible. Owing to the high sensitivity of these screening tests, immunoblotting or analogous methods have additionally been introduced as confirmatory tests to exclude non-specific results. Quantitative RT-PCR for detection of viral RNA genomes is the most important method used today, and is usually applied as a confirmatory test, especially since this method immediately provides the level of the viral load. In general, serum or plasma is used as the source material; liver biopsies are used only in exceptional cases. In addition, the genotype is usually determined by PCR and hybridization tests, as this is crucial for determining the duration of therapy. Since the phase in which an acute hepatitis C infection cannot be serologically diagnosed with certainty lasts several months, automated tests have been introduced for the detection of viral C proteins in order to shorten this phase.

In ELISA or in immunoblotting, recombinant viral proteins are used to detect specific antibodies. IgM antibodies against the NS4 and C proteins can be found in acute infections. However, since these may persist or correlate with the level of liver injury and also with the genotype, IgM diagnostic tests are not of great importance. This is further indication that both viral gene expression and protein synthesis are constantly occurring in chronic infections. IgG antibodies against the C protein can be detected a few days to a few weeks after the onset of symptoms; IgG antibodies against non-structural proteins (NS3, NS4, NS5) are detectable later. Immunoglobulins against envelope proteins E1 and E2 are detected at an early stage in only about 10 % of acute infections. It is unknown whether these antibodies are not formed or whether they cannot be detected owing to the variability of the amino acid sequence and the lack of sensitivity of the test systems. Cytotoxic T lymphocytes can be detected in the blood of patients after stimulation by peptides that are derived from viral proteins.

Therapy and Prophylaxis

There is no vaccine against hepatitis C virus because of the quasispecies problem. The use of IFN-α, and especially the use of IFN-α compounds which act as a depot (pegylated interferon), in combination with ribavirin has proved to be successful for the treatment of chronic infections. In acute infections, curing rates above 90 % can be achieved even with IFN-α alone. In many cases, treatment of chronic infections leads to a significant reduction of the viral load in the peripheral blood, where it is no longer detectable even with ultrasensitive methods. However, there are many treatment failures: especially infections with genotypes 1 and 4 have proven to be largely resistant. Therefore, patients infected with genotypes 1 and 4 are currently treated for 48 weeks, with testing for treatment success after the 12th week (corresponding decrease of viral load in quantitative PCR). If the expected decrease of viral load does not occur, the therapy is stopped. In contrast, in cases of infection with other genotypes, patients are treated for 24 weeks. Inhibitors of the viral protease NS3 have recently been introduced as alternative drugs, e.g. boceprevir, a substance derived from peptides. Despite these advances in drug therapy, the effects of chronic hepatitis C virus infections are responsible for about 20 % of all liver transplants.

Hepatitis G Virus (human pegivirus, GB virus C)

Hepatitis G virus was originally isolated by Friedrich Deinhard. In 1967, he inoculated marmosets with the serum of a surgeon who had hepatitis (G. Barker, according to his initials the virus is sometimes also referred to as GB virus), and was able to isolate a virus from the infected monkeys. In 1995, the genome of this virus was sequenced by Scott Muerhoff and co-workers; it was classified into the family *Flaviviridae* owing to the arrangement of its genes. Since an IRES sequence was identified at the 5′ end of the RNA genome, and genes are present coding for two glycoproteins (E1 and E2), it is related to hepatitis C virus. Subsequently, infections with this pathogen, which had been designated hepatitis G virus and is named human pegivirus today, were identified in many people who responded to the infection with the formation of specific antibodies. Meanwhile, different subtypes of this virus have been identified. The initial presumption that hepatitis G viruses cause liver inflammation in humans has not been verified. Occasionally, these viruses were also isolated from patients with hepatitis because of the high prevalence rates – up to 4 % of blood donors are viraemic. Both the acute and the persistent infection are apparently asymptomatic. There is even evidence that in patients who are infected with human immunodeficiency virus and human pegivirus, the human immunodeficiency virus infection shows an attenuated clinical course. This effect may be a result of the properties of the E2 protein of human pegivirus, which inhibits the replication cycle of human immunodeficiency virus, possibly on the basis of similar epitopes, and leads to cross-reacting immune responses.

14.5.6 Human and Animal Pathogenic Flaviviruses

14.5.6.1 West Nile Virus

West Nile virus is a virus with zoonotic potential. It was originally widespread only in the Old World (Asian and African countries, Romania). In 1999, it also appeared on the American continent, and spread throughout the entire North American continent in subsequent years. West Nile virus falls into the Japanese encephalitis complex, which includes Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus and Kunjin virus. The latter are widespread on the Australian continent.

Epidemiology and Transmission

West Nile virus is transmitted to birds by ornithophilous mosquito species, particularly *Culex univittatus* and *C. pipiens*. It is believed that different bird species exhibit significant differences in their susceptibility for infection with West Nile virus; corvids seem to be particularly susceptible. It has been shown that the pathogen can also be directly transmitted among mosquitoes, at least under laboratory conditions, when two mosquitoes (an infected and an uninfected mosquito) simultaneously suck the blood of the same bird. In addition, the virus can also be transmitted by aerosols among birds. The virus proliferates in susceptible birds and is present in the blood at concentrations that allow transmission by mosquitoes, which occasionally transmit the virus to other hosts, such as horses and humans. Whether birds can establish an infection is unknown. The intra- and intercontinental spread of the virus is done by infected migratory birds. People who are infected with West Nile virus can transmit the pathogen through blood and organ donations, as well as via human milk.

Clinical Features

Humans After an incubation period of 3 days to 2 weeks, patients develop flu-like symptoms such as fever, headaches and back, joint and muscle pains. Nausea, diarrhoea and general lymph node swelling are also observed in some cases; there are also signs of a skin rash especially among children. In severe clinical courses, which are frequently found in elderly patients, the initial symptoms are followed by liver and heart muscle inflammations as well as encephalitis. About 5–10 % of patients with neurological symptoms die.

Animals Avian infections are systemic and also result in encephalomyelitis; extraneuronal lesions are very common. Therefore, myocarditis, muscle degeneration and lymphocytic infiltrations are found in various organs, such as pancreas, lung and liver. An atrophy of the bursa of Fabricius is also frequent. In addition, clinically asymptomatic infections are typical. Among birds, there are considerable differences in susceptibility. Corvids and raptors are considered to be highly susceptible and often have severe disease patterns; thus, monitoring programmes should especially include these birds.

Although in the USA there are a large number of infected horses, clinically inapparent infection is the rule in this species. Experimental infections with

mosquitoes infected with West Nile virus resulted in clinical symptoms in only about 10 % of the exposed and infected horses. In contrast to infections in birds, almost exclusively neurological symptoms are found in horses as a result of poliomeningoencephalitis; other manifestations are practically not observed.

Pathogenesis

During infection of humans, West Nile virus is introduced into the bloodstream through a mosquito bite, and binds to integrins ($\alpha_v\beta_3$) on the surface of monocytes, macrophages and endothelial cells, and invades the whole organism; furthermore, interaction with the proteins ICAM-3 and dendritic-cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) has also been described. There are only a few data concerning the details of the pathogenesis. Similarly to TBEV, for West Nile virus, patients with genetic defects in the chemokine receptor CCR5 have increased risk of developing neurological symptoms and thus severe diseases from infection. Possibly, this receptor and its interaction with the ligand are responsible for the regulation of migration of macrophages and T lymphocytes into the infected tissues.

The Receptor CCR5: Friend or Foe in Viral Infections?

The chemokine receptor CCR5 attracted attention a few years ago: it was identified as a coreceptor, to which human immunodeficiency virus binds on the surface of monocytes and macrophages to initiate the infection (see ► Sect. 18.1). The importance was additionally underscored by the finding that people with genetic defects in the CCR5 gene cannot be infected by human immunodeficiency virus; they are largely resistant. These data constitute the basis for the development of new therapeutics which are aimed at blocking the interaction between human immunodeficiency virus and the CCR5 protein on the cell surface, and should prevent infections. However, the use of such inhibitors could increase susceptibility for developing severe infections with West Nile virus. In using them, we would possibly combat one evil, while accepting another.

Immune Response and Diagnosis

The diagnosis is done by RT-PCR by cultivating the pathogen in chicken eggs or in cell cultures and subsequent isolation of the virus. The virus agglutinates goose erythrocytes, and thus can be detected by haemagglutination or haemagglutination-inhibition tests. IgM and IgG antibodies can only be detected by ELISA.

Control and Prophylaxis

An immunoprophylaxis is not available. In large cities, water accumulations are treated with pesticides to reduce the numbers of mosquitoes. However, the virtue of this measure is controversially debated. Because of deaths, donated blood is examined for the presence of West Nile virus in the USA.

West Nile Virus as a "New Virus" on the American Continent

West Nile virus caused a series of fatal encephalitis cases in humans in New York in 1999. This attracted much attention at that time, especially because the virus was considered as a classic pathogen of the Old World and it was entirely unknown on the American continent and in the urban area of New York. West Nile virus usually infects birds (songbirds, crows), which constitute the reservoir for the pathogen. They can transport the virus over long distances. The virus can be transmitted to horses and humans by mosquitoes of the genus *Culex*. The virus spread in just 1 year throughout the eastern USA. Diseases and deaths due to West Nile virus infections were reported especially in the eastern USA in the years after 1999. In 2002, the pathogen had spread into more than 39 states and 4,156 infections were documented in humans, 248 of whom died. The deaths occurred primarily in people of advanced age (the average age of the people who died was 79 years). Furthermore, during this period more than 3,400 confirmed infection cases were recorded in horses, which develop a clinical picture similar to that in humans. The infection wave reached its climax in 2003, when West Nile virus infections were diagnosed in almost 10,000 patients in all states of the USA. Since then, dead birds are extensively examined to determine whether they are infected with West Nile virus, especially the highly susceptible crows. In subsequent years, the number of infections decreased to 3,000–4,000 per year. To minimize the risk of infection, a very intensive and costly monitoring programme has been implemented, which includes five levels: mosquitoes, sentinel chicken flocks, diseased birds, other sick animals and diseased humans. Because of the infection wave and the risk of transmission of infection through contaminated blood donations, all blood donations have been tested by PCR for the presence of West Nile virus in the USA since 2003; along with the decrease in the number of new infections, the number of detections of West Nile virus in blood donations decreased from 818 in 2003 to below 200 in 2008. In European blood donors, West Nile virus is detected much less frequently; in Germany, for example, it was found with an antibody prevalence of only 0.03 % without evidence of viral RNA in blood donations.

14.5.7 Animal Pathogenic Flaviviruses

The animal pathogenic flaviviruses are divided into two groups: one group is composed of viruses that are transmitted by arthropods (West Nile virus, louping ill virus and TBEV); infections by viruses of the second group occur independently of arthropods (pestiviruses). As animal pathogens, pestiviruses are economically significant, particularly classical swine fever virus and BVDV. West Nile virus can cause fatal infections in humans (see Sect. 14.5.6). TBEV, in rare cases, can also infect ruminants or dogs, causing clinical pictures similar to those of human

infections (Sect. 14.5.5). The distribution of louping ill virus is confined to Great Britain. Louping ill virus, like TBEV, is transmitted by ticks and causes encephalitis in sheep. Humans can also be infected by this virus and become ill, although this occurs extremely rarely. Because of the minor importance of louping ill virus, a more extensive description is not given here.

14.5.7.1 Classical Swine Fever Virus

There are a number of pathogenic flaviviruses within the genus *Pestivirus* which cause economically important diseases in swine and ruminants. These include primarily classical swine fever virus, whose infections cause the classical ("European") swine fever in pigs. Clinically, it cannot be distinguished from the similar African swine fever. The latter is induced by African swine fever virus, a DNA virus from the new family *Asfarviridae* (▶ Sect. 19.7).

Epidemiology and Transmission

The clinical picture of classical swine fever is characterized by severe haemorrhagic and general symptoms. In addition to this severe form, clinically atypical infections occur very frequently (nowadays almost exclusively), and exhibit only few clear and partially mild symptoms. This complicates the rapid clinical diagnosis and can contribute to fast dissemination, considering the high contagiousness of the virus.

Transmission occurs primarily by direct animal contact, particularly as a result of the purchase of pigs in fattening farms which have a subclinical persistent infection or exhibit only attenuated symptoms. A further infection source is contaminated animal food. Frequently, infection occurs via kitchen waste containing the meat of infected animals; therefore, feeding of kitchen waste to pigs is strictly prohibited. Recently, outbreaks of swine fever have occurred in which the virus was transmitted by wild boars. In those cases two factors played a crucial role: direct contact (between pasture or grazing pigs and wild boars) and feeding of pigs with wild boar meat (swine-holding hunters and poachers).

Of particular epidemiological importance are piglets, which develop persistent infections and contain the pathogens in blood after intrauterine transmission by infected mother pigs. Such animals excrete the virus permanently (chronic carrier), and can remain asymptomatic for several months. However, they eventually develop clinical symptoms, and do not reach the age of more than 16 months.

Clinical Features

Classical swine fever is characterized by a peracute to acute disease pattern, which may be accompanied by respiratory or gastrointestinal disorders. Central nervous system symptoms have also been described, such as tremor, paralysis and convulsions. The morbidity can be up to 100 % in livestock. The atypical or chronic infections are less dramatic, and thus can be easily overlooked. In pregnant sows, classical swine fever can cause miscarriages of mummified piglets, or farrowing of weak piglets.

Pathogenesis

Infections with the pathogen of classical swine fever usually occur by oral transmission. During a viraemia, viruses replicate primarily in the tonsils and from there reach nearly all endothelial cells and lymphatic organs, including the bone marrow. Viral replication is associated with significant cellular destruction, which is manifested in multiple haemorrhages, a massive lymphopenia and thrombocytopenia, and disseminated intravascular coagulopathy. Characteristic are multiple splenic infarctions and a severe atrophy of lymphoid organs, which progresses with increasing duration of the disease. Frequently, an encephalitis can also develop. The viruses are transmitted in the intrauterine way. Depending on the gestational age at the time of infection, premature re-entry or return to heat (re-entering oestrus), abortion or birth of malformed piglets can occur. Live-born piglets of infected sows develop a persistent viraemia, like calves during BVDV infections in cattle. They excrete the virus permanently and play a major epidemiological role.

Immune Response and Diagnosis

The diagnosis of classical swine fever is initiated by the veterinary authority after notification. Diagnosis is possible by detection of antibodies using ELISA or neutralization tests. In this context, it is important to distinguish between antibodies against classical swine fever virus and antibodies against BVDV, which can also infect swine, but does not induce any disease, and hence is not subject to compulsory supervision for animal health purposes. Differentiation requires the parallel titration of sera which contain the proteins of BVDV or classical swine fever virus as antigens. However, more important is the direct detection of the virus, which can be achieved by isolating the pathogen in permanent porcine kidney cell cultures, by serological characterization by monoclonal antibodies in immunofluorescence tests (if necessary by flow cytometry) or by genetic characterization by means of PCR.

Control and Prophylaxis

In most countries the regimen that is used to control classical swine fever is rather strict. If cases occur, all pigs on the farm are culled, and extensive epidemiological investigations are implemented to determine the spread of the virus.

Recently, genetically engineered vaccines against swine fever have been developed. They contain glycoprotein E2, which is produced by means of recombinant baculoviruses. These marker vaccines induce an immune reaction exclusively against glycoprotein E2, which allows differentiation between vaccinated and infected swine. However, they are not suitable for emergency vaccinations because a robust protection can be developed only a few days after vaccination.

Interestingly, a highly effective live vaccine has long been available, and has been used in many European countries for many years. It is based on a virus that was attenuated by numerous passages in rabbits (C strain, “lapinized virus”). This virus is now used only for oral immunization of wild boars with bait vaccines in the wild. It is no longer used for immunization of the well-controlled pig populations

because trade of meat from vaccinated animals is accompanied by strict requirements, which make its application economically (currently) unattractive.

Controlling Classical Swine Fever in the European Union

The countries of the European Union work together to control economically important animal diseases, and to adopt binding regulations for all member states. These include the eradication of classical swine fever. Infections at the flock level can be confirmed simply and quickly by antibody detection. Vaccination is not permitted because its application would make the simple serological detection of infected pig herds impossible. Therefore, all animals of an affected herd will be killed in cases of classical swine fever outbreaks owing to the high contagiousness of the virus. Their carcasses have to be destroyed and disposed of, possible movements of animals from the herd must be followed in comprehensive epidemiological surveys, and animals suspected of being contaminated have to be put into quarantine and under official veterinarian surveillance. This expensive method of sanitation is extremely effective and ultimately more cost-efficient than vaccination, which would only save individual pigs within a farm for a short time, but would entail trade restrictions on pigs and pork within and outside the European Union. Restricted trade would result in enormous economic losses. However, the killing of large numbers of animals, especially the killing of non-infected animals, is a subject of debate regarding ethical aspects. The European Union is considering reversing the non-vaccination policy, which would allow vaccination of non-infected flocks when there are outbreaks. The sale of meat from vaccinated animals is currently a problem because it is not internationally accepted.

14.5.7.2 Bovine Viral Diarrhoea Virus

Epidemiology and Transmission

BVDV is an economically important animal pathogen. For years, there has been discussion of whether there are two separate pestiviruses, namely BVDV-1 and BVDV-2, or whether they are different genotypes of a single species of virus. Although they cause the same symptoms, they can be distinguished by their genome sequences and the antibody response induced in cattle. The virus is excreted in the faeces and via mucosal secretions, and is ingested orally.

Clinical Features

In adult animals, BVDV causes a subclinical infection or mild, self-limiting diarrhoeas. Peracute haemorrhagic symptoms are rarely observed. Whether in such cases particular strains or biotypes of BVDV are responsible is being discussed. After infection, the animals develop lifelong immunity. Economic damage is caused by the virus if susceptible, i.e. immunologically unprotected, pregnant cows are infected. Depending on the time of infection, infection leads to abortion,

birth of malformed calves or birth of persistently infected, viraemic calves. These animals can develop mucosal disease, a fatal disease form.

Mucosal disease is a generalized disorder of chronically infected cattle. The virus proliferates lytically in the cells of all mucous membranes and endothelial tissues. The infected animals develop the severe clinical picture of haemorrhagic fever, which invariably ends fatally. There is bleeding in all mucous membranes, and the virus is found in almost every organ.

Pathogenesis

The viral persistence is based on a BVDV-type-specific central immune tolerance, which is established in the embryo against a non-cytopathogenic biotype in the course of infection. These calves are completely immunocompetent against other viral infections, even against BVDV infections with a serologically distinct virus type. During persistence of BVDV, mutations occur in the viral genome, which lead to the development of a virus with a new biotype. This cytopathogenic virus then causes the lethal clinical picture of mucosal disease.

The molecular basis for the emergence of the cytopathogenic virus resides in genetic mutations that lead to different processing of the non-structural protein NS3. Whereas it is produced as the NS2–NS3 fusion protein in non-cytopathogenic BVDV, NS3 proteins do not possess the NS2 fusion part in the corresponding cytopathogenic viruses. The altered processing occurs when cellular genome sequences are integrated into the NS2 coding region within the viral genome. This process changes the pattern of proteolytic cleavage in the precursor protein. It has been shown by homologous RNA recombination that a number of cellular genes have been integrated into the viral genome (Fig. 14.16). Besides the integration of cellular gene sequences, e.g. by incorporating ubiquitin-coding sequences, rearrangements or deletions of viral genome segments have also been described. All these processes lead to the production of free NS3 proteins, which are generated by cleavage of the precursor protein by the cellular ubiquitin hydrolase or the viral protease N^{pro} (Fig. 14.16). The underlying mechanisms that lead to the enhanced virulence are unclear. Classical swine fever virus and border disease virus of sheep (another pestivirus) also produce free NS3 proteins during infection, but without inducing the severe symptoms which are developed in mucosal disease.

Immune Response and Diagnosis

BVDV infections are usually diagnosed by isolating the virus in cell culture, by RT-PCR or by detection of the BVDV antigen in peripheral blood lymphocytes using immunofluorescence tests (flow cytometry) or ELISA. Maternal antibodies can interfere with these tests, and their usage yields reliable results only from the sixth month of life. Therefore, the detection of the viral E^{rns} protein in serum is usual today. It is secreted by virus-infected cells. Maternal antibodies against the E^{rns} protein are hardly present in calves, so verification of infection is possible from the 20th day of life by detection of E^{rns} proteins using ELISA.

The infection produces long-lasting immunity, which probably lasts for life.

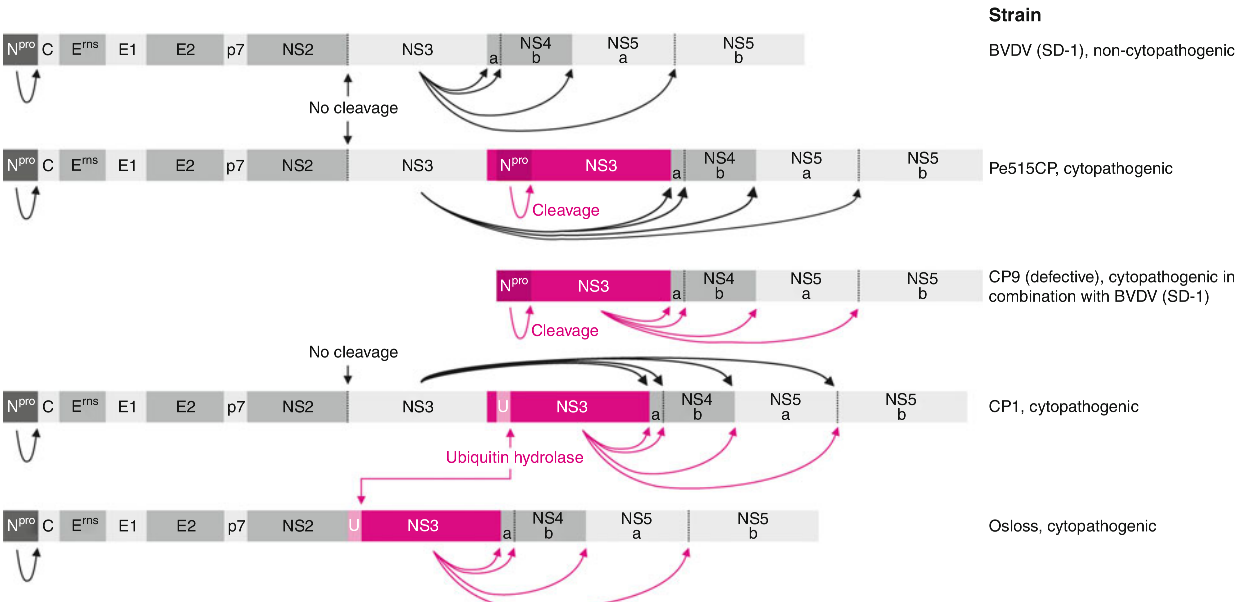


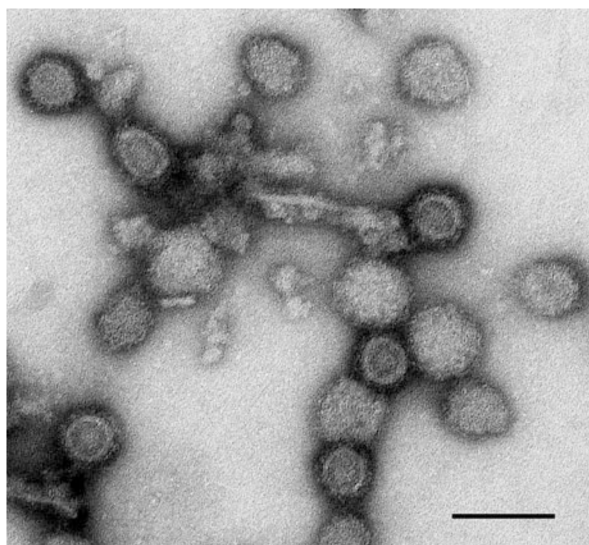
Fig. 14.16 Recombination events in the genome of BVDV. In the non-cytopathogenic strains (BVDV SD-1), no cleavage occurs between proteins NS2 and NS3, and both remain fused and act as a protease to cleave the non-structural part of the precursor protein. In cytopathogenic strains, various recombinations with cellular DNA are found. In such cases, ubiquitin sequences (*U*) are integrated into the viral RNA genomes upstream of the NS3 region (strains CP1 and Osloss). Cleavage and release of NS3 proteins is performed by the cellular ubiquitin hydrolase. Alternatively, recombination events may occur in the cytopathogenic strains, which rearrange the N^{pro} sequences amino-terminally to the NS3 region. The proteolytic activity of N^{pro} leads subsequently to autocatalytic cleavage and the formation of an NS3 protein in addition to the NS2–NS3 fusion product (strain Pe515CP). Furthermore, there are defective viral genomes lacking the regions for the structural and NS2–NS3 proteins but which have rearranged the N^{pro} region upstream of the sequences of NS3 (strain CP9) similarly to strain Pe515CP. These defective viruses become pathogenic when they are present in animals in combination with non-cytopathogenic, infectious strains (SD-1)

Control and Prophylaxis

The principal objective is to avoid the birth of calves with persistent viraemia. In principle, this is possible through two measures. The first measure is that all female offspring are vaccinated before sexual maturity. This prevents the animals being susceptible to a viral infection during pregnancy. Transplacental transmissions of the virus and, ultimately, to new calves with persistent infections are avoided. The other measure is the early identification of animals with persistent viraemia, and their removal from the livestock. This is only possible by individualized virus detection. The serological analysis of representative samples from young animals of a herd to be examined provides, however, good indications for the presence of persistent virus carriers. Owing to nationwide measures, the number of persistently infected cattle is declining. It is assumed that less than 1 % of all cattle exhibit a persistent BVDV infection today.

A series of vaccines are available. They contain either inactivated or attenuated viruses with reproduction capacity. The former are generally not able to prevent intrauterine transmission of the virus. Live vaccines, however, are based on a cytopathogenic BVDV strain. In persistently infected animals, the live vaccine triggers mucosal disease immediately after vaccination or after recombination between the wild type and the vaccine strain. These vaccines are therefore problematic. Furthermore, persistently infected calves can be produced after inoculation of pregnant animals with the live vaccine. It is hence desirable to develop potent inactivated vaccines which reliably prevent intrauterine transmission and protect against the known BVDV genotypes.

14.6 Togaviruses



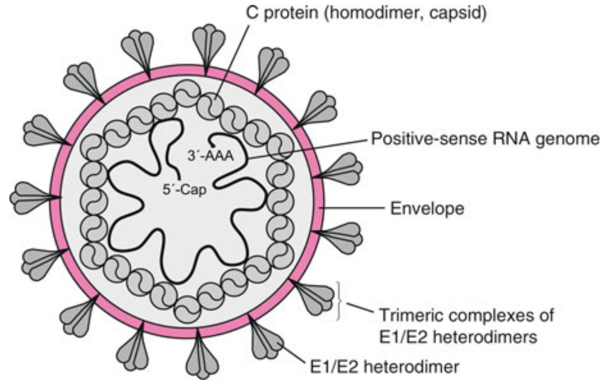


Fig. 14.17 Structure of a togavirus particle. The icosahedral capsid is composed of the C protein; the viral RNA genome is attached to the inner side of the capsid. The capsid is enclosed by an envelope, in which the viral surface proteins are embedded

Togaviruses were originally classified into a common virus family along with flaviviruses owing to their morphological similarity. However, as details about the replication mechanisms became known, significant differences were revealed that led to the current classification into two separate families. With regard to evolution, flaviviruses can be considered as the precursor of togaviruses because they are also enveloped viruses which synthesize polyproteins during their replication cycle. However, togaviruses have developed the synthesis of a subgenomic RNA for translation of structural proteins, which confer the capability of adjusting the quantity of different proteins to the respective requirements, thus resembling astroviruses, caliciviruses and hepeviruses. The name of this virus family is derived from the Latin term *toga* (meaning "mantle" or "shell"): the first electron micrographs revealed the image of a capsid that is surrounded by a wide envelope (Fig. 14.17).

14.6.1 Classification and Characteristic Prototypes

The togavirus family (*Togaviridae*) comprises two genera (Table 14.13). The alphaviruses, which are transmitted by insects, are especially known as pathogens of animal encephalitis and arthritis in America, Africa and Asia. They are classified according to their antigenic similarity in different complexes that are not host-specific and are transmitted by different mosquito species between animal species (horses, rodents, various birds such as pheasants and cranes) and humans, where they proliferate. In humans, they occasionally cause symptomatic infections; infections with chikungunya virus have been occasionally described during the summer months in southern Europe (Italy) since 2005. On the other hand, the members of the second genus, *Rubivirus*, are distributed worldwide. The pathogen of rubella infection belongs to this genus. These viruses are not transmitted by insects.

Table 14.13 Characteristic prototypes of togaviruses

Genus	Human virus	Animal virus	Vector/carrier
Alphavirus	Semliki Forest complex Chikungunya virus	Semliki Forest complex Semliki Forest virus (rodents)	*Aedes* spp.
		Chikungunya virus (non-human primates)	*Aedes* spp.
	O'nyong-nyong virus Ross River virus	O'nyong-nyong virus (animal host unknown)	*Anopheles funestus, A. gambiae*
		Ross River virus (marsupials)	*Culex annulirostris*
		Western equine encephalitis complex Western equine encephalitis virus (birds, horses) Sindbis virus (rodents)	*Culex tarsalis, C. quinuefasciatus*
		Eastern equine encephalitis complex Eastern equine encephalitis virus (bird horses)	*Culex* spp.
		Venezuelan equine encephalitis complex Venezuelan equine encephalitis virus (rodents, horses) Everglades virus	*Culex* spp., *Aedes* spp. *Culex* spp.
Rubivirus	Rubella virus	–	–

Semliki Forest Virus and Sindbis Virus: Two Well-Studied Prototypes of Alphaviruses

Semliki Forest virus and Sindbis virus are the best studied representatives of togaviruses with regard to their molecular biology and replication mechanisms; therefore, they have long been considered as prototypes of this virus family. Sindbis virus is related to western equine encephalitis virus, which is common on the American continent (Sect. 14.6.6). Both species of virus can be easily reproduced in cell cultures and show a pronounced cytopathic effect. Sindbis virus is widespread in Africa, eastern Europe and Asia. It is transmitted by mosquitoes of the genus *Culex*, and only in rare cases causes a febrile illness with rash and joint pain, which is similar in terms of clinical features and pathogenesis to a disease caused by some flaviviruses in terms of the clinical picture and pathogenesis. Neurotropic isolates have previously been described only in individual cases. Semliki Forest virus is endemic in Africa, India and Southeast Asia. It is transmitted by *Aedes* spp. and is largely non-pathogenic in humans. Therefore, it is now frequently used as a genetic engineering vector for heterologous gene expression in eukaryotic cell cultures.

14.6.2 Structure

14.6.2.1 Virus Particle

The infectious particles of togaviruses have a diameter of 60–80 nm and consist of icosahedral or spherical capsids (diameter 40 nm), which are enclosed by an envelope. Glycoproteins E1 and E2 are embedded in the envelope. They are present as E1/E2 heterodimers, which further associate to form trimeric protein complexes. There are about 80 trimers per virion. They form spike-like protrusions of 6–8 nm in length on the surface of the virus (Fig. 14.17). The trimers mediate attachment to cellular receptors and are binding targets of virus-neutralizing antibodies. In rubella virus, there are also E1 homodimers on the particles. The capsid is composed of 240 dimers of the C protein. It contains the RNA genome, and is associated with amino acids on the inner side of the capsid.

14.6.2.2 Genome Organization and Structure

The togavirus genome consists of a single-stranded, positive-sense RNA molecule, which is capped at the 5′ terminus and polyadenylated at the 3′ end. It has a length of 9,762 (rubella virus, strain Therien), 11,703 (Sindbis virus), 11,675 (eastern equine encephalitis virus) or 11,442 (Semliki Forest virus) nucleotides. The genome encompasses two open reading frames: the open reading frame beginning at the 5′ terminus encodes the precursor protein of the four non-structural proteins NSP1–NSP4 in alphaviruses, as well as p150 and p90 in rubiviruses; the open reading frame in the 3′-terminal half of the genome contains the genetic information encoding the structural proteins, C, E1 and E2 (Fig. 14.18). The two open reading frames are separated by a few nucleotides in Sindbis virus and Semliki Forest virus, whereas the intergenic region of rubella virus has a length of 124 nucleotides. The polyprotein of the structural proteins is translated from another open reading frame. There is a short UTR at the 5′ end of the genome (41 nucleotides in rubella virus, 60–80 nucleotides in Sindbis virus). Between the stop codon of the second open reading frame and the poly(A) tail at the 3′ terminus of rubella virus there are 61 nucleotides (264 nucleotides in Semliki Forest virus, 322 nucleotides in Sindbis virus), and these are folded into defined secondary structures.

14.6.3 Viral Proteins

14.6.3.1 Polyprotein of Non-Structural Proteins

The precursor protein of the up to four non-structural proteins NSP1–NSP4 differs among the various togaviruses. Two different precursors are synthesized in Sindbis virus; one includes proteins NSP1 – NSP3, and ends at an opal stop codon (UGA), which is located between coding regions NSP3 and NSP4 (Fig. 14.18a). However, this termination signal for translation is skipped in 20 % of cases, and the translation is continued until the end of the NSP4 protein. In contrast, only one full-length non-structural polyprotein is synthesized in Semliki Forest virus and in rubella virus

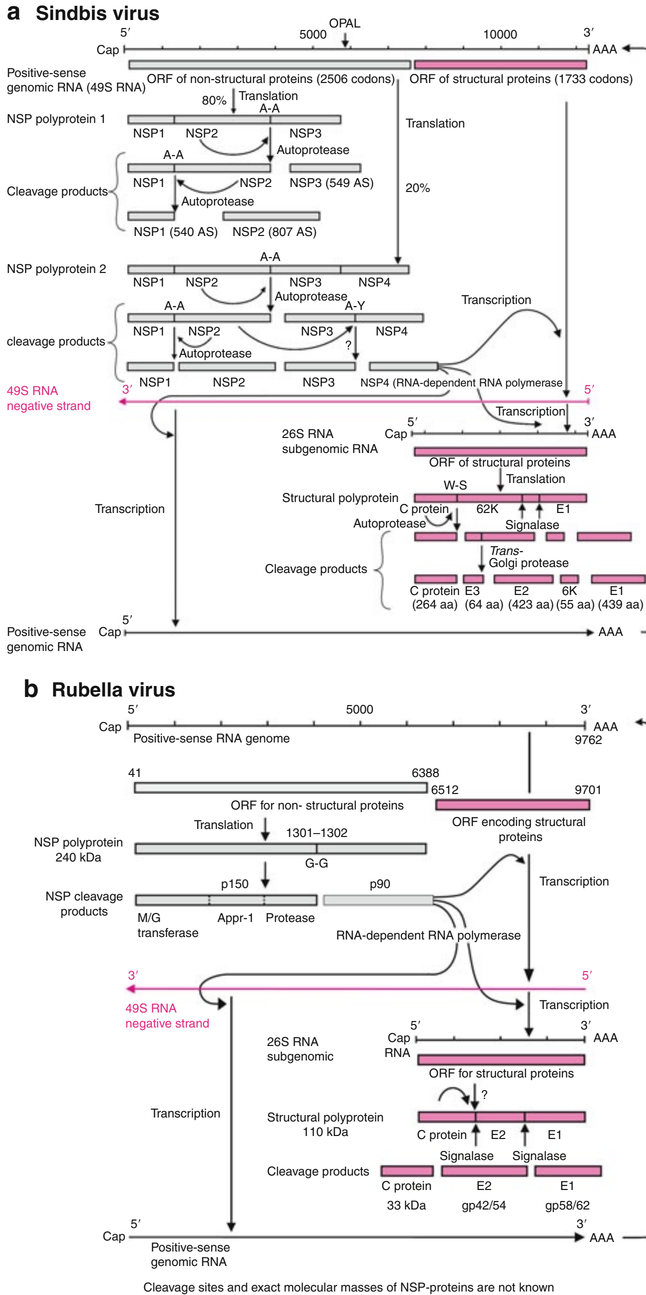


Fig. 14.18 Genome organization and replication of togaviruses. (**a**) Sindbis virus. (**b**) Rubella virus. The RNA genomes encode a single version (rubella virus) or two versions of the non-structural polyprotein as well as one structural polyprotein. First, the precursor of the

(240 kDa in rubella virus; Fig. 14.18b). Whether similar two-stage translation processes play a role in other togaviruses is not known. Table 14.14 provides a summary of the size and function of togavirus proteins.

14.6.3.2 Non-Structural Proteins

Alphaviruses

The data on the cleavage reactions of the precursor which generate non-structural proteins NSP1–NSP4 have principally emerged from studies in Sindbis virus and Semliki Forest virus. It is expected that the non-structural proteins in the other togavirus species fulfil identical functions in the infection cycle. Their activities play an important role in viral transcription and replication.

The NSP1 protein is a methyltransferase/guanylyltransferase involved in the formation of methylated 5′-cap structures in viral RNA molecules. Togaviruses need to encode this enzyme function, because they can not use the corresponding cellular functions that are localized in the nucleus, as their replication cycle occurs exclusively in the cytoplasm. The sequences of the NSP1 protein are highly conserved among the different togaviruses. The protein is modified with palmitic acid at cysteine residues 418–421 (in Semliki Forest virus). This determines, together with an amphipathic α-helix in the centre of the protein, the association with intracellular membrane compartments.

The amino-terminal region of the NSP2 protein displays RNA helicase activity, which is necessary for transcription and genome replication. A proteolytic activity is localized in the carboxy-terminal region and autocatalytically cleaves the precursor protein between the NSP2 and NSP3 moieties. This proteolytic processing step generates the products NSP1–NSP2 and NSP3–NSP4 (or NSP3 in Sindbis virus). Two consecutive alanine residues serve as a recognition sequence in Sindbis virus. The NSP1–NSP2 protein is further cleaved into NSP1 and NSP2 by the NSP2 protease. Whether the NSP2 protease is also involved in the processing of the other precursor protein (NSP3–NSP4), whose cleavage at the amino acid sequence alanine–tyrosine proceeds only very slowly, has not been elucidated yet.

The NSP3 protein is required for viral genome replication; however, it is not known how it works. It has a short half-life, is present in a phosphorylated form and is partially linked to intracellular membranes in the cell.

Fig. 14.18 (continued) non-structural proteins is synthesized and autocatalytically cleaved into the individual components by the intrinsic protease activity of the polyprotein. As a result, RNA-dependent RNA polymerase is generated, which uses the positive-sense RNA genome as a template and synthesizes a negative-sense RNA. This in turn serves as a template for the synthesis of both new positive-sense RNA genomes and subgenomic RNA molecules. The latter is used as mRNA for translation of the polyprotein for the structural components and which is processed into the various individual components by the activity of cellular signalases. *Appr-1* protein domain with homology to the cellular protein ADP-ribose 1-monophosphate processing enzyme, *aa* amino acids, *M/G transferase* methyltransferase/guanylyltransferase, *NSP* non-structural protein

Table 14.14 Function and size of togavirus proteins

Protein	Sindbisvirus	Semliki Forest virus	Rubella virus	Function
NSP polyprotein	2,506 aa	2,431 aa	2,116–2,205 aa 240 KDa	Precursor of non-structural proteins
NSP1	540 aa	537		Methyltransferase/ guanylyltransferase, 5′-capping enzyme; palmitoylated
NSP2	807 aa	798 aa		Protease, helicase (nucleotidase)
NSP3	549 aa	482 aa		Active during replication
NSP4	610 aa	614 aa		RNA-dependent RNA polymerase
p150	–	–	1,300/1,301 aa 150 kDa	Methyltransferase/ guanylyltransferase, 5′-capping enzyme; cysteine protease
p90	–	–	815–905 aa 90 kDa	Helicase, RNA-dependent RNA polymerase
Structural polyprotein	1,733 aa	1,739 aa	1,063 aa 110 kDa	Precursor of structural proteins
C	264 aa	267 aa	260–300 aa 33 kDa	Capsid protein, dimeric, protease
E3	64 aa	64 aa	–	Cleavage product, N-terminal domain of E2
E2	423 aa	418 aa	42–54 kDa	Glycosylated, palmitoylated, neutralization in Sindbis virus and Semliki Forest virus; haemagglutination and fusion in rubella virus
6K	55 aa	60 aa	–	Cleavage product, signal sequence at the N-terminus; ion channel protein?
E1	439 aa	438 aa	58–62 kDa	Glycosylated, palmitoylated, neutralization in rubella virus, haemagglutination and fusion in Sindbis virus and Semliki Forest virus

The protein succession order in the table corresponds to the real localization in the corresponding polyprotein
aa amino acids

The NSP4 protein is an RNA-dependent RNA polymerase. It is active in the synthesis of both negative-sense RNA molecules and genomic and subgenomic RNA species.

Rubiviruses

In the case of rubella virus, only two cleavage products are generated by proteolytic processing of the precursor of the non-structural proteins: p150 corresponds to the

amino-terminal region; p90 is equivalent to the carboxy-terminal region. The proteolytic activity of a Zn^{2+}-dependent, papain-like cysteine protease resides in the carboxy-terminal domain of p150, which cleaves the precursor polyprotein between two glycine residues at positions 1301 and 1302. The amino-terminal region of p150 is active as a methyltransferase/guanylyltransferase, which is necessary for 5′-capping of viral RNAs. The amino acid sequence between both of these enzyme domains is homologous to the cellular protein ADP-ribose 1-monophosphate processing enzyme (Appr-1). Whether this function is necessary for viral infection is still not clear. The protein p90 possesses both RNA helicase and RNA-dependent RNA polymerase activity.

14.6.3.3 Polyprotein of Structural Proteins

In all togaviruses the structural polyprotein is significantly smaller than that of the non-structural proteins. In rubella virus, it has a molecular mass of 110 kDa (Fig. 14.18b). It contains the sequences of the C, E2 and E1 proteins. In alphaviruses, between the respective protein segments there are connecting amino acid sequences which are removed during polyprotein processing and viral maturation. The synthesis of the structural polyproteins occurs in the ER membrane. Amino acid sequences that are analogous to signal peptides have been found in alphaviruses directly after the carboxy-terminal end of the C protein (i.e. at the amino terminus of p62 protein, from which E2 arises) and within the 6K protein upstream of the E1 domain (Fig. 14.18a). In rubella virus, they are located at the carboxy-terminal ends of the C and E2 proteins, respectively. These regions are responsible for the transport of the nascent polyprotein and its anchoring in the ER membrane. Membrane-associated proteases (signalases) cleave the precursor proteins after the signal-peptide-like sequences, thus leading to the generation of the individual components. In alphaviruses, an additional autocatalytic protease activity has been identified within the C protein. It contributes to the cleavage of the capsid protein from the precursor and resembles a serine protease. The cleavage occurs at the amino acid sequence tryptophan–serine. Similar functions have also been postulated for the C protein of rubella virus.

14.6.3.4 Capsid Protein (C Protein)

Depending on the type of virus, the C protein has a length of 260–300 amino acids and a molecular mass of approximately 33 kDa. After cleavage from the nascent polyprotein chain by its autoproteolytic activity in alphaviruses or by the signalase-mediated release in rubella virus, C protein dimerizes and associates with the viral RNA genome to form nucleocapsids. The RNA-binding protein domain resides between amino acid residues 28 and 56. This interaction is very strong because only very few free C proteins can be found in the cytoplasm of infected cells. After their synthesis, C proteins are subject to complex phosphorylation and dephosphorylation processes. The degree of modification seems to influence the interaction with the RNA genome: non-phosphorylated C proteins bind to the genomes much more strongly than phosphorylated C proteins. The dephosphorylation of C proteins in the late stage of the replication cycle, presumably catalysed by the cellular protein

phosphatase 1A, seems to promote the interaction with the RNA genomes and their packaging. This dephosphorylation reaction may prevent premature interactions of RNA genomes and C proteins.

14.6.3.5 Glycoprotein E2

In alphaviruses, the E2 protein is formed by cleavage of a precursor protein of molecular mass 62 kDa. This protein, p62, is anchored in the ER membrane by hydrophobic sequences in its carboxy-terminal domain, and is transported to the cell membrane by the Golgi apparatus. Thus, the carboxy-terminal end is oriented towards the cytoplasm. It possesses amino acids that specifically interact with the C proteins of the nucleocapsid. As a result, the assembly process is induced in the late phase of the infection cycle, during which the ER membrane envelops the preformed capsids. On the way to the cell surface, p62 is modified with sugar and fatty acid groups and cleaved by a trypsin-like protease into the amino-terminal E3 moiety and the E2 protein in the *trans*-Golgi region. In Sindbis virus, E3 is released from the cell surface, whereas in Semliki Forest virus, it remains associated with the E2 protein and is detectable in various amounts in the virion. In alphaviruses, the vast majority of neutralizing antibodies are directed against the E2 protein, which is present in the virion as a heterodimer with E1. Three important epitopes have been characterized.

The E2 protein (gp42–54) of rubella virus is also glycosylated, modified with fatty acids and anchored in the membrane by its carboxy-terminal amino acids. An E3 moiety, which is similar to that of alphaviruses, has not been identified in rubella virus. The E2 protein of rubella virus has haemagglutination and membrane-fusion activities, and is present predominantly as a heterodimer with E1. However, in this case, most of the neutralizing antibodies are not directed against the E2 protein, but are directed against the E1 protein.

14.6.3.6 Glycoprotein E1

In alphaviruses, a short, hydrophobic region of 55–60 amino acids is located between the carboxy terminus of the E2 protein and the beginning of the E1 sequences. It is referred to as 6K protein owing to its size of about 6 kDa. It contains signal-peptide-like sequences, which are recognized and cleaved by signalases, and mediates translocation of the amino acid chain of the E1 protein through the ER membrane during translation. It is also found in small quantities in infectious particles. However, it has also been ascertained that 6K protein has independent functions: it seems to be a pore-forming ion channel protein, which affects the membrane permeability of infected cells. It belongs to the viroporin protein family, which also includes the 7K proteins of hepaciviruses and pestiviruses (Sect. 14.5). The E1 protein, which is anchored to the membrane by a hydrophobic transmembrane region at the carboxy terminus, is glycosylated and modified by fatty acids. In alphaviruses, it appears to be associated with the haemagglutination und fusion activities. In contrast to the E2 protein, only a few E1-specific antibodies with virus-neutralizing function have been found.

In rubella virus, neutralizing antibodies are predominantly directed against the E1 protein. Two protein domains which are recognized by protecting antibodies

have been identified. The monomeric protein has a molecular mass of 58–62 kDa, it is glycosylated and is modified with palmitic acid. The E1 protein of rubella virus is responsible for attachment of the particle to cellular receptors. Mutations in the regions of the genome which code for the hydrophobic regions of the E1 protein reduce the infectivity of the virus.

14.6.4 Replication

The cellular receptors of rubella virus and most alphaviruses are not known. In Sindbis virus, laminin and the laminin receptor precursor have been identified as receptors on the surface of chicken fibroblasts, but other cellular proteins also bind the virions; in the case of rubella virus, specific phospholipids on the cell surface seem to be involved in binding the virus. The virus particles penetrate into the cells by receptor-mediated endocytosis. The interior of endocytotic vesicles (endosomes) is acidified in an energy-dependent process through the import of H^+ ions. This generates conformational changes of the viral envelope proteins which lead to the fusion of the viral envelope with the endosomal membrane, resulting in the release of the capsid. How the tight interaction of C proteins with the RNA genome is abolished is not known. However, the polarity of the positive-sense RNA allows binding of the cellular cap-binding complex via the cap structure at the 5′ terminus and the association with ribosomal subunits, which initiate translation of the sequences encoding the non-structural polyprotein. This polypeptide is synthesized on ribosomes in the cytoplasm, it associates with the ER membrane by hydrophobic amino acid sequences and the palmitoylation in the NSP1 moiety, and is cleaved by the cysteine protease present in NSP2 (alphaviruses) or p150 (rubella virus) into the individual components. As soon as the activity of the RNA-dependent RNA polymerase is present in the form of functionally active NSP4 protein, the negative-sense RNA is synthesized. In Sindbis virus, the NSP4 protein forms a complex with the uncleaved NSP polyprotein (Fig. 14.18a). The initiation occurs at the 3′ end in a highly conserved nucleotide sequence, which is located directly upstream of the poly(A) tail. In addition, nucleotides from the untranslated sequences at the 5′ terminus seem to be involved. These are partially complementary to sequences at the 3′ end and can form a partial double-stranded RNA, thus leading to circularization of the genome. Cellular proteins influence the initiation of RNA synthesis at the 3′ end: phosphorylated forms of the cellular protein calreticulin bind to the 3′ end of the rubella virus genome. The details of initiation of RNA synthesis are unknown; however, it is clear that in the further course of the replication process an RNA product which is complementary to the whole genome is generated. This process occurs on the ER membrane.

From the negative-sense RNA, new full-length complementary RNA genomes and an additional subgenomic RNA molecule are synthesized. The latter is initiated at the transition region between the two reading frames. It contains the sequences encoding the structural polyprotein. RNA synthesis is catalysed by the RNA-dependent RNA polymerase of the NSP4 protein and the other cleavage products

of the non-structural proteins. The subgenomic RNA is capped and methylated at the 5′ end, thus facilitating translation of the structural proteins. It is also referred to as 26S RNA according to its sedimentation behaviour – in contrast to the genomic 49S RNA (Fig. 14.18). In infected cells, many more subgenomic RNA molecules than genomic RNA molecules are synthesized. Similar replication processes that lead to the synthesis of a subgenomic mRNA have also been found in astroviruses, caliciviruses and hepeviruses (Sects. 14.2–14.4).

If sufficient amounts of dephosphorylated C proteins are present, they associate with nucleotide sequences in the 5′ region of the newly synthesized genomic positive-sense 49S RNA molecules and assemble into nucleocapsid precursors. These initial packaging steps also prevent interaction with the cap-binding complex and ribosomal subunits and that genomic RNA is translated, thus interrupting the synthesis of further NSP polyproteins. This relatively simple regulatory mechanism ensures that viral structural components are preferentially produced in the late phase of infection because they are required in much larger quantities for the formation of virus particles than the enzymatically active non-structural proteins at this time.

Subsequently, the preformed nucleocapsids associate with the carboxy-terminal regions of E2 proteins, and are then surrounded by the membrane and the viral glycoproteins embedded in it. These budding complexes can arise on the membranes of the ER and the Golgi apparatus as well as on the plasma membrane. The enveloped virions are either transported through Golgi vesicles to the cell surface or directly released there. During infection, apoptosis is also induced in the cells. This process, which results in cell death, has been found particularly in infections with rubella virus, whose C proteins also act proapoptotically.

14.6.5 Human Pathogenic Togaviruses

14.6.5.1 Rubella Virus

Epidemiology and Transmission

Rubella (German measles), which was frequently an epidemic disease, was described in detail by the German physician George de Maton in 1814. The virus was transmitted by ultrafiltrates to humans and monkeys for the first time in 1938. During an epidemic in Australia in 1940, the ophthalmologist Sir Norman Gregg discovered that the mothers of children with congenital cataracts, hearing loss and heart malformations (Gregg syndrome) had experienced a rubella infection during pregnancy (Table 14.15). Therefore, the virus causes not only the harmless rubella, but also severe embryopathies. The virus was cultivated for the first time in 1962. It generates a cytopathic effect in kidney cell lines of rabbits (RK-13) or monkeys (Vero cells). After a major epidemic in the USA in 1964, an attenuated live vaccine was developed in 1967. Its application led to the result that rubella occurs only very rarely today; since 2001, it has been considered as eradicated in the USA. Acute rubella infections are also rare in Europe owing to vaccination; there are estimated to be 150–400 cases per year in Germany, for example.

Table 14.15 Rubella embryopathy and its symptoms

Syndrome	Organ	Symptoms
Gregg syndrome	Heart	Persistent duct of Botallo Aortic stenosis
	Eyes	Cataract Glaucoma Retionopathy
	Ears	Inner ear defects
Expanded rubella syndrome		Mental retardation Low birth weight Dwarfism, osteopathy Encephalitis Hepatosplenomegaly Pneumonia Thrombocytopenia Purpura
Late rubella syndrome		Chronic exanthem Growth arrest Interstitial pneumonia IgG and IgA Hypogammaglobulinaemia Persistence of IgM
Late manifestation		Ear lesions Diabetes mellitus Progressive panencephalitis Convulsion disorder

The rubella virus is serologically uniform. With respect to its nucleic acid sequences, all isolates can be classified into two strains (clades 1 and 2), which can also be subdivided into ten genotypes (1a–1g, 2a–2c); the rubella virus of clade 2 has been isolated only in the Eurasian region. Rubella virus is found only in humans, but can be transmitted to some monkey species. Transmissions occur by droplet infection, and lead to infections during fleeting contact in about 20 % of cases. Carriers are infected individuals in the viraemic phase, which starts just 6 days before the onset of the exanthem and lasts 1–2 weeks (Fig. 14.19), infected infants who were born with congential rubella syndrome, and rarely adults with asymptomatic reinfections, who, nevertheless, can still transmit virus. Viruses are also found in the lacrimal fluid, in urine, in cervical secretions, in the stool, in the lungs, in cerebrospinal fluid and in synovial fluid.

Clinical Features

Postnatal Infections

Rubella is a relatively harmless, not very febrile illness. About half of infections are asymptomatic in children. The symptoms associated with the disease, mottled and not confluent exanthem, appear approximately 1–2 weeks after contact with the rubella virus and remain for up to 5 days (Fig. 14.19). The disease is often uncharacteristic and therefore indistinguishable from other viral diseases. Cold-like symptoms are absent; swollen neck lymph nodes are frequently observed. Especially in young women, the infection is associated in part with arthralgia of

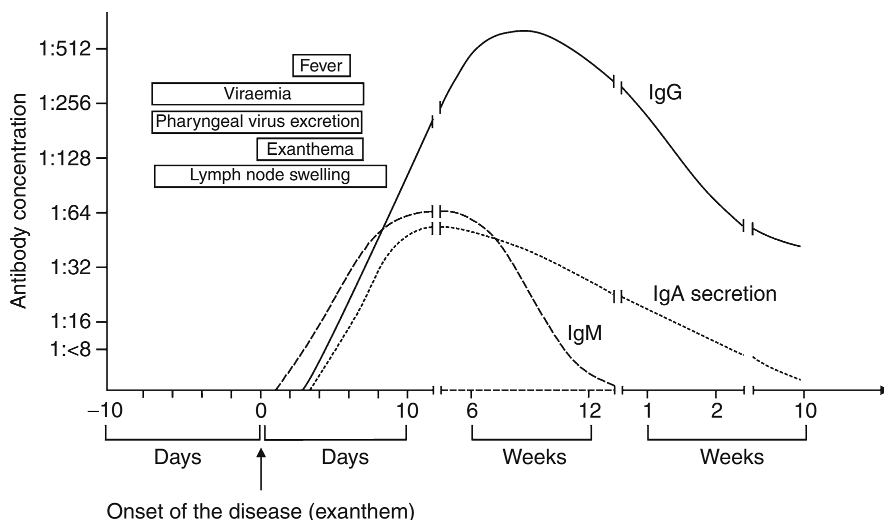


Fig. 14.19 Time course of antibody formation during rubella virus infection. Day 0 on the scale indicates the time at which the first appearance of the exanthem is observed. The incubation period lasts up to 10 days. As early as before the appearance of the exanthem, swollen lymph nodes are found, and rubella virus is detectable in the blood and pharyngeal lavage. Immediately after the onset of symptoms, IgM can be detected in the blood; its concentration decreases within 3–6 months. IgG antibodies appear after IgM antibodies and remain detectable for life

small joints, which usually subsides within a few weeks. A thrombocytopenia is rarely observed and its pathogenesis is still unclear. Encephalitis occurs with an incidence of 1:6,000. About 20 % of rubella infections with postinfectious encephalitis are fatal.

Prenatal Infections

If infections with rubella virus occur during the first trimester of pregnancy, abortion, stillbirth and malformations of the embryo can occur. By contrast, the mother shows no or only mild symptoms. A large number of embryos are particularly damaged during the first 20 weeks of pregnancy, the time of organ differentiation. Multiple defects occur primarily after an infection in the first 2 months of gestation; however, the complications are reduced to nearly zero after the third month of pregnancy. During the viraemic phase, the placenta is infected in 80–90 % of cases, whereas the embryo is infected with a probability of 60–70 %. Cardinal symptoms include eye damage, heart defects and inner-ear defects. Hearing defects, panencephalitis, diabetes and epilepsy or convulsion disorder are observed as long-term effects (Table 14.15).

Pathogenesis

The virus is transmitted by droplet infections. It penetrates into the organism through the mucous membranes of the upper respiratory tract. The primary replication occurs in the epithelium of this region. There, macrophages and lymphocytes

are infected, and carry the virus to local lymph nodes. There, the virus finds further target cells, where it replicates. Subsequently, the lymph nodes become extremely swollen. The viraemia probably originates from the infected lymph nodes, and rubella viruses are present in the blood (as free particles and in cell-bound form). They spread throughout the organism and can be detected in lacrimal fluid, in the nasal–pharyngeal region, in cervical secretions, in cerebrospinal fluid and in synovial fluid. The exanthem appears together with the first virus-specific antibodies. Immune complexes, i.e. antibody–virus complexes, are responsible for this response. They attach to the endothelium of blood capillaries in the skin and cause local inflammations.

Even the acute arthritis which is associated with the infection has been attributed to virus–antibody complexes that are present in synovial fluid. In cells of the synovial membranes, IL-1 is synthesized in large quantities, an indication that inflammation processes are occurring. Evidence that rubella virus can proliferate and persist in synovial cells has been found in infants with congenital rubella syndrome, i.e. in children who were infected during the embryonic stage. In such infants, rubella virus can be detected in the growth areas of the bones, the epiphyses and diaphyses. It is presumed that in these children persistent viruses trigger the production of interferons, which inhibit the proliferation of bone cells, resulting in retardation of growth of the extremities. In the rare cases of postinfectious encephalitis, viral proteins can occasionally be detected in brain tissue. It is suspected that a cellular autoimmune response is triggered against basic myelin protein in the spinal cord and nerve sheaths because lymphocytes of such patients proliferate after addition of this protein.

In infections during pregnancy, the viruses are transported into the placenta and the chorionic villi by blood. They proliferate there, invade the endothelium of placental blood vessels and reach the circulatory system of the child. The infected and detached endothelial cells form so-called emboli, which spread the virus in the organism. After birth, production and excretion of the virus last a long time (up to 1 year). How injury to the differentiating organs and impairment of embryonic cell division is achieved is unknown. It is thought that interferons and possibly other cytokines with cytotoxic properties or apoptosis mechanisms may play an important role.

Immune Response and Diagnosis

Postnatal Rubella

During infection, IgM, IgA and IgG antibodies are produced against the viral E1, E2 and C proteins. E1-specific immunoglobulins are neutralizing. Antibodies are detected by haemagglutination-inhibition tests or ELISA. Virus-specific IgM is detectable for about 4–6 months after infection; IgG is detectable lifelong (Fig. 14.19). Acute rubella infections can be diagnosed by the common presence of virus-specific IgM and IgG as well as by detection of viral RNA by PCR. The sole presence of IgG antibodies indicates a previous infection. In immune individuals, cytotoxic T cells can be detected, and these preferentially recognize epitopes of the C protein.

Prenatal Rubella

Embryonal infections can be diagnosed early by detecting viral RNA by PCR analyses of chorionic villi samples. Virus-specific IgM cannot be detected in umbilical cord blood before the 22nd and 23rd weeks of pregnancy. However, the mere detection of IgM and viral RNA does not provide evidence for whether there is an embryopathy. Rubella-virus-specific IgM antibodies and viral RNA remain detectable after birth for a long time. A peripheral tolerance, possibly in combination with a disturbance in switching the synthesis of antibody classes from IgM to IgG, may be responsible for the persistence of rubella infection. The stimulation of lymphocytes by viral proteins is significantly reduced.

Prenatal rubella is a medical indication for abortion. Because of the currently applicable regulations for maternity guidelines in many countries, immunity against rubella must be examined and documented in the maternity record book. In the case of suspected rubella exposure of a non-immune pregnant woman, the antibody titre has to be determined. If IgG antibodies are present at levels of 10 IU/mL and above, the patient is protected against the infection and there is no danger of damage to the embryo. If the level is below 10 IU/mL, and there is also no clear reactivity in alternative tests, rubella-virus-specific IgG should be administered within 3 days after exposure because a fresh infection is possible. Subsequently, it must be ascertained by further antibody tests whether an infection is actually ongoing or has finished in the pregnant woman, and whether the embryo has been infected. A further complication is that IgM reactivity in the ELISA may be unspecific during gestation. If rubella-virus-specific IgM antibodies are present in the umbilical cord blood and viral RNA is detected by PCR, this is an indication for termination of pregnancy because of the high risk of possible harm to the unborn child by the infection. The retrospective determination of the probable date of infection of the mother is of crucial importance since the risk of embryopathy decreases significantly in the course of the first trimester of pregnancy. This includes immunoblot analysis of avidity for and reactivity against individual proteins.

Therapy and Prophylaxis

There is an attenuated live vaccine against rubella virus infections. Strain RA 27/3 has been used since 1979. This vaccine induces high antibody titres, and its application has led to a dramatic decrease in the incidence of rubella in Europe and North America, where it occurs only sporadically. Today, the rubella vaccine is administered as a combined vaccine with attenuated live vaccine strains against measles, mumps and varicella at the beginning of the second year of life; a two-time application is considered as protective. Since the introduction of vaccination, both the number of infections and the number of congenital rubella embryopathies have substantially decreased. In most countries both congenital and acute postnatal rubella are notifiable.

In cases of proven exposure of non-immune pregnant women to rubella virus, a passive immunization with virus-specific IgG may be performed. The protective effect is the greater, the sooner the passive immunization is performed; even 3 days

after exposure, there is only a slight protection. The use of the live vaccine is not allowed during pregnancy. An antiviral chemotherapy does not exist.

Chikungunya Virus Has Recently Caused Human Diseases in Europe

Chikungunya virus was isolated from a patient with a febrile illness in the former Tanganyika (now part of Tanzania) in 1953. Infections with this virus have likely been noticed since the late eighteenth century but incorrectly documented as epidemic outbreaks of dengue fever. Between 1960 and 2003, chikungunya virus was repeatedly ascertained to be the cause of regionally restricted epidemics in the countries of eastern, southern and western Africa as well as in Southeast Asia (among others, in India, Pakistan, Malaysia, Thailand, Indonesia and Vietnam). Since 2004, the virus has repeatedly been found also in patients in different European countries, particularly in Italy, but also in Germany, Belgium, France, Spain, the UK and Norway. Infections induce severe fever, arthralgia and rash, and have a high morbidity. In particular, joint swelling and inflammations can last for months – they gave the virus its name: *chikungunya* means "that which bends up". The natural hosts and reservoirs for the pathogens are non-human primates in Africa and Southeast Asia. The virus is transmitted to a number of different *Aedes* mosquito species, which further spread it to and also between humans. The tiger mosquito (*A. albopictus*) is considered to be particularly responsible for the chikungunya virus infections that have been observed in Europe in the last few years, as these mosquitoes can also exist in subtropical and cooler regions. Presumably the virus was originally introduced to European countries by tourists or mosquitoes, resulting in local outbreaks. In particular, further warming of the climate could have the effect that the *Aedes* mosquitoes increasingly feel at home in Europe, which could thus lead to increased infections with chikungunya virus.

14.6.6 Animal Pathogenic Togaviruses

14.6.6.1 The Various Equine Encephalitis Viruses

Epidemiology and Transmission

The genus *Alphavirus* encompasses pathogens that are transmitted by arthropods, essentially by mosquitoes of the genera *Culex* and *Aedes*, and can cause various diseases in humans and animals. In addition to the neurotropic alphaviruses that can cause encephalitis, in particular the different types of equine encephalitis viruses and some other alphaviruses of the New World, alphaviruses play a role in Europe, Africa and Asia, and usually provoke mild symptoms or moderate arthritis.

Besides their significance as zoonotic pathogens, equine encephalitis viruses are also important because of their epidemiology and molecular evolution. All have an enzootic cycle that includes small rodents and birds. They are transmitted between

their hosts by mosquitoes, and the host specificity of the mosquitoes determines the nature of the infection. Horses and humans are merely accidental hosts from which the virus cannot spread further. Only certain epizootic subtypes of Venezuelan equine encephalitis virus are able to cause viraemia in horses or humans, during which they proliferate in the large quantities that are necessary to infect a sucking mosquito. Infection of horses and humans contributes to dissemination of the viruses solely in such cases.

Eastern equine encephalitis virus is endemic in the southern USA and in many countries of South America. It asymptomatically infects different bird species, including various songbirds and wading birds. In other introduced bird species such as pheasant and emu, it causes lethal infections and severe economic losses. Certain mosquitoes that suck blood only in birds, particularly *Culiseta melanura*, maintain this endemic bird cycle. If blood of infected birds is sucked by other mosquito species which can also bite mammals and humans, they can cause epidemics which include mammals as hosts. In regions with a temperate climate, there is a seasonal accumulation of diseases in the late summer, whereas in tropical climates, infections and transmissions of the reservoir hosts occur throughout the year.

Western equine encephalitis virus is also widespread on the American continent, and is transmitted by various mosquito species. Whereas in North America the enzootic cycle is largely maintained by infection of various songbirds, in South America, especially rodents play a prominent role as a reservoir. The infection of horses and humans is also accidental.

Unlike the eastern and western equine encephalitis viruses, the switch from an enzootic to an epizootic cycle in Venezuelan equine encephalitis virus depends on a mutation in the coding sequences for the E2 protein of the prevalent enzootic virus. Whereas the enzootic subtypes I-D to I-F and II–VI induce only limited infections in small rodents and are transmitted by mosquitoes of the genus *Culex*, the transmission of the epizootic subtypes I-AB and I-C is performed by mosquitoes of the genera *Aedes* and *Psoropha* and involves the infection of a variety of mammals, including horses and humans. The pathogenetic basis for the epizootic types lies in their ability to cause a high-titre viraemia in their hosts and to facilitate transmission. Phylogenetic studies have revealed that the epizootic subtypes possibly arise from enzootic viruses before each epizootic outbreak. Thus, the epizootic biotype can be generated by replacement of the E2 gene from an enzootic I-D virus by that of an I-AB virus.

In addition to the epizootiology of these viruses, their evolution is also interesting. It has been shown that western equine encephalitis virus emerged from a recombination between eastern equine encephalitis virus and a similar precursor virus with similarity to Sindbis virus (▶ Chap. 12). This recombination resulted in the replacement of the genes encoding the glycoproteins of eastern equine encephalitis virus by those of the Sindbis-like virus.

Clinical Features

Infections with the alphaviruses that are widespread in eastern Europe, Asia and Africa are often asymptomatic (Semliki Forest virus) or are associated with slight

fever, rash and arthritis (Sindbis virus). The American equine encephalitis viruses, however, frequently induce encephalitis in the infected organism.

Pathogenesis

Alphaviruses are transmitted by insect bites directly into the bloodstream and attach, using envelope proteins, to unknown receptors on endothelial and lymphatic cells. They then proliferate in these cells. They are transported through the bloodstream to other target organs. The damaged endothelium allows the transfer of the virus into the central nervous system, where it proliferates in neurons. The mechanisms of neuronal injury by induction of apoptosis have been studied in detail in Sindbis virus infections in mice. Sindbis virus shows an age-dependent pathogenicity in mice. Newborn mice become lethally ill after an intracerebral infection, whereas mice do not become sick at the age of 4 weeks and eliminate the virus 1 week after inoculation. The virus is eliminated by virus-specific antibodies. This phenomenon is based on the fact that the viral infection induces apoptosis in immature neurons, whereas mature neurons can prevent this process by an unknown factor. However, the resistance is not absolute and can be broken by extremely virulent virus strains. Obviously, the viral glycoprotein E2 is of extraordinary importance: the study of mutant viruses that were produced by site-directed mutagenesis revealed a special role in virulence of the amino acid histidine at position 55.

Immune Response and Diagnosis

Equine encephalitis virus infections lead to a stable, long-lasting immunity. The diagnosis can be ascertained by the direct detection of the virus in the blood during the viraemic phase, or after death in brain tissue. Indirect evidence of infection by the examination of serum pairs that are obtained at the time of acute infection and after a further 3 weeks is commonly used. The antibody titres are determined in haemagglutination-inhibition or in virus-neutralization tests.

Control and Prophylaxis

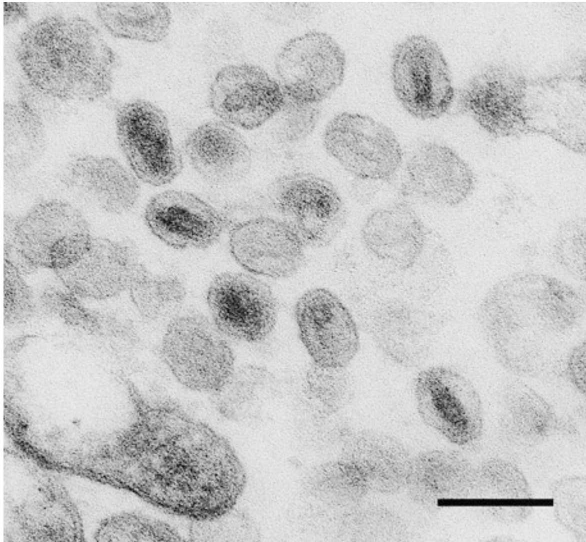
Vaccines are available against infections with all members of equine encephalitis viruses, and are used in humans, horses and birds such as the threatened whooping crane (*Grus americana*). They are based on inactivated viruses that were propagated in cell culture. A consequent vaccination can prevent the emergence of epizootic diseases, or favourably influence its development after an outbreak.

Enzootic and Epizootic Cycles Determine the Epidemiology of Equine Encephalitis Viruses

Eastern equine encephalitis virus, western equine encephalitis virus and Venezuelan equine encephalitis virus are arboviruses (*ar*thropod-*bo*rne), which usually replicate asymptomatically in their hosts, especially songbirds and rodents, and establish a sufficiently high viraemia to be transmitted by mosquitoes among these hosts. This is referred to as an enzootic cycle.

Various factors such as a massive proliferation of not strictly host-specific mosquito species, which serve as reservoir for the virus, can lead to sucking of other species, thus contributing to the transmission to humans, horses and other mammals. This is referred to as an epizootic cycle.

14.7 Arteriviruses



Arteriviruses constitute along with coronaviruses (Sect. 14.8) and roniviruses the order *Nidovirales*. This classification is based on the genome organization, on the use of polycistronic mRNA transcripts for viral gene expression and on the transcription and translation strategies, i.e. on features and processes which are similar in the members of these virus families. The name of the order has its origin in the Latin word *nidus* meaning "nest". It refers to the unique transcriptional strategy of the members of the order *Nidovirales*. During mRNA synthesis, transcripts are synthesized with the same 5′ and 3′ termini, but in which the 5′ terminal sequence region is combined with different RNA segments that are localized farther downstream; therefore, sets of polycistronic (nested) mRNA molecules are generated. On the other hand, the differences in the size of the viral genomes and particles as well as in the sequence and nature of the structural proteins are so clear that they have been classified into separate families. Compared with the particles and the genomes of coronaviruses and roniviruses, those of arteriviruses are much smaller. Members of the Roniviridae and the Mesoniviridae, a recently designed virus family, infect only invertebrates and insects; therefore, they will not be discussed in more detail in this book.

Table 14.16 Characteristic prototypes of arteriviruses

Genus	Animal virus
Arterivirus	Equine arteritis virus Lactate dehydrogenase elevating virus of mice Porcine reproductive and respiratory syndrome virus Simian haemorrhagic fever virus

14.7.1 Classification and Characteristic Prototypes

In the family of arteriviruses, which comprises exclusively animal pathogens, there is only one genus (Table 14.16). The characteristic prototype is equine arteritis virus, which induces persistent asymptomatic infections in horses and donkeys; however, it can also cause miscarriages or haemorrhagic fever in these animals. Lactate dehydrogenase elevating virus (LDV) and simian haemorrhagic fever virus infect mice and various African and Asian monkeys, respectively. These pathogens were described for the first time in the nineteenth century. By contrast, infections of swine with porcine reproductive and respiratory syndrome virus (PRRSV) occurred for the first time in Europe and the USA nearly simultaneously between 1983 and 1988.

14.7.2 Structure

14.7.2.1 Virus Particle

The infectious particles of arteriviruses have a diameter of 40–60 nm and consist of spherical or icosahedral nucleocapsids (diameter 25–35 nm), which are surrounded by an envelope (Fig. 14.20). In contrast to coronaviruses, arteriviruses possess envelope proteins protruding only slightly from the surface of the particle (10–14 nm). Four viral glycoproteins are inserted in the envelope: GP_2, GP_3, GP_4 and GP_5; in the case of LDV, GP_2, GP_3, GP_4 and GP_7. In addition, the M and E proteins are associated with the viral membrane. The nucleocapsid inside the envelope is composed of N proteins and the single-stranded RNA genome.

14.7.2.2 Genome Organization and Structure

The genomes of arteriviruses have an organization similar to that of coronaviruses; however, the succession of coding regions is much more densely packed than in the latter. Therefore, they are shorter and comprise between 12,704 nucleotides in equine arteritis virus and approximately 15,000 and 15,700 nucleotides in PRRSV and simian haemorrhagic fever virus, respectively. They consist of single-stranded, positive-sense RNA containing a methylated cap structure at the 5′ terminus and a poly(A) tail the 3′ end. The coding regions of the genome are flanked by 156–221 and 59–117 untranslated nucleotides at the 5′ and 3′ termini, respectively. Two large reading frames (1a and 1b), of which 1a begins at the 5′ terminus, have overlapping ends and cover approximately two thirds of

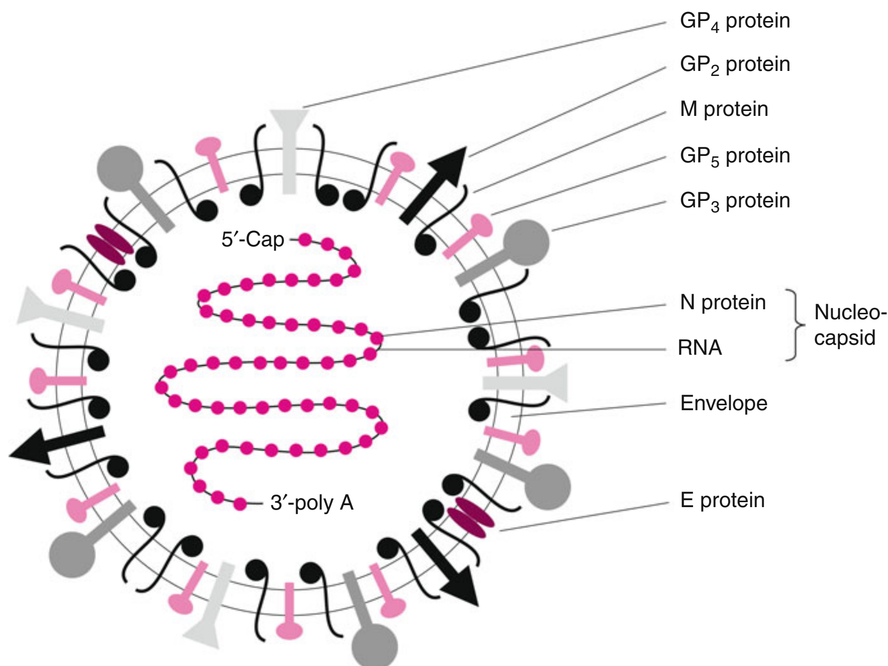


Fig. 14.20 Structure of an arterivirus particle represented by porcine reproductive and respiratory syndrome virus. Inside the particle, the RNA genome is associated with N proteins in a helical nucleocapsid, which is enclosed by a membrane envelope, in which four viral glycoproteins (GP_2–GP_5) and the non-glycosylated M protein are embedded

the genome (Fig. 14.21). They contain the genetic information for the synthesis of two polyproteins (1a and 1ab), from which the non-structural proteins are generated by proteolytic cleavage. A ribosomal frameshift event during translation leads to skipping the stop codon at the end of reading frame 1a, resulting in the synthesis of the larger protein 1ab. Autocatalytic cleavage leads to the generation of three proteases (NSP1, NSP2, NSP4), RNA-dependent RNA polymerase, a helicase and several other non-structural proteins of unknown function. Reading frames 2–7 (or 9 in PRRSV) encoding the structural proteins are located downstream of the genes of non-structural proteins. Towards the 3′ end, they encode the different glycoproteins and the M and N proteins; the E protein is encoded by an internal reading frame located on the second longest mRNA.

14.7.3 Viral Proteins

14.7.3.1 Non-Structural Proteins

Two major precursor products of the non-structural proteins are synthesized during translation of the genomic mRNA: protein 1a has a molecular mass of 187–260 kDa

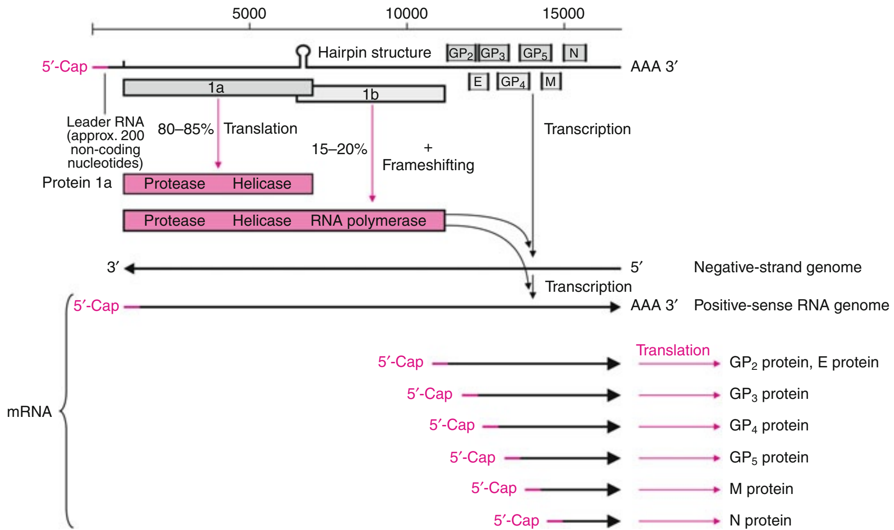


Fig. 14.21 Genome organization and replication in arteriviruses. The viral RNA genome functions as mRNA, and is translated in the cytoplasm. The two overlapping reading frames encode non-structural polyproteins 1a and 1ab. A hairpin structure induces a ribosomal frameshift during translation that leads in approximately 15–20 % of all translation processes to the synthesis of non-structural polyprotein 1ab, which contains the RNA-dependent RNA polymerase domain in its carboxy-terminal region. Both polyproteins are cleaved by the autocatalytic activity of two cysteine proteases and a serine protease that are located in the amino-terminal regions of the precursor proteins. The RNA-dependent RNA polymerase transcribes the positive-sense RNA genome into a negative-sense RNA strand. This serves both as a template for the synthesis of new positive-sense RNA genomes and for transcription of a series of subgenomic mRNA species, which are modified at the 5′ end with a cap group and contain identical leader sequences in all mRNA molecules. The structural proteins are translated from the different subgenomic mRNAs, whose reading frames are localized in the 3′-terminal third of the positive-sense RNA genome. They partially overlap

among the different members of arteriviruses, the amino-terminal half of protein 1ab (345–421 kDa) is identical to that of protein 1a. The three proteases (NSP1, NSP2, NSP4) that reside in the amino-terminal region of both proteins cleave the precursor proteins into a total of 12 non-structural proteins (NSP1–NSP12), whose functions are in some cases not resolved.

The NSP1 and NSP2 proteins are cysteine proteases which are autocatalytically cleaved from the polyproteins (Table 14.17). NSP4 is a serine protease and is similar to the 3C protease of picornaviruses (Sect. 14.1). Its activity is required for subsequent processing of the precursor proteins 1a and 1ab at up to eight cleavage sites. This proteolytic processing generates, among others, RNA-dependent RNA polymerase, metal ion binding protein MP and a helicase (NSP10). The latter has a Zn^{2+} ion binding domain, which is needed for the synthesis of viral mRNAs, but is not necessary for genome replication. NSP11 constitutes the endoribonuclease

Table 14.17 Functions and properties of arterivirus proteins

Protein	Size (kDa)	Properties	Function
GP_5/GP_7 (LDV)	24–44	Glycosylated	Membrane protein; major structural protein neutralizing antibodies, forms heterodimers with M protein, apoptosis induction?
GP_2	20–35	Glycosylated	Membrane protein, minor structural protein, heterotrimer with GP_3 and GP_4
GP_3	27	Glycosylated	Membrane protein; minor structural protein, heterotrimer with GP_2 und GP_4
GP_4	20	Glycosylated	Membrane protein; minor structural protein, heterotrimer with GP_2 und GP_3
M	16–20	–	Membrane protein; major structural protein, forms heterodimers with GP_5 and GP_7
N	12–15	Phosphorylated	Nucleocapsid protein; major structural protein, homodimer
E	7–8	Very hydrophobic	Ion channel protein? Homooligomers
NSP1	29		Papain-like cysteine protease
NSP2	61		Cysteine protease
NSP4	21		Serine protease, homology to chymotrypsin and 3C proteases of picornaviruses, main protease
NSP9	?		RNA-dependent RNA polymerase
NSP10	?		RNA helicase
NSP11	?		Endoribonuclease (NendoU)
MP	?	Zn^{2+}-binding	?

(nidoviral uridylate-specific endoribonuclease, NendoU) which is present in all members of the order *Nidovirales*. Its activity seems to be especially necessary for the synthesis of subgenomic RNA molecules. In most cases, the functional assignments have been made by comparative sequence analysis and not by direct purification and biochemical characterization of protein activities.

XendoU and NendoU

The cellular endoribonuclease XendoU, originally discovered in *Xenopus laevis*, is responsible for processing of nucleic RNA species and belongs to a small protein family, into which the nidoviral endoribonuclease NendoU is also classified. The enzymatic activity of NendoU is dependent on Mn^{2+} ions. It preferentially cleaves double-stranded RNA before or after uridine residues in the sequences GUU and GU, generating molecules with 2′-3′-cyclic phosphate ends. Furthermore, NendoU enzymes, which have only been found in nidoviruses so far, seem to possess some additional, but not yet characterized activities.

14.7.3.2 Structural Proteins

The structural proteins of arteriviruses can be subdivided into major and minor proteins. Major proteins are GP_5 (GP_7 in LDV), M and N. The other group includes GP_2, GP_3, GP_4 and E proteins. The function of the structural E protein has not been definitively resolved, but it is essential for infectivity, and there are indications that it might be an ion channel protein. The glycoproteins are present in complex arrangements, GP_5/GP_7 and M proteins form heterodimers, whereas GP_2, GP_3 and GP_4 form heterotrimers. In addition, intermediates of the GP_2 and GP_4 heterodimers can be observed, and associate with GP_3 by cysteine bonds in the subsequent step. This complex formation with GP_3 is a prerequisite for their installation in the viral envelope. The neutralizing epitopes are located primarily in the GP_5 or GP_7 proteins. However, the presence of heterodimeric structures with the M protein seems to be essential for their correct conformation.

The M protein is the structural protein of arteriviruses with the highest degree of conservation. It resembles the M protein of coronaviruses. The carboxy-terminal domain is located inside the particle, whereas the short amino-terminal region is localized on the viral surface and is flanked by hydrophobic sequences that anchor the protein in the envelope. Through a cysteine residue in the amino-terminal domain, the M protein forms a disulphide bond with glycoprotein GP_5, or GP_7 in LDV.

The phosphorylated N protein (12–15 kDa) is bound to the RNA genome and forms the nucleocapsid.

14.7.4 Replication

Arteriviruses preferably infect macrophages and gain access to the cell through receptor-mediated endocytosis. The receptors used by the virus for attachment are not definitively known. PRRSV appears to interact with heparan sulphate on the surface of macrophages. In addition, it has also been found that the virus binds to CD163 and to the amino-terminal variable immunoglobulin-like domain of sialoadhesin, a member of the immunoglobulin superfamily on the surface of macrophages. Which viral glycoprotein mediates this interaction is not clear. The replication cycle is very similar to that of coronavirus; also in arteriviruses, all replication steps occur in the cytoplasm of the cell. As already mentioned, non-structural polyproteins 1a and 1ab are primarily translated from the genomic RNA by induction of a ribosomal frameshifting mechanism. Protein 1ab comprises the RNA-dependent RNA polymerase (Fig. 14.21).

In the next step, the complementary strand is synthesized by the polymerase activity of the enzyme using the genomic RNA as a template. It encompasses the entire genome and has a negative polarity. Later, it has two functions in the replication cycle: it serves as a template for the synthesis of new viral genomes and for the production of subgenomic mRNA species, from which the various structural proteins of arteriviruses are translated. All subgenomic mRNAs have

the same 5′ and 3′ termini, which correspond to those of the viral genome. At the 5′ ends there is a uniform sequence, the leader RNA. It is capped at the 5′ terminus and corresponds to the sequences in the 5′ UTR of the genome. The leader sequence serves as a primer for the synthesis of subgenomic mRNA species. Near its 3′ end, the leader RNA exhibits a conserved sequence (UCAAC in equine arteritis virus). Sequences complementary to this motif are found in the negative-sense RNA at different regions. They are located upstream of the different initiation sites for the synthesis of subgenomic mRNA species in the region of the genome between the end of the reading frame encoding polyprotein 1ab and the 3′ end of the genome: they can hybridize with the leader RNA, thus providing a small double-stranded region with a free 3′-OH end to continue polymerization. As in coronaviruses, the RNA polymerase can itself probably not initiate the synthesis of subgenomic mRNA species at the different sites. Only the reading frames adjacent to the 5′ end of these nested transcripts are usually translated into proteins; transcription of an internal reading frame has only been observed in the case of the E protein.

The N protein interacts with genomic RNA to form the nucleocapsids and binds to the carboxy-terminal domain of the M protein, which is embedded in the ER membrane. This interaction triggers the budding process, during which the nucleocapsid is surrounded by the membrane containing M protein and glycoproteins. The particles formed are released into the ER lumen, and are further transported via Golgi vesicles to the cell surface, where they are released into the environment.

14.7.5 Animal Pathogenic Arteriviruses

No arteriviruses that are able to infect or to cause disease in humans are known. The most important animal pathogenic arteriviruses include equine arteritis virus and PRRSV.

14.7.5.1 Equine Arteritis Virus

Epidemiology and Transmission

Equine arteritis virus has a worldwide distribution. It induces a well-known disorder that is now known as equine arteritis. It is manifested in oedema of the head and extremities. This manifestation of the disease has also led to the synonyms “pink eye” and “equine influenza”. An economically important form of the disease is abortion in pregnant mares, which can occur epidemically in stud farms.

Only horses seem to be susceptible to the infection. The virus is transmitted by direct contact and through aerosols. The infection occurs more frequently at tournaments, fairs or exhibitions, where many horses from different regions come together. The virus can cause a persistent infection; some stallions excrete viruses in the ejaculate for many years. This is epidemiologically very important and constitutes an animal health problem because the virus can be spread in this way among the population even when artificial insemination is used.

Clinical Features

After an incubation period of about 3 days to 2 weeks, the animals develop fever and oedemas in the head, limbs and abdomen (preputial and scrotal oedema in stallions), as well as conjunctivitis. The disease is normally transient, and deaths are rarely observed. In pregnant mares, abortion can occur 10–30 days after infection. In a seronegative, and therefore susceptible livestock population, up to 80 % of gestating mares can abort ("abortion storms"). Temporary infertility can also be observed in stallions as a complication of equine arteritis virus infections. However, the infection usually develops subclinically and diseases are rarely observed.

Pathogenesis

The main target cells of the virus are macrophages and endothelial cells. This tropism explains both the symptoms and the pathological aspects of the disease. With the exception of the central nervous system, the virus can very quickly reach virtually every organ system, where it induces vascular perturbations and pathological alterations. Local infarctions and bruises (oedema) are combined with systemic hypovolaemic symptoms. Intrauterine transmission leads to an infection of the fetus, which also dies, and is aborted owing to generalized oedemas. Abortion is habitually associated with the complete detachment of the placenta.

Immune Response and Diagnosis

The diagnosis can be made by neutralization tests. The examination of serum pairs allows a retrospective diagnosis of acute infections. Detection of the virus is achieved by cultivation of the pathogen in equine cell lines or by using RT-PCR.

Control and Prophylaxis

In several countries, vaccines based on both inactivated and attenuated viruses have been licensed. Both vaccines have proven to be basically effective.

14.7.5.2 Porcine Reproductive and Respiratory Syndrome Virus

Epidemiology and Transmission

PRRSV has only been known since around 1985, when it emerged almost simultaneously in Europe and North America. Sequence analyses of the first viruses isolated on both continents revealed a sequence identity of only about 60 %. The isolates are referred to as genotypes owing to that divergence. Genotype I includes the isolates of the European type, whereas genotype II includes those of the North American type. The origin of these genotypes is uncertain. One hypothesis assumes a host switch from mice to swine which occurred independently in Europe and North America. Alternatively, it is also discussed that wild boars might have been infected by mice with an LDV-like virus in Europe, and were then transported from Europe to North America. Inasmuch as PRRSV is considered as the virus with the

highest known mutation rate, it is quite conceivable that it might have developed in separated swine populations into the known genotypes.

The virus seems to infect only swine and is transmitted by direct contact between animals. Epidemiologically important are clinically healthy swine with persistent PRRSV infection which spread the virus in susceptible herds. The complete viral contamination occurs within a few weeks.

Clinical Features

PRRSV infections cause a disease in pigs which is very similar to equine arteritis. Typical symptoms include fertility problems, which are principally manifested as late abortions after a gestation period of more than 110 days (the normal gestation period in pigs is115 days). In addition, dead and mummified fetuses can also be aborted. In contrast to infection with porcine parvoviruses (▶ Sect. 20.1.6), pathological changes can be observed in aborting sows. These include endometritis and myometritis, frequently accompanied by haemorrhagic placenta. In non-pregnant animals, the infection is present as a febrile systemic disease that is often accompanied by respiratory symptoms. A classic symptom is an abnormal blue discoloration of the ears, the snout and the vulva owing to reduced blood flow.

Pathogenesis

The target cells of the virus are macrophages and endothelial cells. The virus persists in macrophages despite the presence of neutralizing antibodies. These antibodies may contribute to the pathogenesis of the disease by an antibody-dependent cytotoxicity (▶ Chap. 7). There are indications that PRRSV prevents the induction of IFN-β: it inactivates IFN-β promoter stimulator 1 (IPS-1), an adapter molecule for RIG-I helicase, and thus inhibits the RIG-I-mediated signalling cascade.

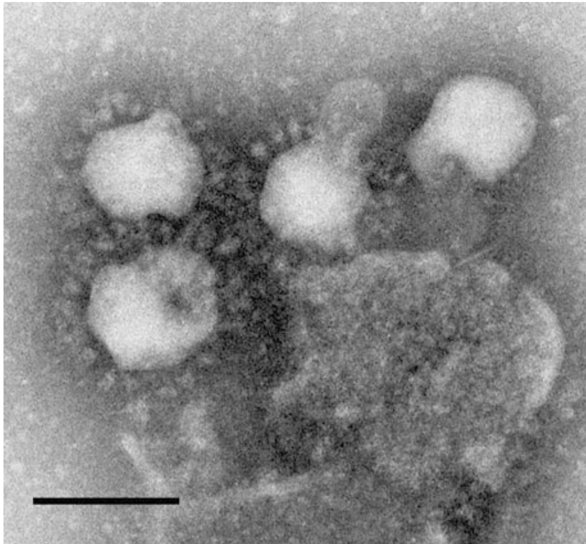
Immune Response and Diagnosis

The virus can be detected by immunofluorescence in stillborn piglets. Cultivation of the virus in cell culture is possible in porcine macrophages or in the monkey kidney cell line MA-104. Antibodies are detectable using commercial ELISAs. However, serology is of only limited diagnostic significance owing to the widespread vaccination of pigs.

Control and Prevention

There are live vaccines for protection against infections with the two genotypes as well as inactivated vaccines, the effectiveness of which is controversially discussed. The currently available inactivated vaccines seem to be considerably less efficient than the attenuated vaccines. A very lively debate is being conducted about the effectiveness of the vaccines beyond the limits of the genotypes and the possible back mutation of attenuated vaccine viruses of both genotypes into virulent pathogens.

14.8 Coronaviruses



The family *Coronaviridae* is classified, together with the families *Arteriviridae* (Sect. 14.7) and Roniviridae, into the order *Nidovirales*. Human coronaviruses were discovered by David A.J. Tyrrell and colleagues during cold epidemics in 1965. They were originally defined as their own family on the basis of morphological differences from other viruses in 1968. Electron micrographs revealed virus particles that are surrounded by an envelope containing embedded proteins, which confer on them the appearance of a "halo" (Latin, *corona*). When the molecular details of the genome structure and replication mechanisms became known later, they confirmed the original classification based strictly on morphological analyses.

Coronavirus infections cause predominantly harmless colds and infections of the upper respiratory tract in humans. Infections with coronaviruses are also known in some domestic mammals. Essentially, they are associated with acute gastroenteritis in cattle, swine, cats and dogs. However, there are also other disease patterns, such as encephalitis in pigs and a fatal systemic general infection in cats, known as feline infectious peritonitis, which is caused by the feline coronavirus. The mouse hepatitis virus, which provokes liver inflammation and bronchitis in rodents, is an important model system for unravelling pathogenetic mechanisms. Besides the different coronaviruses that infect mammals, there are some types that cause severe infections in poultry. Most notable is avian infectious bronchitis virus.

Infections with the severe acute respiratory syndrome (SARS)-related coronavirus emerged in humans for the first time in the winter of 2002–2003, mainly in Southeast Asian countries (China, Hong Kong, Taiwan), but also in Canada. Worldwide, this unique outbreak caused more than 8,000 manifest infections and 700 deaths, corresponding to a mortality rate of about 10 %. The origin of this novel virus was

unclear for a long time until an almost identical SARS-related coronavirus was found in China in bats (*Rhinolophus* spp., greater horseshoe bat). They transmit the pathogen to civet cats, which in turn can pass the pathogen on to humans in animal markets.

14.8.1 Classification and Characteristic Prototypes

Coronaviruses are subdivided into two subfamilies, *Coronavirinae* and *Torovirinae*. In the subfamily *Torovirinae*, white bream virus is a member of the genus *Bafinivirus* and is pathogenic for fish, whereas bovine torovirus equine torovirus, human torovirus and porcine torovirus belong to the genus *Torovirus* and cause gastrointestinal infection in the respective animals. The biology of toroviruses and the diseases that they cause have barely been explored, however. The subfamily *Coronavirinae* is classified into four genera according to differences in genome organization and sequence (Table 14.18). Coronaviruses that infect humans as well as many mammals such as ungulates, carnivores and bats have recently been assigned into the genera *Alphacoronavirus* and *Betacoronavirus*. SARS-related coronavirus has been classified into the latter genus along with various related coronaviruses isolated from civet cats and bats. The genera *Gammacoronavirus* and *Deltacoronavirus* contain species that cause disease in various birds.

14.8.2 Structure

14.8.2.1 Virus Particle

The enveloped virions of coronaviruses have a diameter of 80–180 nm. The single-stranded, positive-sense RNA genome is associated with the N protein as a nucleocapsid in the interior of the particle (Fig. 14.22). The nucleocapsid has a helical shape. The helix has a diameter of 10–20 nm. Particular amino acids of the N protein interact with the carboxy-terminal domain of the M protein that is inserted in the envelope. In this way, the nucleocapsid is associated through protein interactions with the inner side of the envelope. Apart from the M protein, an amino-terminally glycosylated protein of 20–30 kDa, two other viral proteins are embedded in the envelope: the glycosylated S protein (180–200 kDa) is present in club-shaped trimers, which protrude about 20 nm from the envelope surface and are responsible for the appearance of the corona, and the E protein (9–12 kDa), which is present in only small amounts. Another membrane-associated protein, haemagglutinin esterase (HE), is present only in members of the genus *Betacoronavirus*. It has a molecular mass of 65 kDa, is present as a dimer and exhibits haemagglutination activity.

14.8.2.2 Genome Organization and Structure

Coronaviruses possess the largest genome of all known RNA viruses: it has a length of 27,000–32,000 nucleotides (human coronavirus 229E, 27,317 nucleotides; avian infectious bronchitis virus, 27,608 nucleotides; porcine

Table 14.18 Characteristic prototypes of coronaviruses

Subfamily	Genus	Human virus	Animal virus
Coronavirinae	**Alphacoronavirus**	**Human coronavirus 299E**	**Alphacoronavirus 1**
			Porcine transmissible gastroenteritis virus
			Feline coronavirus (feline enteric coronavirus, feline infectious peritonitis virus)
		Human coronavirus NL63	**Miniopterus bat coronavirus 1**
			Miniopterus bat coronavirus HKU8
			Rhinolophus bat coronavirus HKU2
	Betacoronavirus	**Betacoronavirus 1**	**Betacoronavirus 1**
		Human coronavirus OC43	Bovine coronavirus
			Equine coronavirus
			Porcine hemagglutinating encephalomyelitis virus
		Human coronavirus HKU1	**Murine coronavirus**
			Mouse hepatitis virus
			Rat Coronavirus
		SARS-related coronavirus	**SARS-related coronavirus**
			SARS coronavirus (Bat coronavirus HKU3, SARS coroanvirus civet)
			Pipistrellus bat coronavirus HKU5
			Rousettus bat coronavirus HKU9
	Gammacoronavirinae		**Avian coronavirus**
			Avian infectious bronchitis virus
			Turkey coronavirus
			Beluga Whale coronavirus SW1
	Deltacoronavirus		**Bulbul coronavirus HKU11**
			Thrush coronavirus HKU12
Torovirinae	**Bafinivirus**		**White bream virus**
	Torovirus	**Human torovirus**	**Bovine torovirus**
			Equine torovirus
			Porcine torovirus

SARS-Severe acute respiratory syndrome. The names for virus species are given in bold, in combination with selected virus types

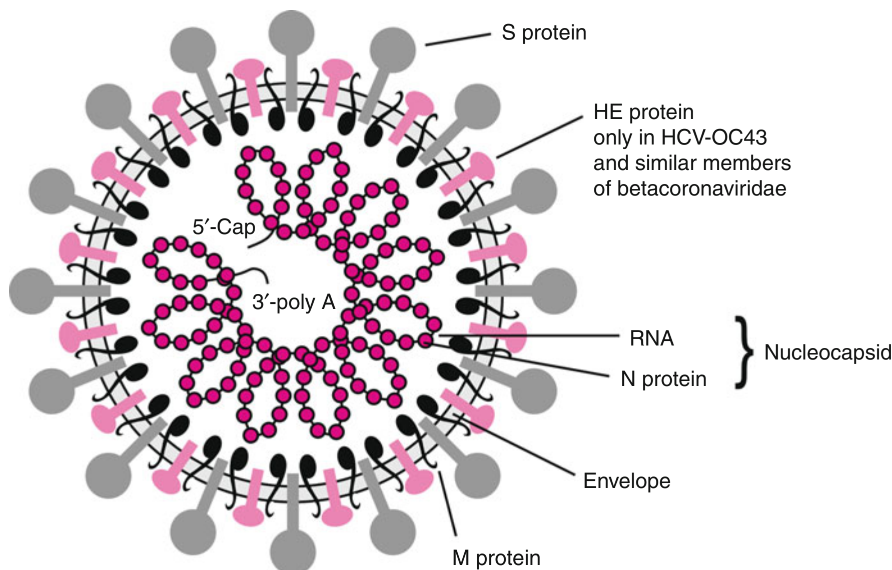


Fig. 14.22 Structure of a coronavirus particle. The RNA genome is complexed with N proteins into a helical nucleocapsid on the inner side of the particle. It is surrounded by an envelope, in which the S and HE glycoproteins and the non-glycosylated M protein are integrated. *HCV* human cornonavirus

transmissible gastroenteritis virus, 28,580 nucleotides; SARS-related coronavirus, 29,727 nucleotides; mouse hepatitis virus, 31,357 nucleotides), is single-stranded and has a positive-sense polarity, and is modified at the 5′ end with a cap-structure and at the 3′ terminus with a poly(A) tail (Fig. 14.23). The RNA is infectious. The genome contains multiple coding regions: two relatively large open reading frames, 1a and 1b, overlap at their ends by 40–60 nucleotides (1a starts immediately adjacent to the 5′ terminus). They span approximately 20,000 nucleotides and code together for a polyprotein, 1ab (pp1ab), of theoretically 700–800 kDa. A ribosomal frameshifting during translation leads to skipping the stop codon at the end of open reading frame 1a, facilitating the continuous synthesis until the end of open reading frame 1b. This process occurs in 20–30 % of all translation events and renders possible the synthesis of pp1ab, the precursor of the non-structural proteins. If this ribosomal frameshifting does not occur, then the translation will end at the stop codon at the end of open reading frame 1a, resulting in polyprotein 1a (pp1a; 450–500 kDa). These polyproteins contain the genetic information for two (in SARS-related coronavirus and avian infectious bronchitis virus) or three (in other coronaviruses) proteases, which autocatalytically cleave the precursor proteins pp1a and pp1ab, as well as a functionally active RNA-dependent RNA polymerase and an RNA helicase. The reading frames for the structural proteins are situated within the last third of the genome. In the 5′ to 3′ direction, they encode the envelope proteins S, HE (only in most betacoronaviruses, not in SARS-related coronavirus), E, M and, just

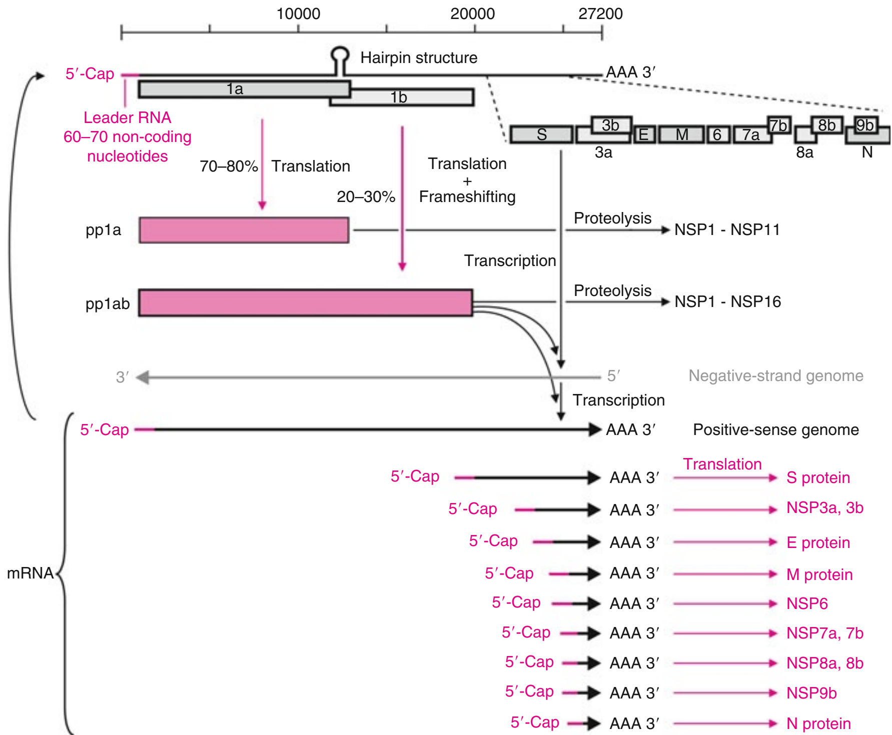


Fig. 14.23 Genome organization and replication in coronaviruses (here SARS-related coronavirus). The viral RNA genome functions as a mRNA, and is translated in the cytoplasm. The two overlapping reading frames encode non-structural polyproteins 1a and 1ab (*pp1a* and *pp1ab*). A hairpin structure induces a frameshifting during translation that leads in about 15–20 % of translation processes to the synthesis of non-structural polyprotein 1ab, which harbours in its carboxy-terminal region the RNA-dependent RNA polymerase activity. It is cleaved by the autocatalytic activity of a cysteine protease in the centre of the precursor protein. Papain – like protease sequences reside within the N-terminal domains of proteins 1a and 1ab, they are autocatalytic active and perform their own cleavage from the precursor proteins. The RNA-dependent RNA polymerase transcribes the positive-sense RNA genome into a complementary negative strand. It serves as a template for the synthesis of new positive-sense RNA genomes and for the transcription of various subgenomic mRNA species, which are modified at the 5′ end with a cap structure, and include identical sequences of the leader region in all mRNA molecules. The structural proteins are translated from the different subgenomic mRNAs, whose reading frames are localized in the 3′-oriented part of the positive-sense RNA genome. They are partially overlapping. Besides the genes specified here, there are some additional small reading frames in this region of the genome in the different coronavirus types which commonly encode non-structural proteins of unknown function. They are not shown here

before the 3′ end, N. In addition, there are various small open reading frames (2a, 3a, 3b, 6, 7a, 7b, 8a, 8b and 9b) in the part of the genome encoding the structural proteins. The coronaviruses of different genera differ significantly in the occurrence of these small open reading frames that predominantly encode very small accessory proteins; most of them are not essential for viral replication.

14.8.3 Viral Proteins

14.8.3.1 Non-Structural Proteins

The viral genome serves as mRNA for the synthesis of the precursor proteins pp1a and pp1ab (486 and 790 kDa, respectively, in SARS-related coronavirus). To synthesize pp1ab, a ribosomal frameshift is necessary to skip the stop codon at the end of open reading frame1a. This occurs because of a defined secondary RNA structure, which forms a stem loop at the end of the first reading frame. The large, experimentally not isolable precursor protein pp1ab is autocatalytically cleaved by the activity of the proteases into 13–16 cleavage products, depending on the various virus types. The function of the resulting non-structural proteins has not been ascertained in all cases. The best investigated activities are those of the non-structural proteins NSP1–NSP16 in SARS-related coronavirus; here, there is an RNA-dependent RNA polymerase (NSP12) that is required for both replication of the viral RNA genome and synthesis of subgenomic mRNAs. It constitutes part of the large precursor protein pp1ab along with the RNA helicase (NSP13) that probably binds Zn^{2+} ions, an exoribonuclease (NSP14), an endoribonuclease (NSP15) and a 2′-*O*-ribose methyltransferase (NSP16). SARS-related coronavirus includes in precursor protein pp1a (486 kDa), which is cleaved in this virus into 11 proteins (NSP1–NSP11), the sequence of papain-like protease 2 ($PL2^{pro}$; NSP3) and an enzyme that resembles the 3C protease of picornaviruses ($3CL^{pro}$, NSP5). The enzyme $3CL^{pro}$ constitutes the most active protease and is responsible for 11 of the cleavage reactions in the precursor polyprotein. These two proteases are present in all coronaviruses. Most of them contain an additional papain-like cysteine protease ($PL1^{pro}$) at the amino terminus of pp1a and pp1ab, which autocatalytically cleaves an amino-terminal domain from these polyproteins. Its function is not completely understood (Table 14.19). In SARS-related coronavirus, instead of this proteolytic activity of non-structural protein NSP1, there is a function that interferes with the metabolism of the cells and causes the degradation of cellular mRNAs, including the transcripts that are necessary for the synthesis of class I interferons. In addition, the different coronaviruses encode some other non-structural proteins which are produced during the infectious cycle. The genetic information resides in the gene region encoding the structural proteins, but not regularly in all virus types. The function of these small ancillary proteins is largely unknown.

14.8.3.2 Structural Proteins

Three types of envelope proteins are found in all coronaviruses: the M protein (formerly also known as E1 protein), the S protein (also called E2 protein) and the E protein (also called sM protein). An additional glycoprotein (HE) is only present in most betacoronaviruses (Table 14.19).

The S protein (“S” for “surface” or “spike”) has a molecular mass of 180–200 kDa. It is glycosylated and anchored in the envelope of the virus, but also in the cytoplasmic membrane by a transmembrane domain at the carboxy terminus, which is modified by an aliphatic acid. The S protein exists as a dimer or trimer and forms

Table 14.19 Functions and properties of coronavirus proteins

Reading frame	Protein	Size	Properties	Function
ORF1a	NSP1	180 aa		Virulence factor; activity described only in SARS-CoV, causes degradation of cellular RNA and allows the virus to replicate unimpaired, blocks the synthesis of IFN-α and IFN-β
	NSP2	638 aa	?	Function unclear; interaction with the cellular proteins prohibitin 1 and prohibitin 2; deletion has no effect on replication
	PL1^{pro}	?		Papain-like cysteine protease; autocatalytic cleavage of the N-terminal domain from pp1a and pp1ab (not in SARS-CoV)
	NSP3/PL2^{pro}	1,922 aa	Zinc finger motif, 180–200 kDa	Papain-like cysteine protease; cleaves pp1a and pp1ab between NSP2 and NSP3; causes protein deubiquitylation; ADP phosphatase activity
	NSP4	500 aa	Membrane-associated	Influences formation of intracellular membrane vesicles, active in virus morphogenesis?
	NSP5/3CL^{pro}	306 aa	30 kDa, dimer	Serine protease with homology to 3C protease of picornaviruses; principal protease
	NSP6	290 aa	Hydrophobic, transmembrane domain	?
	NSP7	83 aa	Forms supercomplexes with NSP8	?
	NSP8	198 aa	Forms supercomplexes with NSP7, nucleic acid binding properties	Alternative RNA-dependent RNA polymerase to NSP12; primase activity for primer synthesis in genome replication and translation?
	NSP9	113 aa	Forms homodimers, interaction with NSP8	?
	NSP10	139 aa	Zinc finger motif, nucleic acid binding properties	?
	NSP11	13 aa	?	?

ORF1b	NSP12/RNA-dependent RNA polymerase	932 aa	106 kDa	Synthesis of genomic and subgenomic RNA species
	NSP13/RNA helicase	601 aa	Zn^{2+} ion-binding, 67 kDa	ssRNA/dsRNA helicase; NTPase, dNTPase; necessary for genome replication
	NSP14/3–5′ exoribonuclease (ExoN)	527 aa		Exoribonuclease, active during RNA synthesis, possibly involved in recombination and repair processes
	NSP15/endo-ribonuclease (NendoU)	346 aa		Uridylate-specific endoribonuclease; active during RNA synthesis
	NSP16	298 aa		Active during 5′ capping of mRNAs and viral genomes
ORF2a	2a (mouse hepatitis virus)	30 kDa	Non-structural protein	Cyclic phosphodiesterase, RNA processing
ORF2	S	180–200 kDa	Glycosylated membrane protein; trimer; located in virus and plasma membrane of infected cells	Viral attachment/receptor binding Induces membrane fusion Induces production of neutralizing antibodies ADCC response
ORF2.1	HE	65–70 kDa	Glycosylated membrane protein, present in most betacoronaviruses (not in SARS-CoV)	Haemagglutinin and esterase responsible for secondary adherence to acetylated neuramic acid residues
ORF3	3a	274 aa	Membrane protein, O-glycosylated; tetramer; component of virus particles	Ion channel protein; induces production of proinflammatory cytokines in SARS-CoV
	3b	154 aa	Transport into nucleus	Function unclear; inhibits production of IFN-α and IFN-β?
ORF4	E	9–12 kDa	Membrane protein	Necessary for particle assembling, ion channel protein (viroporin), in some coronaviruses proapoptotic
ORF5	M	20–30 kDa	Glycosylated membrane protein; localized in ER and viral membranes of infected cells	Interaction with N protein Initiates viral morphogenesis by budding within the ER lumen

(*continued*)

Table 14.19 (continued)

Reading frame	Protein	Size	Properties	Function
ORF6	ORF6	63 aa	Betacoronaviruses (SARS-CoV): associated with ER membrane and Golgi vesicles, component of the virus particle Alphacoronaviruses and gammacoronaviruses: non-structural protein, function unclear	Function unclear; interaction with karyopherin-α_2, whereby there is inhibition of nuclear transport of Stat1?
ORF7	7a	122 aa	Localized in ER/ERGIC/Golgi, also component of the virus particle, interaction with M/E proteins, and with other ancillary or cellular proteins	Apoptosis induction, activation of cellular kinases, interaction with BiP and proteasome
	7b	44 aa	Membrane protein (SARS-CoV), detectable in virus particles, in infected cells in Golgi vesicles	Function unclear
ORF8	8ab 8a 8b		SARS-CoV isolated from animals (civet cats, bats) contain a continuous ORF8 producing a single 8ab protein; in human isolates ORF8 contains a deletion of 29 nucleotides, thus generating ORFa and ORFb	Function unknown
ORF9	N	60–70 kDa	Phosphorylated, dimeric, strongly basic, localization cell type-specific, also in the nucleus	Binding to the RNA genome forming helical nucleocapsids Interaction with the cytoplasmic domain of M protein Interaction with smad3, interferes with cellular transcription and cell cycle regulation
	9b			Function unclear; morphogenesis?

Data and indications refer to SARS-CoV, unless otherwise noted. The protein succession order in the table corresponds to the real order of their reading frames in the viral genome

ADCC antibody-dependent cellular cytotoxicity, *BiP* binding immunoglobulin protein, *ERGIC* ER–Golgi intermediate compartment, *pp1a* polyprotein 1a, *pp1ab* polyprotein 1b, *ssRNA* single-stranded RNA

club-like protuberances on the surface of the virus. Neutralizing antibodies are produced against the S protein in the course of an infection. Three important epitopes have been identified within the amino acid sequence. The virus attaches to cell surface molecules by specific domains of the S protein. The fact that the S protein is also present in the cytoplasmic membrane of infected cells makes them targets for the antibody-mediated cytotoxic cell lysis by killer cells. Furthermore, the viral fusogenic activity resides in the S protein. This refers to the ability of the viral envelope to merge with the cytoplasmic membrane and to induce the fusion of the membranes of infected and uninfected cells to form polykaryocytes. To induce the fusogenic activity, the S protein of some coronaviruses must be cleaved by a cellular trypsin-like protease at a highly basic amino acid sequence in the middle of the protein (mouse hepatitis virus, infectious bronchitis virus of birds and similar viruses). This process probably occurs in the Golgi apparatus during late stages of viral maturation. It results in an amino-terminal moiety S_1 that is non-covalently linked to the carboxy-terminal half S_2, and can be detached from the surface of the virus. The fusion effect is not mediated as in paramyxoviruses, in which a new hydrophobic amino terminus in the S_2 moiety is generated by cleavage (▶ Sect. 15.3). The molecular mechanism of membrane fusion in coronaviruses has not been conclusively unravelled. Moreover, virus types whose S protein is not proteolytically cleaved can also induce cell fusions in spite of all that. Two hydrophobic segments of the S_2 protein seem to be involved in this process, as shown in the mouse hepatitis virus. If they are altered by mutation, the fusion activity is lost.

The HE protein is present only in betacoronaviruses; SARS-related coronavirus does not possess such a gene. HE protein is glycosylated, has a molecular mass of about 65 kDa and forms dimers by disulphide bonds. The viruses that encode and express the HE protein have the capability of haemagglutinating and binding to erythrocytes. In this process, the HE protein interacts with the 9-O-acetylated neuraminic acid (sialic acid), which is a modification of lipid and protein components on cell surfaces. The HE protein has an esterase activity, which enables the virus to remove acetyl groups from sialic acid molecules. The HE protein of coronaviruses has a strong sequence homology to the HEF protein of influenza C viruses (▶ Sect. 16.3).

The E protein (9–12 kDa) is found within infectious virus particles in different concentrations; it is necessary for particle assembly and morphogenesis. In some coronaviruses (mouse hepatitis virus and SARS-related coronavirus), the E protein has a proapoptotic function. Furthermore, it seems that E protein acts as viroporin, forming ion channels and altering the membrane permeability.

M protein ("M" for "matrix") is a surface protein with a molecular mass of 20–30 kDa and is glycosylated at the amino-terminal domain. The sugar groups are mainly linked to serine or threonine residues. In contrast to the normal N-glycosylation at asparagine residues, the glycosylation is an O-glycosylation. Only a few amino-terminal regions of this protein are exposed on the surface, and it has three transmembrane domains. The carboxy terminus resides inside the virus particle and interacts with the N protein of the nucleocapsid.

The M protein is not transported to the plasma membrane via the Golgi apparatus, but it remains in the ER membrane throughout the infection cycle. By the interaction of theM protein with the nucleocapsid, the first steps of viral assembly occur at those sites, which initiate the budding process into the ER lumen. The E protein is also involved in viral morphogenesis. If M and E proteins are produced by genetic engineering methods in eukaryotic cells, they self-assemble into virus-like particles.

The N protein (“N” for “nucleic acid binding”) interacts with the viral genome; it is rich in basic amino acids and phosphorylated. It can also interact specifically with the carboxy-terminal regions of the M protein. Phylogenetic trees based on the nucleic acid sequences of the N genes correlate well with the classification of coronaviruses into different genera. N proteins are detectable in both the cytoplasm and the nucleolus of infected cells. Because of the cell-type-specific localization in the nucleus/nucleolus, it is assumed that N proteins interfere with various cellular processes.

14.8.3.3 Accessory Proteins

Besides the classical structural proteins, there are many products that are also detectable in the virus particles. They are encoded by the small reading frames within the structural gene region at the 3′ half of the genome. These include proteins 3a, 7a, and 7b, and the ORF6 protein. These are predominantly gene products with accessory functions: deletions of the corresponding genes do not fundamentally affect the infectiveness or the replicative capacity in vitro. Presumably, these proteins have important roles as virulence factors for infecting humans or animals. Little is known with regard to the function of these proteins (Table 14.19).

14.8.4 Replication

Human coronaviruses 229E attach to target cells by interaction with some still uncharacterized domains of the S protein with the zinc metalloprotease CD13 (aminopeptidase N) on the cell surface. There is no evidence that the protease CD13 cleaves the S protein during binding. Feline coronavirus uses also an aminopeptidase as a receptor. On the other hand, SARS-related coronavirus and human coronavirus NL63 bind by a domain of the S_1 protein to another metalloprotease, angiotensin-converting enzyme 2 (ACE-2). This protein is present on the surface of pneumocytes, but also on enterocytes and cells of other tissues and organs (heart, kidney, endothelium). In addition, binding to some lectins such as DC-SIGN, L-SIGN and LSECtin, facilitates entry of SARS-related coronavirus into the cell. Mouse hepatitis virus uses different isoforms of the carcinogenic embryonic antigen as cellular receptors; these isoforms belong to the immunoglobulin superfamily. Coronaviruses, which in addition to the S protein contain the HE protein in the membrane, can also interact with 9-O-acetylated neuraminic acid residues on the cell surface. However, this initial interaction of HE protein with

sugar groups is not enough to infect a cell. It must be strengthened by specific binding of the S protein with unidentified cellular proteins.

Penetration of the particle seems to occur by receptor-mediated endocytosis, and subsequent fusion of the endosomal membranes with the viral envelope (as in flaviviruses and togaviruses; Sects. 14.5 and 14.6). Binding between S_1 and ACE-2 proteins induces conformational changes in the S proteins of SARS-related coronavirus, whereby a fusogenic domain in the S_2 moiety develops its activity at low pH, promoting fusion between the viral envelope and the cell membrane. A protease, cathepsin L, which is associated with endosomal membranes, promotes the infectivity of the virus by processing still uncleaved S polypeptides into S_1 and S_2 proteins.

All replication steps are executed in the cytoplasm. The RNA genome of coronaviruses contains a cap structure at the 5′ terminus and this mediates binding of ribosomes. As already mentioned, the non-structural polyproteins pp1a and pp1ab are primarily translated from the genomic RNA; pp1ab arises by induction of a translational frameshifting mechanism, and contains the RNA-dependent RNA polymerase. The precursor polyprotein pp1ab has a molecular mass of 700–800 kDa; however, it has not yet been directly detected in the cell during viral replication. Nevertheless, in vitro translation experiments in which the genomic RNA was used as a template revealed that this translation product is actually synthesized. The proteases that are encoded in its sequence ($PL1^{pro}$, $PL2^{pro}$ and $3CL^{pro}$) cleave pp1ab into 16 non-structural proteins, one of which is the RNA-dependent RNA polymerase (NSP12).

In the next step, the complementary strand is synthesized by the concerted activities of RNA-dependent RNA polymerase (NSP12), RNA helicase (NSP13), exoribonuclease (NSP14) and endoribonuclease (NSP15) using the genomic RNA as a template. The newly synthesized complementary strand comprises the entire genome and has a negative polarity. It has two functions in the replication cycle: it serves as a template for the synthesis of new viral genomes and several subgenomic mRNA species. Eight subgenomic mRNAs have been found in SARS-related coronavirus infections, from which the various accessory and structural proteins are translated. They are characterized by their discontinuous synthesis: all have the same 3′ terminus, but their initiation sites are different and are located in the genomic region between the end of the reading frame of pp1ab and the 3′ end of the genome. Despite their different initiation sites, all subgenomic mRNA molecules possess a standard sequence of approximately 60–90 nucleotides at the 5′ terminus: the leader RNA. It has a capped 5′ end and is complementary to the 3′ terminus of the negative strand. It is believed that the leader sequence serves as a primer for the synthesis of subgenomic mRNA species; whether the activity of NSP8 is required for the synthesis of these short RNA molecules is unclear. The leader RNA has a conserved sequence (UCUAAAC) at the 3′ terminus. Sequences complementary to this motif are found in the negative-sense RNA at different regions. They are located upstream of the different initiation sites for the synthesis of the subgenomic mRNA species: the leader RNA can hybridize with them, thus providing a small double-stranded region with a free 3′-OH end to resume

polymerization. Probably, the RNA polymerase is not capable of initiating synthesis of the subgenomic mRNA species at the different start sites. Therefore, it was probably necessary for the virus to develop the transfer mechanism of a leader RNA. The finding that multiple elements of the conserved heptamer sequence are present upstream of some of the transcription start sites, e.g. upstream of the start of the coding sequences for the N protein, suggests that RNA synthesis is preferably initiated at these sites, thus facilitating regulation of the quantity of the various proteins.

The nested transcripts are modified by the ribose methyltransferase (NSP16) with a cap structure at the 5′ terminus; this mediates binding of ribosomal subunits and translation of the respective reading frames into proteins. However, in the different coronavirus types, many of the different subgenomic RNAs are bicistronic or tricistronic, and from these two or three proteins are translated. Among the subgenomic RNAs, a total of five bicistronic RNAs have been found in SARS-related coronavirus, and in avian infectious bronchitis virus there is one tricistronic mRNA. The translation of the reading frames, which are not localized adjacent to the 5′ end of the capped subgenomic transcripts, is performed by an alternative mechanism, frequently by translational frameshift. However, in the case of subgenomic RNAs 3 and 5 of avian infectious bronchitis virus and mouse hepatitis virus, an IRES-like secondary RNA structure facilitates binding of the ribosomes before the start of the downstream reading frames. In addition, it seems that there are other ways which allow translation initiation of alternatively used reading frames. However, they are usually not very effective and result in a low expression of the corresponding proteins. Which mechanisms also play a role in the regulation of protein synthesis has not been clarified. In infected cells, the most abundant polypeptide is the N protein, which is translated from the shortest transcript.

During virus morphogenesis, N proteins interact with genomic RNA molecules to shape the helical nucleocapsids and bind to the carboxy-terminal domains of the M and E proteins, which are integrated in the ER membrane. This triggers the budding process, during which the nucleocapsid is surrounded by the envelope containing M proteins and glycoproteins. The particles produced are released into the ER lumen and are transported by Golgi vesicles to the cell surface, where they are released into the environment.

14.8.5 Human Pathogenic Coronaviruses

14.8.5.1 Human Coronaviruses 229E and OC43

Epidemiology and Transmission

Human coronaviruses 229E and OC43 have been known since 1966 and 1967, whereas the species NL63 and HKU1 together with some additional virus types were identified only a few years ago during an intensive search for human coronaviruses as a result of the SARS epidemic. All these coronavirus types have a worldwide distribution: between 75 % and 65 % of children of 3.5 years of age

and up to 90 % of adults have antibodies against coronaviruses – an indication of their wide dissemination. It is estimated that globally 10 % of all infections of the upper and lower respiratory tract are caused by them. Coronaviruses are transmitted by infected people through droplet infection. Like other droplet infections, these viruses can also be transmitted by smear infections due to poor hygiene. Infections occur more frequently during the winter months. Reinfections – even with the same strain of the virus – are common and usually have an asymptomatic course.

Clinical Features

Coronaviruses cause cold diseases of the upper respiratory tract, rarely infecting the lower airways. Infections are often asymptomatic, or have only mild symptoms. The incubation period lasts 2–5 days; the duration of the illness with coryza, cough, sore throat and headache associated with low-grade fever lasts for about 1 week. In infants and young children, the infection can take a much severer course and can be associated with croup-like symptoms. It can lead to asthma attacks and in some individual cases to bronchitis and pneumonia. In cases of pre-existing respiratory diseases such as asthma and chronic bronchitis, these symptoms can be strengthened in both children and adults. Human coronaviruses have also been associated with disorders of the gastrointestinal system. This appears to occur only in immunologically compromised individuals, e.g. in AIDS patients, who can also have prolonged diarrhoea.

Pathogenesis

The human coronaviruses multiply in the ciliated epithelial cells of the respiratory tract, which express the corresponding receptor molecules ACE-2 or CD13. Electron microscopy studies suggest that they can also proliferate in the intestinal epithelium. The infection is usually restricted to the epithelial cells of these organs.

Diseases in which the virus infects macrophages and lymphocytes, proliferates in these cells and then spreads via the bloodstream into liver, endothelial, glial and kidney epithelial cells and infects these organs and tissues are only known from animal coronaviruses and SARS-related coronavirus. Whether the HE protein plays a role in the infection of these cell types and in the pathogenesis of the disease is not known. Mutations in the S protein alter both virulence and tropism, i.e. the specificity for different cell types. In addition to virus-specific factors, genetic factors appear to be important for the establishment of coronavirus infections: e.g. the susceptibility to human coronavirus 229E infections is apparently determined by a factor encoded on chromosome 15.

Immune Response and Diagnosis

During a coronavirus infection, IgM, IgG and IgA antibodies are produced. Immunoglobulins against the S protein are neutralizing. IgA antibodies and interferons, which are secreted in the nasal secretion, seem to be important for protecting against infections. Very little is known about the importance of the cellular immune response in human infections. From mouse hepatitis virus is known that cytotoxic T cells are involved in the clearance of the virus from the organism.

Coronaviruses can be cultivated only to a limited extent in cell culture. Human coronavirus 227E can be propagated in tissue cultures of embryonic trachea, human rhabdomyosarcoma cells or in the cell line MA-177, a diploid cell line from the intestinal epithelium. The cultivation of human coronavirus OC43 is much more difficult.

The diagnosis of coronavirus infections is usually done retrospectively by detecting virus-specific IgM and IgG antibodies in ELISA. Alternatively, the occurrence of viral nucleic acids can now be demonstrated by PCR from appropriate clinical materials.

Therapy and Prophylaxis

Since coronavirus infections are largely harmless, no attempts have ever been undertaken to develop a vaccine. There is no antiviral therapy.

14.8.5.2 SARS-Related Coronavirus

Epidemiology and Transmission

The first infections with SARS-related coronavirus emerged in Foshan and Heyuan in the Chinese province of Guangdong in November 2002. Most patients had direct or indirect contact with animal markets. Until January 2003, the infections spread through human-to-human transmissions to Guangzhou, the capital of Guangdong province, where they affected mainly people who were working in public health services. There was talk of an "infectious atypical pneumonia", and a number of infectious agents were suspected as possible causes, among others, chlamydia. When the number of patients increased to over 300, including 100 nurses and physicians, and five of them had died, the WHO was informed of this new infection on 11 February 2003. In March 2003, an infected nephrologist from Guangdong province stayed in a hotel in Hong Kong, and verifiably infected at least ten other hotel guests, who in turn carried the infection to various countries, including Singapore, Vietnam, Ireland, the USA and Canada. During a second infection wave in Hong Kong and all the afore-mentioned countries, particularly physicians and nurses were infected, and these in turn passed on the infection in the hospitals and to their families, thus exporting it to other countries. The pathogen arrived in central Europe (Germany) via Singapore. On 10 March 2003, this new disease received the name severe acute respiratory syndrome (SARS). In the following months until 5 July 2003, the SARS disease emerged worldwide in 8,400 patients in 29 countries, and about 800 of them died. As a result of massive countermeasures by the WHO, it was possible to control the infection relatively rapidly. These measures included isolation/quarantine, entry bans/travel restrictions and checks at airports, and an open and timely information policy. In March 2003, Christian Drosten of the Bernhard Nocht Institute in Hamburg and other international research groups independently identified a coronavirus as the aetiologic agent of the disease, and determine its entire genome sequence. This facilitated the development of specific diagnostic test systems. At that time, a peculiarity was that all information was made immediately accessible and divulged, e.g. via the Internet.

Inasmuch as it was assumed that this new human virus had been transmitted to humans by zoonotic means, various animal species which are traded on

animal markets in Guangdong were examined for the presence of analogous infections. Coronaviruses were found in nasal swabs and faecal samples from civet cats (*Paguma larvata*) and raccoons (*Nyctereutes procyonoides*). Their genomes were almost identical to that of human SARS-related coronavirus. Animal traders frequently exhibited antibodies that reacted with the proteins of SARS-related coronavirus. It was found that the virus can infect a number of other animals, such as cats, mice, ferrets and macaques; however, wild civet cats and raccoons were not infected. This suggested that these animals do not represent the natural hosts. The natural hosts are probably bats of the genus *Rhinolophus* (greater horseshoe bat), in which the SARS virus was detected in high concentrations. They excrete the pathogens in their faeces. The bats transmitted the virus to civet cats under the limited space conditions at the animal markets, and from these the virus was further transmitted to animal dealers, buyers and exploiters (cooks, furriers). The occurrence of further mutations facilitated the adaptation to human hosts, leading to the rapid transmission from infected patients to other people. The transmission among humans occurs preponderantly by airborne infection, whereby it was primarily assumed that every infected person develops symptoms within 10 days. However, there are clear indications that the virus is also excreted in the stool, and faecal–oral smear infections may contribute to its dissemination. With the exception of a few infections in Guangdong in January 2004 which were associated with animal contacts again, and some cases that could be attributed to laboratory contacts with SARS-related coronavirus, no more SARS infections and illnesses have occurred since the SARS outbreak of 2003.

The Physician Who First Identified SARS Died of It

Carlo Urbani was one of the first people to recognize that SARS is a new, unusual pneumonia, from which a considerable risk to human health may emanate. He was an expert of infectious diseases who worked at the WHO. Urbani along with the WHO and the governments of the SARS-affected countries initiated the implementation of appropriate measures in Vietnam and Southeast Asia in February/March 2003 that were aimed at preventing and controlling the dissemination of the infection. Unfortunately, he infected himself as a result of his investigations, and died from SARS on 29 March 2003.

Clinical Features

The virus is transmitted from person to person by droplet or smear infections. The incubation period lasts 2–10 days, and the disease begins with flu-like symptoms, swollen lymph nodes and fever. Patients develop a dry cough associated with colds, limb pain, muscle pain, neck ache and headaches. At this stage, the disease is clinically virtually indistinguishable from an influenza infection. In addition to the severe respiratory symptoms, it is frequently also associated with disorders of the gastrointestinal system (diarrhoea, nausea) as well as with thrombocytopenia.

About 1 week after the appearance of the first symptoms, a subset of patients develop a severe pneumonia, which is often associated with pulmonary fibrosis, myocardial infarction, acute renal failure and ultimately multiple organ failure.

Pathogenesis

Probably, SARS-related coronavirus initially proliferates in the epithelial cells of the respiratory tract, like other pathogenic coronaviruses. The ACE-2 receptor proteins, to which the virus attaches by S proteins, are found on the surface of cells in many tissues; they are an integral part of the renin–angiotensin system and regulate it. The interaction of S proteins with ACE-2 decreases its concentration on the cell surface. This possibly induces a particular susceptibility to inflammations and lung failure in infected patients. In addition to ACE-2, the S proteins have the ability to attach to DC-SIGN. They are receptor proteins that are found on the surface of dendritic cells. However, they do not directly permit the entry of the virus to these cells, but they can transport the virus to the lymph nodes and other tissues containing ACE-2-positive cells, thus mediating infection in trans. The virus seems to spread via the bloodstream throughout the body, and subsequently infects – like animal coronaviruses – endothelial, lung, kidney and intestinal epithelial cells. Many patients have a myocardial infarction during the disease. Possibly, this is associated with binding of viral S proteins to ACE-2 proteins on the surface of myocytes.

In the early infection phase, a rapid activation of non-specific immune responses with increased production of various CC chemokines and chemokine receptors, proinflammatory interleukins and Toll-like receptor 9 are found in patients. This causes a rapid mobilization of monocytes and macrophages, which migrate into the infected organs, especially the lungs, and initiate the inflammatory process. Simultaneously, a rapid decrease in $CD4^{+}$ and $CD8^{+}$ T lymphocytes can be observed in patients during the acute phase of infection. The cause of this decline is unclear. SARS-related coronavirus is not able to infect lymphocytes; therefore, destruction of T cells cannot be a direct consequence of infection. Possibly, apoptotic processes induced by the infection may be involved. It has been found that several SARS-related coronavirus proteins, such as protein 7a, are able to induce a caspase-dependent apoptosis mechanism in cell lines from different tissues (lung, liver, kidney) in vitro.

Genetic factors in infected patients also seem to be important for the establishment of symptomatic SARS-related coronavirus infections. Individuals who can produce only small amounts of mannose-binding lectin appear to be predisposed to the establishment of a symptomatic infection. Mannose-binding lectin belongs, together with the pulmonary surfactant proteins A and D, to the collectins. They are proteins that attach to glycosylated regions of S proteins on the surface of the virus, facilitating penetration of the virus into granulocytes, and potentially preventing interactions with the target cells. In other studies, it has been found that SARS patients frequently exhibit haplotypes HLA-B*0703, HLA-B*4601 and HLA-DRB1*0301. However, these data are in part contradictory.

Immune Response and Diagnosis

During a SARS-related coronavirus infection, IgM, IgG and IgA antibodies are produced against N and S proteins. They are detectable at the earliest on the fourth day after the onset of symptoms; however, in many patients they are detectable considerably later. Mainly immunoglobulins against the S protein are neutralizing antibodies. They are directed predominantly against epitopes of the domain determined by amino acid residues 441–700. Little is known about the relevance of the cellular immune response in humans during infection.

ELISAs for detection of specific antibodies against the S and N proteins are available. Isolation and cultivation of the virus is time-consuming and not always successful, and must be performed only in biosafety 3 (or higher) laboratories. Therefore, acute SARS-related coronavirus infections are usually diagnosed by detecting viral genomes through RT-PCR in sputum, pharyngeal lavage, stool or serum. In such cases, a region of reading frame 1b, which encodes for the viral RNA polymerase, is usually amplified. A positive detection has to be verified in additional tests and reference laboratories. SARS-related coronavirus is well cultivable in a number of cell lines. The viruses exhibit strong lytic or rather persistent infections, depending on the cell type.

Control and Prophylaxis

There is no vaccine against SARS infections. During the SARS outbreak in 2003, infected patients were treated with ribavirin. In severe cases, corticosteroids were used for treatment because, at least in part, the symptoms are immunopathogenetically determined. It was also attempted to reduce the viral load in patients by use of high doses of immunoglobulins. Inhibitors of viral proteases are currently being developed.

14.8.6 Animal Pathogenic Coronaviruses

The family *Coronaviridae* encompasses many viruses that cause illnesses in animals. The most important are the pathogen of porcine transmissible gastroenteritis and feline coronavirus, which can cause peritonitis and polyserositis in cats. These viruses will be discussed in detail. Furthermore, there is also porcine haemagglutinating encephalomyelitis virus, which induces encephalitis in newborn piglets. Occasionally, it is also associated with diarrhoea. The infection of the central nervous system occurs via peripheral nerves, to which the viruses are transported from the initial reproduction site, the mucosa of the respiratory and gastrointestinal tract, without a viraemia developing. An immunoprophylaxis is not available. A globally important disease of poultry is caused by avian infectious bronchitis virus. Apart from an acute disease of the respiratory tract, substantial economic loss can arise from lesions of the fallopian tubes and the associated decline of egg production. Mouse hepatitis virus is widespread in mouse populations. It represents an important model system for studying the biology and pathogenesis of coronavirus infections. Mouse hepatitis virus causes a wide spectrum of clinical symptoms, which can range from gastrointestinal, hepatic and respiratory symptoms to central

nervous system disorders. Introduction into laboratory animal colonies occurs by inclusion of mice with persistent infections. The diagnosis is performed histopathologically or by cultivation of the virus. The elimination of the virus from infected colonies is almost impossible. Usually, it is necessary to annihilate the colony and to replace the infected livestock with new, virus-free animals. Therefore, regular examinations of mouse colonies are essential to detect infections with mouse hepatitis virus.

Coronavirus infections have been described in other species, particularly in dogs, but they do not have veterinary importance owing to their low clinical relevance.

Bovine coronavirus infections are a major cause of calf diarrhoea in the first few days of life. The infection is local and viral replication is restricted to enterocytes. A vaccine is available in the form of a so-called maternal prophylaxis, in which the cow is vaccinated twice before parturition. The actual immunization consists in the intake of antibodies with the colostrum.

14.8.6.1 Porcine Transmissible Gastroenteritis Virus

Epidemiology and Transmission

Transmissible gastroenteritis is a disease of swine with high morbidity but low mortality. However, if animals become infected at the age of only a few days, the mortality rate can be very high. The virus is excreted over a period of about 14 days with the faeces. Persistent infections and chronic carriers are very rare. In the latter, the virus seems to persist in lung macrophages. The virus is usually transmitted by direct contact, but airborne transmissions have also been demonstrated.

A natural infection results in a durable immunity, which lasts approximately 1–2 years. It is based on a local mucosal immunity. Parenteral inoculation induces systemic antibodies which do not protect against an infection. Moreover, a systemic vaccination of sows does not stimulate the release of immunoglobulins with the milk.

Clinical Features

Infections with porcine transmissible gastroenteritis virus provoke severe diarrhoea and vomiting as its main symptoms. Other symptoms are rarely observed and other organ systems are normally not affected, except for the small intestine.

Pathogenesis

The virus is ingested orally, and infects enterocytes after gastric passage. They are lytically infected, destroyed and replaced by enterocytes from the Lieberkühn's crypts. This process explains the typical symptoms of transient, non-bloody diarrhoea. Histologically, the classic clinical picture is that of acute enteritis with atrophy of villi (in the posterior sections of the small intestine). The gastric passage of the acid-labile coronavirus is achieved by the pH-buffering effect of milk and is additionally supported by the slightly acidic pH in the stomach of young animals.

There is a naturally occurring mutant of transmissible gastroenteritis virus which carries a deletion within the gene encoding the S protein. This mutant virus has

completely lost its enterotropism, and replicates primarily in macrophages of the respiratory tract. It causes no or only mild symptoms, but interferes significantly with the serological surveillance during the control of transmissible gastroenteritis.

Immune Response and Diagnosis

The virus can easily be isolated. Alternatively, it can be detected by immunofluorescence in intestinal sections of dead animals. Examination of antibody levels in serum pairs allows indirect diagnosis. The infection status of a herd can be determined serologically by ELISA or neutralization tests in epidemiological studies.

Control and Prophylaxis

The successful control of infection is based on the elimination of seropositive animals and the establishment of and compliance with strict hygiene and husbandry requirements. A vaccine is available on the basis of inactivated viruses, which are propagated in cell culture; however, it is not very efficient. The local immune defence in the intestine of piglets is important for effective protection, which is most effectively established and transmitted to the piglets by the colostrum from naturally infected sows.

14.8.6.2 Feline Coronavirus (Feline Infectious Peritonitis Virus)

Epidemiology and Transmission

So-called feline infectious peritonitis is caused by infection of cats with feline coronavirus. Infections with this pathogen are widespread in cat populations. The virus is able to persist in cats, and is excreted primarily in the faeces. The cats are usually infected at a very early age by social contact with the mother or other persistently infected cats.

The exact host range of this virus is unknown. It infects domestic and large cats and probably also dogs, as some isolates of feline coronavirus have been identified as recombinants between feline and canine coronaviruses.

Clinical Features

The virus commonly causes a subclinical infection or mild and transient enteritis. The enteritis-causing virus can change by mutations, which create a new biotype with altered tissue tropism. This virus variant replicates no longer exclusively in enterocytes, but is also able to infect macrophages, thus inducing a systemic infection. The virus variant is scattered by macrophages in virtually all organs, where it can induce, starting from a vasculitis, pseudo-granulomatous inflammations. This clinical picture is referred to as feline infectious peritonitis. From a pathological point of view, this disease pattern is a generalized polyserositis and vasculitis/perivasculitis.

Pathogenesis

The virus binds to aminopeptidase N on cells of cats, dogs and pigs. The peritonitis-causing feline virus variant apparently emerges anew in each individual outbreak. In the course of this, the persisting virus mutates to a virulent variant. Transmission of the mutated virus from cat to cat is occasionally observed, but it

does not seem to be of significant epidemiological significance. Deletion of some nucleotides in gene 3c has been found in the virus mutant. This gene codes for a non-structural protein of unknown function during the viral replication cycle, which, however, probably influences the virulence of the isolates. Nevertheless, the deletions are not the same in all mutants that cause feline infectious peritonitis, but differ slightly in all viruses isolated from different outbreaks. The molecular-biological bases of these changes are largely unknown.

Immune Response and Diagnosis

The virological diagnosis is extremely difficult because a distinction between both the benign and the virulent virus variants is not possible with currently available techniques. Therefore, the unambiguous diagnosis of feline infectious peritonitis can only be performed by histopathologic examination.

Control and Prophylaxis

A vaccine is available on the basis of an attenuated vaccine containing a temperature-sensitive mutant of feline coronavirus; however, its efficacy is controversial. There are fundamental safety concerns regarding the use of live vaccines for the prevention of an infectious disease with unclarified (immuno) pathogenesis whose attenuated virus has scarcely been investigated and which possesses a broad host range. Furthermore, it has also been shown that coronaviruses from different species can recombine their genetic material to generate new virus variants. In the case of recombination with the vaccine virus, the emergence of virus variants with altered receptor binding is possible, and these in turn might infect other animal species.

14.8.6.3 Avian Infectious Bronchitis Virus

Epidemiology and Transmission

Infectious bronchitis of chickens is also caused by a coronavirus. It is distributed worldwide. Chickens are the only natural host for the virus. Avian infectious bronchitis virus causes a disease only in chickens. Similar coronaviruses have also been isolated from turkeys and pheasants, but they are considered to be their own species. Of particular importance is the huge antigenic diversity of this virus. On the basis of differences in the sequence of the S protein, a number of serotypes and genotypes are distinguished. The virus is very contagious, and it spreads very quickly within a flock through virus-containing faeces and nasal secretion. Between different flocks, the virus is predominantly transmitted indirectly by the farm staff.

Clinical Features

Avian Infectious bronchitis virus causes lesions in the kidney, in the oviduct and in the respiratory tract. The nature of the clinical manifestation depends on the virus strain and the host. In particular, the age and breed of the chicken influences the clinical picture. The disease is especially pronounced in few-day-old chicks. The morbidity is very high; thus, virtually all birds of a flock are infected. The mortality, however, is generally low (0–25 %).

Pathogenesis

The virus initially replicates in epithelial cells of the respiratory tract or the gastrointestinal tract and invades, after a subsequent viraemia, many organs, especially kidneys and fallopian tubes. Generally, the virus is eliminated by the immune response, but in rare cases the infection can persist for weeks.

Immune Response and Diagnosis

An infection produces a durable and protective immunity against the homologous virus, which is normally based on antibodies against the S protein. The degree of cross-immunity is different for individual strains. Diagnosis is performed by detection of the virus (isolation of the virus in cell culture, PCR) or immunofluorescence with identification of viral proteins in tissues of infected animals, or, at the livestock level, by detection of virus-specific antibodies by neutralization and haemagglutination-inhibition tests or ELISA.

Control and Prophylaxis

Infectious bronchitis of chickens is not subject to animal health regulations. Several live and inactivated vaccines are available and are generally applied according to the specific serotype.

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15 Viruses with Single-Stranded, Non-Segmented, Negative-Sense RNA Genomes

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Viruses with a continuous, single-stranded, negative-sense RNA genome are classified in the order *Mononegavirales*. They include the families *Rhabdoviridae*, *Bornaviridae*, *Paramyxoviridae* and *Filoviridae*.

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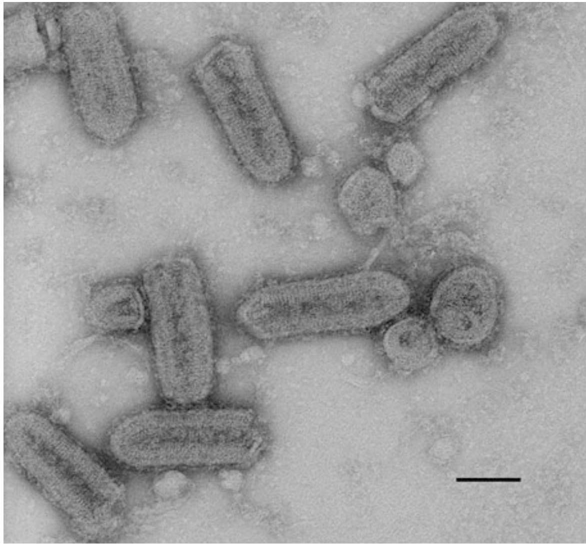
15.1 Rhabdoviruses



The family *Rhabdoviridae* comprises pathogens with a single stranded, non-segmented, negative-sense RNA genome. Their infectious particles are rod-shaped and resemble a rifle cartridge. Many different viruses belong to the family *Rhabdoviridae*, and approximately half of them are plant pathogens. The animal pathogenic rhabdoviruses have a broad host range and are able to infect various organisms. They are transmitted either by insect stings or by bites of infected animals. The rabies viruses, which belong to this virus family, cause severe, fatal diseases in both animals and humans.

15.1.1 Classification and Characteristic Prototypes

The animal rhabdoviruses – of which more than 70 types are known – have been divided into seven genera: *Vesiculovirus*, *Lyssavirus*, *Ephemerovirus*, *Novirhabdovirus, Tibrovirus, Perhabdovirus* and *Sigmavirus*. Members of Perhabdo- and Novirhabdovirus cause diseases in fish those of Sigmaviruses in insects (Table 15.1).

The molecular biology and replication mechanisms of vesicular stomatitis virus are well studied. It is transmitted by mosquitoes and sandflies, and causes serious epizootic diseases (vesicular disease) in horses and cattle on the American continent (Sect. 15.1.6). This virus can easily be cultivated in well-established cell culture systems such as human HeLa cells or the murine L cell line. Transmissions to humans have been observed only in combination with laboratory contaminations during autopsies of infected animals, whereby flu-like symptoms appeared.

Table 15.1 Characteristic prototypes of rhabdoviruses

Genus	Human/animal virus	Animal virus	Plant virus
Vesiculovirus		Vesicular stomatitis virus Cocal virus Piry virus Springviraemia of carp virus Chandipuva virus	
Lyssavirus	Rabies virus Mokola virus Duvenhage virus European bat lyssaviruses types 1 and 2 Australian bat lyssavirus	Lagos bat virus Aravan virus Irkut virus Khujand virus	
Ephemerovirus		Bovine ephemeral fever virus Adelaide river virus Berrimah virus	
Novirhabdovirus		Infectious hematopoietic necrosis virus of salmoias	
		Infectious hemarrhagic septicemia virus Hirame rhabdovirus	
Tibrovirus		Tibrogargan virus Coastal plains virus	
Perhabdovirus		Sea trout rhabdovirus	
		Anguillid rhabdovirus	
Sigmavirus		Drosophila melanogaster Sigmavirus	
		Drosophila obscura Sigmavirus	
Nucleorhabdovirus			Sonchus yellow net virus Maize mosaic virus
Cytorhabdovirus			Lettuce necrotic yellows virus Strawberry crinkle virus

The members of the genera *Ephemerovirus* and *Tibrovirus* are also transmitted by insects. They are common in tropical areas of Africa, Asia and Australia; they infect animals and cause a febrile disease. The human pathogenic lyssaviruses include rabies virus, Mokola virus and the European and Australian bat lyssaviruses

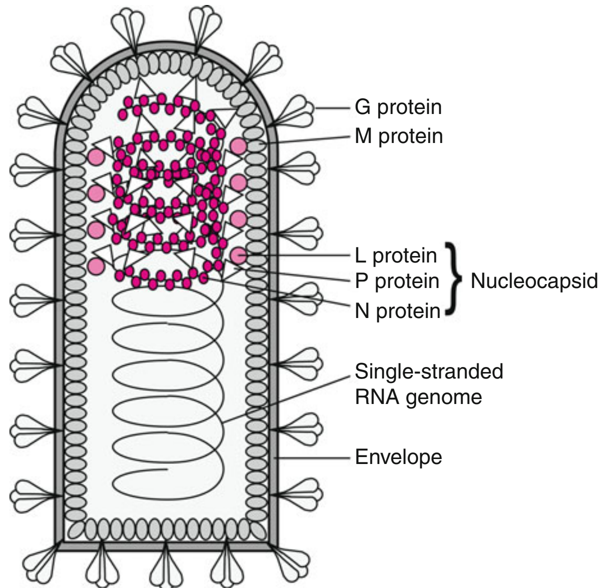


Fig. 15.1 Structure of a rhabdovirus particle. The genome is constituted of a single-stranded RNA, which interacts with the N, P and L proteins to form a helical nucleocapsid. The nucleocapsid is surrounded by an envelope, in which G proteins are inserted. The M protein is associated with the inner side of the envelope and interacts concurrently with the components of the nucleocapsid

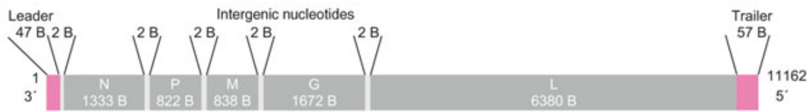
as well as the African Duvenhage virus, which occur in bats, including fruit bats. So far, no infections by Lagos bat, Aravan-, Irkut- and Khujand virus have been reported in humans. Lyssaviruses are transmitted through animal bites to humans and almost always cause fatal diseases of the central nervous system. The virus types can be distinguished by serological differences in their G surface proteins.

15.1.2 Structure

15.1.2.1 Virus Particle

The virions of animal rhabdoviruses are bullet-shaped, have a length of approximately 180 nm and a diameter of 65 nm; virions of plant rhabdoviruses can be twice as long. The structure of vesicular stomatitis virus is well known, and is probably similar in other animal rhabdoviruses. The infectious particles are composed of two components: a nucleocapsid and an envelope. The nucleocapsid is folded into tight helical coils – 35 in vesicular stomatitis virus – and consists of the viral proteins N (60 kDa), P or NS (40 kDa) and L (190 kDa) as well as the RNA genome (Fig. 15.1). The L protein is the RNA-dependent RNA polymerase, which is a component of the virion in negative-sense RNA viruses. The nucleocapsid is surrounded by an envelope, in which the viral glycoproteins G (64–68 kDa) are embedded as trimeric complexes, protruding about 10 nm from the particle surface. The matrix protein M (25–26 kDa) is associated with the inner side of the envelope membrane and interacts simultaneously with the proteins of the nucleocapsid via basic amino acids, hence ensuring its tight encapsidation inside the particle. The envelope of the virus exhibits a higher content of cholesterol,

a Vesicular stomatitis virus



b Rabies virus (strain PV)

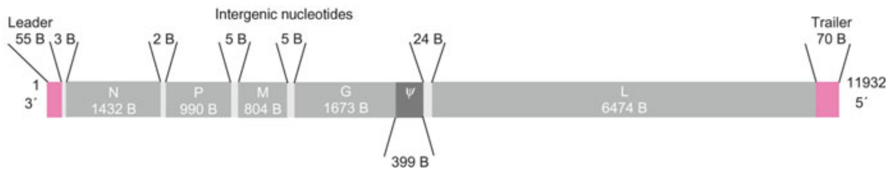


Fig. 15.2 Genome organization of rhabdoviruses. (**a**) Vesicular stomatitis virus. (**b**) Rabies virus (strain PV). The lengths of the various genes refer to the messenger RNAs (mRNAs) which are synthesized during the replication process. The non-coding regions, i.e. the intergenic sequences that are skipped during the synthesis of mRNAs, are pictured in *light grey*, and *red bars* represent the leader and trailer regions at the 3′ and 5′ ends, respectively. In the rabies virus genome, a non-coding region is located within the gene for the G protein, which is referred to as pseudogene or ψ gene (*dark grey*). It is separated from the G sequences by a transcriptional stop signal, which, however, is not used in all cases. Therefore, read-through transcripts can also be found, which reach to the end of the pseudogene. *B* base

aminophospholipids and sphingomyelin in comparison with the cell membrane and is significantly more flexible owing to this composition. The size of the genome determines the length of the virions. In all preparations, there are non-infectious particles (also known as defective-interfering particles), which, having the same diameter, are only 60–90 nm long. The genome of defective viruses can be shortened by up to 80 %.

15.1.2.2 Genome Organization and Structure

The single-stranded RNA genome of most rhabdoviruses is composed of about 12,000 nucleotides (11,162 in vesicular stomatitis virus, 11,932 in rabies virus, strain PV) and has negative-strand polarity; therefore, it cannot be used directly as messenger RNA (mRNA), and is not infectious. It has five open reading frames, which are arranged in 3′–5′ orientation in the order N, P, M, G, L (Fig. 15.2). At the 3′ end of the N gene, there is a short, non-coding leader region (47 nucleotides in vesicular stomatitis virus, 55 nucleotides in rabies virus). At the 5′ end there is also a short non-coding region (57 and 70 nucleotides in vesicular stomatitis virus and rabies virus, respectively). Both regions contain *cis*-active sequence elements that are essential for the initiation of transcription and replication. At their 3′ and 5′ ends, the individual genes have conserved nucleotides, which signal the start and the end of transcription.

Non-coding, intergenic segments of variable length are located between the individual genes: in vesicular stomatitis virus they are composed of guanosine and adenosine dinucleotides in all cases. The genome of this virus is highly condensed, and has very few non-coding regions. In rabies viruses, two nucleotides can be

Table 15.2 Molecular properties of rabies virus proteins

Proteins	Molecular mass (kDa)	Modification	Function
N	60	Phosphorylated	Component of the nucleocapsid, about 1,800 units per particle, RNA-binding protein, responsible for genome condensation in the virion; superantigen
P	40–45	Phosphorylated	Component of the nucleocapsid, about 900 units per particle, transcription cofactor
L	190	Unknown	Nucleocapsid component, approximately 30–60 units per particle, RNA-dependent RNA polymerase, capping and polyadenylation of viral messenger RNA species; methyltransferase, protein kinase for modification of P protein?
M	25–26	–	Matrix protein; located on the inside of the capsid; important for viral morphogenesis
G	64–68	N-glycosylated	Envelope protein; mediates attachment, fusion, haemagglutination; induces synthesis of neutralizing antibodies

found only between the N and P genes; in other intergenic regions they are pentanucleotides. Between the genes for the G and L proteins, there is a relatively large non-coding section of 423 nucleotides, whose function is unknown and is referred to as pseudogene ψ. A mRNA is synthesized from this region in infectious haematopoietic necrosis virus of salmonid fishes (a member of the genus *Novirhabdovirus*). Its function is unclear and it is unknown whether it codes for a sixth viral protein. The gene has length similar to that of the HN gene of paramyxoviruses (Sect. 15.3), which is located at a comparable genomic position in these viruses. Thus, it has been inferred that infectious haematopoietic necrosis virus of salmonids represents an evolutionary link between vesiculoviruses and paramyxoviruses. Comparative sequence analyses of the pseudogenes, which as a non-coding region are particularly vulnerable to mutations, enable one to reconstruct the evolution of the various rhabdoviruses.

15.1.3 Viral Proteins

15.1.3.1 Nucleocapsid Proteins

N Protein

The N protein is the main component of the nucleocapsid. It is associated with the RNA genome over its entire length and is responsible for folding of the helical nucleocapsid. On the basis of electron-microscopic data, it is estimated that 1,800 N proteins per particle are associated with the RNA genome. The N protein has a molecular mass of approximately 60 kDa and is phosphorylated. Investigations by Monique Lafon and colleagues demonstrated that the N protein of rabies virus can acts as a superantigen (Table 15.2). Independently of the epitope, it can connect MHC class II proteins with certain V_{β} chains of T-cell receptors on the surface of

$CD4^+$ cells, and is also able to induce their proliferation and release of cytokines (▶ Chap. 7, Sect. 15.1.5). In addition, the N protein is the group-specific antigen of rhabdoviruses: antibodies and T-helper (T_H) cells, which are directed against N-protein-specific epitopes of rabies viruses, can cross-react with the N proteins of other members of the genus *Lyssavirus*, but not with those of vesiculoviruses and vice versa. This indicates that many amino acids in the N proteins are conserved among the members of the respective genera.

P Protein

In the older literature, this protein is referred to as NS (non-structural) or M1 protein. It has a molecular mass of 40–45 kDa, and approximately 900 molecules are associated with the nucleocapsid. The P protein is phosphorylated at about 20–30 serine and threonine residues. This high degree of modification with acidic phosphate groups is responsible for the unusual running behaviour of the protein in sodium dodecyl sulphate–polyacrylamide gels: accordingly, its molecular mass is only approximately 33 kDa. The P protein interacts with both N and L proteins, and seems to reduce the interaction of N proteins with the RNA genome, thus making them accessible to the polymerase complex. Rabies virus mutants carrying a defective P gene which were generated by reverse genetics can perform only the primary transcription steps in infected cells – they bring the necessary P proteins as part of the virus particles into the cells; however, they are not able to replicate their genomes, and to form infectious virus progeny because the de novo synthesis of P proteins does not occur.

P proteins are not only components of the nucleocapsid and actively participate in transcription and replication of the viral genome, they are also involved in suppressing non-specific immune responses, and inhibit interferon regulatory factor 3 (IRF-3) phosphorylation. Thereby they suppress the expression of interferon genes, and prevent the nuclear transport of STAT1 proteins, which induce the non-specific immune response as part of the interferon-mediated signalling cascade (▶ Chap. 8).

L Protein

The L protein is an RNA-dependent RNA polymerase. Approximately 30–60 copies of the enzyme interact with the P proteins of the nucleocapsid. The very large L protein contains 2,142 amino acids, and the corresponding coding region comprises more than half of the genome in some rhabdovirus types. It has a theoretical molecular mass of more than 244 kDa; however, a molecule of 190 kDa has been detected experimentally. It corresponds to the active enzyme. Whether proteases lead to the formation of a truncated form, or whether modifications are responsible for the different molecular mass, remains to be elucidated. The sequences of L proteins are highly conserved among vesiculoviruses and lyssaviruses; the central region of the protein exhibits a homology of 85 %. In addition to its RNA polymerase activity, the L protein has an RNA:GDP polyribonucleotidyltransferase activity and is involved in RNA capping. Presumably, it is also involved in polyadenylation of primary transcripts, as well as phosphorylation of P proteins.

15.1.3.2 Envelope Proteins

M Protein

The M protein ("M" for "matrix") is not an integral envelope protein. It is not glycosylated, and has no hydrophobic sequence with the characteristic features of transmembrane regions. Its molecular mass is 25–26 kDa. The M protein contains many basic amino acids, by which it binds with its amino-terminal domain to the phosphorylated N and P proteins of the nucleocapsid. The carboxy-terminal region of the M protein seems to interact with the acidic phosphate groups of membrane lipids through similar ionic interactions. Thus, the M protein acts as an adhesive between the envelope membrane and the nucleocapsid. M proteins spontaneously accumulate in the cytoplasmic membrane, and are able to form virus-like membrane-containing vesicles, which are released from the cell surface. They are essential for assembling the various viral components into infectious particles, and by their intracellular localization determine the site of the budding process.

G Protein

The G protein is glycosylated and anchored in the envelope membrane by a hydrophobic domain at the carboxy-terminal region. A short amino acid sequence of 44 residues at the carboxy terminus, which is oriented towards the cytoplasm in the membrane-anchored protein, is probably involved in the aggregation of G proteins to trimers. Depending on the extent of its glycosylation, the monomeric G protein has a molecular mass of 64–68 kDa. The amino acid sequences of G proteins of various rhabdoviruses are very heterogeneous, but there are indications that they are similar in respect of their conformation and structural properties. The G protein is biologically active only as a trimer: it mediates attachment of virus particles to the respective cellular receptors, and is involved in membrane fusion processes that are induced by acidification of the interior of endosomes after penetration into the cell, which finally lead to the release the nucleocapsid into the cytoplasm. To induce the fusion activity, proteolytic cleavage of the G protein is not necessary, as is known from paramyxoviruses, orthomyxoviruses and retroviruses (Sects. 15.3.3, ▸ 16.3.3, and ▸ 18.1.3). Structural analysis revealed that G proteins differ significantly with regard to their conformation in the prefusion and postfusion stages. In the postfusion stage, G proteins of vesicular stomatitis virus exhibit homologies to the gB glycoprotein of herpes simplex virus (▸ Sect. 19.5). Additionally, the G protein is responsible for the haemagglutination property of the virus. Neutralizing antibodies against defined epitopes of the G protein are produced during infection. With use of monoclonal antibodies against different rabies virus isolates, five epitopes have been characterized, and these are responsible for binding of neutralizing immunoglobulins. In this way, the seven different rabies virus types can be distinguished.

15.1.4 Replication

In the first stage of the replication cycle, rhabdoviruses bind to cellular receptors by their G proteins on the particle surface. The cellular structures responsible for the

attachment of both vesicular stomatitis virus and rabies virus have not yet been definitively resolved. In vitro experiments with Vero cells (kidney cell cultures of vervet monkeys) infected with vesicular stomatitis virus provided evidence that binding is mediated by phosphatidylserine molecules in the cytoplasmic membrane; however, recent studies showed that phosphatidylserine does not act as a receptor, but it is only involved in internalization of the virus after receptor binding. Similarly, cell membrane phospholipids and glycolipids have also been identified as interaction partners in rabies viruses. Even in this case, further components of the cytoplasmic membrane seem to be additionally involved in specific binding of G proteins. Participation of nicotinic acid dependent acetylcholine receptors in binding the virus to the surface of neuronal cells has been assumed, because attachment of rabies viruses to neurons can be inhibited by incubation with neurotoxins such as α-bungarotoxin; this notion is also supported by the fact that G protein exhibits homology to neurotoxins at amino acid residues 189–214. In addition, binding of rabies virus to the cell surface protein CD56 (neural cell adhesion molecule) has been described. This protein is produced especially in the nervous system of adult animals. Furthermore, the neurotrophin receptor $p75^{NTR}$, a member of the TNF receptor family, has been described as being involved in the interaction between the cell and rabies virus. Nevertheless, binding of viral G proteins to $p75^{NTR}$ is not sufficient for penetration of the virus.

Following attachment, the subsequent penetration of virus particles into the cell is performed by receptor-mediated endocytosis. The acidification of the vesicle interior by an ATP-dependent H^+ ion pump in the endosomal membrane changes the conformation of the G protein, and activates its fusogenic activity; the membranes merge and the nucleocapsid enters the cytoplasm.

Subsequently, N, P and L proteins that are complexed with the nucleocapsid perform the primary transcription of the negative-sense RNA genome. This process begins at the 3′ terminus of the genome (Fig. 15.3), where the sole initiation site for RNA synthesis is situated. First, a short RNA is synthesized, which is complementary to the leader region. This RNA molecule is not capped and not polyadenylated; it does not encode a protein. The role of the leader RNA, which is produced in large quantities, is not known exactly. In cells infected with vesicular stomatitis virus, these RNA molecules appear to be transported into the nucleus and to interfere with the DNA-dependent RNA synthesis. This blocks the transcription in the host cell, and thus the synthesis of cell proteins. The cellular metabolism is now used exclusively for viral replication. The molecular details of this process have not yet been completely elucidated. In contrast to vesicular stomatitis virus, in rabies viruses the shutdown of cellular functions is not very excessively pronounced.

Transcription of the viral genome stops at the end of the leader region, and the intergenic nucleotides are skipped. The RNA-dependent RNA polymerase resumes its activity at the beginning of the N gene, and synthesizes the complementary mRNA, which is subsequently capped. At the end of the N gene there is a uridine-rich sequence motif that serves as a signal for polyadenylation (5′-AGU$_7$CAUA-3′). This sequence motif has also been discovered at ends of the other protein-coding regions. The polymerase complex reads over the intergenic sequences and resumes

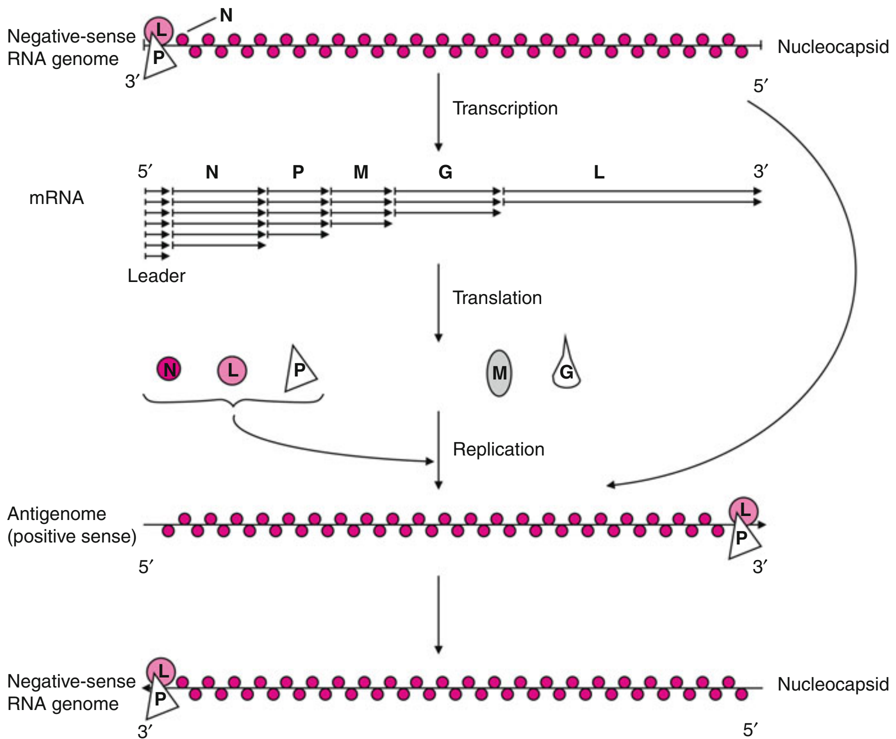


Fig. 15.3 Genome replication in rhabdoviruses. The negative-sense RNA genome of rhabdoviruses is present in the cytoplasm of infected cells as a complex with the viral proteins N, P and L. The first step is the synthesis of mRNAs, which is catalysed by the RNA-dependent RNA polymerase activity of the L protein; subsequently, the mRNAs are translated into the corresponding proteins. The short leader RNA does not code for proteins and probably has regulatory functions. The transcription stops at regulatory sequences between the individual genes, skips the intergenic regions and resumes mRNA synthesis. This process is not always successful. This results in a concentration gradient of transcripts, which steadily decreases with advancing direction of transcription. If a sufficient amount of newly synthesized N proteins is present in the cell, then they together with the P and L proteins cause the regulatory elements not to be used at the transition sites between the genes. The result is a continuous positive-sense RNA molecule that is complexed with N proteins throughout its entire length. It serves as an antigenome, and thus as a template for the synthesis of genomic negative-sense RNA molecules

its activity at the beginning of the P gene, a process that is repeated at all other transition sites. This process of skipping and reinitiation is not always successful, so subsequently a gradient is formed among the newly synthesized mRNA species, which continuously decreases towards the 5′ end of the genome (Fig. 15.3). Since the mRNA species are translated, a protein concentration gradient also arises in infected cells, in which the N protein concentration is the highest and the L protein concentration is the lowest.

The switch from the transcription mode with synthesis of individual monocistronic mRNA species to the replication mode with the formation of a continuous RNA molecule, which serves as an intermediate for the synthesis of new negative-sense viral genomes, depends on the quantity of N proteins that were produced in the cell. The N protein interacts with RNA sequences during their synthesis and prevents termination of transcription at the ends of the respective gene, so a continuous positive-sense RNA molecule results (Fig. 15.3). At its 3′ terminus, i.e. in the sequence following the L gene, the synthesis of new genomes is initiated, and they in turn form complexes with N proteins. P and L proteins bind to the negative-sense RNA that is coated with N proteins and form the nucleocapsids, which in the next step interact with M proteins. The aggregation of M proteins to the nucleocapsid induces the condensation into the helical structure.

The synthesis of G proteins starts with the translation of the amino-terminal signal peptide, and continues on the endoplasmic reticulum membrane, after association with the signal recognition particles. The signal peptide is removed by the cellular signalase and the G protein is translocated into the lumen; it is anchored in the membrane by the carboxy-terminal hydrophobic region and is glycosylated during further trafficking through the Golgi apparatus to the cytoplasmic membrane. Via binding to M proteins, the complex interacts with regions of the membrane of the endoplasmic reticulum and the cytoplasmic membrane, which have a high concentration of G proteins. As a result, the budding process is initiated, and in the meantime the nucleocapsid is enclosed by the membrane in the lumen of the endoplasmic reticulum and is released from the cell surface – especially in the case of rabies viruses.

The shutdown of the host cell metabolism is very pronounced in cells that are infected with vesicular stomatitis virus. The cells show a distinct cytopathic effect, which is a sign of cell damage (▶ Chap. 5). In cell cultures infected with rabies virus it is not nearly as pronounced, possibly indicating a lower replication rate, which results in fewer progeny viruses.

Rabies Has Long Been Known as a Contagious Disease

Rabies was known in ancient times. As early as the third millennium BC it was known that "rabid" dogs could transmit the disease through bites. For example, in the Babylonian statute book of Eshnunna, which was valid before the code of laws of the king Hammurabi (about 2300 BC), it was mentioned that the holder of rabid dogs had to face fines for deaths caused by them. Aulus Cornelius Celsus recognized in the first century BC the importance of wild animals and dogs for the transmission. He recommended the excision and cauterization of the bite wounds. In 1804, Georg Gottfried Zinke transmitted rabies by rubbing the saliva of rabid dogs in fresh wounds of rabbits in Jena. For the first time, in 1882, Louis Pasteur transmitted rabies intracerebrally to rabbits and developed a vaccine by continuous passage of the virus in rabbits. The vaccine virus (*virus fixe*) was characterized by a constant incubation period; by contrast, the incubation time of the wild-type virus

(*virus de rue*) was variable. The inoculum consisted of spinal cord material of infected rabbits, which was crushed and dried for 2 weeks. It was no longer infectious, but was immunogenic and in dogs caused protection from the infection. On 6 July 1885, Joseph Meister from Alsace was the first person vaccinated against rabies. On the basis of Pasteur's vaccine, the Semple vaccine was developed, which was used for many decades; it contained killed viruses, which were inactivated by phenol treatment. The Hempte vaccine, which is still used in developing countries, contains ether-extracted viruses from the spinal cord of infected rabbits. Since 1980, vaccines that contain in vitro cultivated, killed rabies viruses have been are applied in veterinary and human medicine (Sect. 15.1.5). Since 1954, patients with bites from potentially rabid animals have been passively immunized with immunoglobulin preparations containing antibodies against rabies virus, in addition to the active vaccine.

15.1.5 Human and Animal Pathogenic Rhabdoviruses

15.1.5.1 Rabies Virus

Epidemiology and Transmission

Rabies is found all over the world: it is also referred to as hydrophobia or lyssa because of the wolf-like howling of sick dogs. Epidemiologically, in principle, urban rabies and sylvatic or wild rabies must be distinguished from each other. This classification is based on the hosts which carry the endemic disease. Sylvatic rabies predominates in the countries of northern Eurasia and North America. In Europe and Asia, rabies virus infections and transmission of rabies virus to humans are or were primarily observed in terrestrial predators such as foxes, wolves and badgers, whereas skunks and raccoons are affected on the North American continent. By contrast, urban rabies is predominant especially in India, East Asia, Africa and South America. There, stray or roaming dogs are the main source of transmission of rabies virus to humans. Approximately 1,600 rabies cases in humans are reported to the World Health Organization per year. However, the real figure is very high – the actual number is estimated to be between 40,000 and 70,000. In Western countries, dogs do not play a significant role as vectors for transmission of the disease to humans because of widespread vaccination. Virus-free countries include the UK, Sweden, Norway, Finland, Switzerland, France, Belgium and – since April 2008 – Germany. In individual cases, infected dogs which are occasionally taken by travellers from endemic areas can import rabies to central Europe, and transmit it to non-vaccinated animals.

In South America, rabies virus is endemic in bloodsucking and fruit- or insect-eating bats. Vampires – blood-sucking bats – are the main carriers of rabies to cattle and other domestic animals, but they are rarely able to infect humans. Even bats in the Netherlands, Denmark and parts of the Baltic Sea area are infected with European bat lyssavirus (types 1 and 2). Virus isolates from bats can clearly be

distinguished serologically using monoclonal antibodies, and genetically by nucleic acid sequencing. The epidemiology of bat rabies is independent of that of the other types of wild rabies. Here, specific infection chains seem to exist, where transmissions to humans or domestic and wild animals are rarely observed. However, rabies cases caused by bat rabies viruses have been described both in humans and in animals; especially sick, flightless bats are suspected of being infected. Children should be strongly advised of this risk. Infectious viruses have also been detected in bat faeces. Therefore, inhalation of virus-containing dust is a possible way of infection for humans, especially for cavers, and occasionally there are patients without evidence of a bite wound. In Australia, which is considered rabies-free, Australian bat lyssavirus has been isolated from fruit-eating bats and fruit bats. To avoid official restrictions of the status "rabies-free", it has been designated "Australian bat lyssavirus".

Transmission occurs mainly through bites from infected animals, which excrete the virus in the saliva up to 12 days before the onset of the disease. The transmission by virus-containing aerosols (bat caves) is an exception, since here the infectious titre is significantly lower. In principle, rabies virus can infect all warm-blooded animals. However, the various species are differently susceptible to infection with rabies viruses. Foxes (the most important species for the transmission of rabies in Europe), wolves and jackals are particularly vulnerable and transmit the infection very effectively, whereas bats, cats, raccoons and dogs are less susceptible; horses and humans are considered to be not very susceptible and the opossum is considered to be largely resistant.

Transmission of Rabies by Organ Transplant

In 2004 and 2005, a total of seven deaths from rabies virus infection in transplant recipients became known in the USA and Germany. The patients were infected through the transplanted organs (liver, lung, kidney, pancreas), which came from two donors. Both died from clinically less apparent symptoms of rabies. The case in 2004 occurred in the USA, A 20-year-old drug-addicted American was admitted to the emergency department of a hospital with nausea, vomiting and swallowing difficulties. A short time later, he was admitted to another hospital in a disoriented state and with fever and died shortly afterwards. The cause of death was diagnosed as brain haemorrhage due to drug abuse. His organs (lung, kidney, liver, abdominal aorta) were transplanted into five patients: four died of rabies, one due to transplant-related complications. Retrospective investigations revealed that the organ donor had been bitten by a bat some time before the appearance of symptoms, and he was probably infected with the rabies virus by the bite.

In the second case, in Germany, the organs (liver, lung, kidney, pancreas, cornea) of a 26-year-old woman who died and who had had behavioural disorders, headache and fever were transplanted into six patients. Cocaine addiction was also diagnosed in this organ donor, and toxic psychosis was

documented as the cause of death. However, also in this case it could be demonstrated that the patient had had rabies. She was probably infected by a dog bite during a stay in India a few weeks before. Three of the transplant recipients died of rabies in the following weeks, but the recipient of the liver survived because he had been vaccinated against rabies in previous years; accordingly, neutralizing antibodies against the G protein were detected in his serum. The two recipients of the corneas survived as well, but the transplanted corneas were removed as a precautionary measure, although they proved to be virus-free by PCR analysis.

These two cases exemplify that sometimes the symptoms of rabies may be very unclear, and are frequently not recognized by the treating clinicians owing to their rarity. Furthermore, they also show that in organ donation a number of infectious pathogens can potentially be transmitted which cannot be diagnosed in the donors with current detection tests.

Clinical Features

The incubation period of rabies can differ considerably in humans and animals (from 10 to 12 days to several months). Mainly, it lasts between 2 and 7 weeks. The development of symptoms needs more than 3 months in only 10 % of cases. However, individual cases have been described in which the rabies appeared only after a few years. The course of infection is almost invariably fatal, as soon as the first symptoms are detectable.

Compared with other mammals, humans are considered to be relatively insusceptible to rabies. About 15 % of people who were bitten by rabid dogs died before the introduction of passive and active immunization. Frequently, no signs of disease are manifested in spite of the bite of a proven rabid animal; probably, the infection could not be established. Bite wounds to the face and head entail a much higher risk of disease than those to the arms or legs. The distance of the bite wound from the brain, where the disease is manifested, the quantity of virus that is transmitted and the possibly different virulence of various isolates are crucial for the length of the incubation period and the outbreak of rabies.

The first signs of rabies are pain and burning at the bite site, often associated with headaches, joint stiffness and fever. The acute neurological symptoms are hyperactivity, convulsions, hyperventilation and paralysis. These symptoms can develop into a phase of "raging rabies" including hydrophobia (fear of water), swallowing spasms, increased fever, augmented salivation and mental confusion, which lasts about 2–7 days. In 20 % of patients, this phase is followed by the stage of "quiet rabies", which is characterized by paralysis, unconsciousness and coma. Death results from respiratory failure. The course of rabies that is transmitted by bat bites is different from that caused by, for instance, wolf bites. Nonetheless, in such cases patients with overt infections also develop neurological symptoms and die.

In wild and domestic animals, the symptoms are multifaceted. Behavioural changes and frequently aggression are in the foreground at the beginning of the disease ("raging rabies"). In the advanced stage of "quiet rabies", the main symptoms are paralysis and convulsions. Characteristic symptoms are laryngeal convulsions and a subsequent apparent hydrophobicity, intake of stones or other objects (allotriophagy) and very often spasms of smooth muscles (tenesmus) and sustained roar. The symptomatic disease is fatal in all hosts mentioned.

Untreated Rabies Is (Almost) Always Fatal in Humans

It is not clear whether there are asymptomatic forms of rabies. Medical screening of veterinarians and raccoon hunters in the USA revealed that low concentrations of rabies-virus-specific antibodies were present in some healthy individuals. These data may evidence that inapparent rabies infections are possible in rare cases. Since the observed antibody levels were very low, it cannot be excluded that they are attributable to non-specific reactivities. So far, the only verified case is a 15-year-old girl who was not vaccinated against rabies after she had been bitten by a bat, and then developed febrile symptoms. After she had been admitted to hospital, high IgG titres against the viral G protein were found in her blood and cerebrospinal fluid, but no virus was found. This patient was put into an artificial coma; she had apparently controlled the pathogen immunologically and recovered from the infection without further consequences.

Pathogenesis

The pathogenesis of infection is similar in humans and animals. As a result of a bite, the virus enters the skin, subcutaneous tissue and muscle. It replicates initially in the skin, connective tissue or muscle cells at the bite wound or infects directly nerve endings and nerve fibres that supply the pertinent tissue by binding to acetylcholine and CD56 receptors. At least in cases with a short incubation time, it can be assumed that the virus reaches the peripheral nerves directly. Infection experiments in co-cultures of muscle and nerve cells have shown that rabies virus accumulates rapidly at the neuromuscular junctions. This also suggests a direct infection of nerve cells. Once it has reached the peripheral nerves, the virus migrates retrogradely through axons into the spinal cord at a speed of 8–20 mm per day; it proliferates there in secondary neurons, and crosses the synaptic cleft between the secondary and primary motor neurons, thus entering the brain. Thereby, entire particles are not transmitted, but the nucleocapsids are passed on from cell to cell. How viruses overcome the synapses is not known. Infectious viruses must be present for the transsynaptic transition because this process is dependent on the presence of G proteins in the viral envelope. When rabies virus mutants that do not produce G proteins are used, the virus remains restricted to the nerves at the inoculation sited and the transsynaptic transfer into secondary and

primary neurons does not occur. If the bite occurs in the face and the virus has direct access to cranial nerves, the brain is reached much faster.

The main replication sites in the brain are Ammon's horn, the hippocampus and the brainstem. The result is the development of an encephalomyelitis. Tissue changes are slight; perivascular infiltrates occur only towards the end of the disease. Almost exclusively neurons are destroyed. After proliferation in the brain, rabies virus migrates anterogradely along individual cranial nerves into the conjunctiva, salivary glands and cutaneous glands; at this stage, it is present in quantities of 10^6 particles per millilitre of saliva and lacrimal fluid. At the same time, an anterograde spread occurs into many peripheral organs, such as the adrenal glands. Infectious virus particles are produced only in this late phase of infection. It is not known how clinical symptoms arise. It is presumed that dysfunction of neurotransmitter systems is involved. There are lines of evidence suggesting that the paralysis symptoms are caused immunopathologically by cytotoxic T lymphocytes attacking against infected nerve cells. Possibly, this occurs through a interferon-mediated mechanism, as interferon-γ (IFN-γ) and interferon-α (IFN-α) are secreted by stimulated T_H cells and macrophages during the early phase of infection. In addition, apoptotic processes are induced in infected brain cells. Since brain cells are not able to regenerate, the symptoms can also be caused, at least partially, by neuronal death. Furthermore, infected tissue seems to elicit the synthesis of the inducible nitric oxide synthase, which also contributes to the death of brain and nerve cells. If its activity is specifically inhibited by aminoguanidine, the consequences are inhibition of caspase-1 in vitro and delayed apoptosis in vivo, which results in longer survival of infected mice.

The G protein appears to be crucial for the virulence of rabies viruses and their neuroinvasive properties. There are naturally occurring virus variants with mutations at amino acid position 333; such point mutations lead to a replacement of arginine by isoleucine or glutamic acid. These virus mutants do not kill immunocompetent mice; thus, they exhibit an attenuated virulence and a reduced tendency to induce membrane fusions in cell cultures of neuronal origin. Moreover, amino acid substitutions between positions 164 and 303 influence the virulence of virus isolates: non-pathogenic vaccine virus strains gain pathogenicity and spread rapidly in infected animals and in cell culture when asparagine is replaced by lysine at position 194.

$CD4^+$ T lymphocytes are of crucial importance for the immune control of infection. If they are removed in experimental animal systems, the infection cannot be controlled, even after previous vaccination. Therefore, the effect of the N protein as a superantigen seems to be important for the entry into the late phase of the disease with paralysis symptoms. Typical clonal deletions of T_H cells with certain V_β chains of T-cell receptors have been observed in mice. The effect of the N protein is based on an epitope-independent cross-linking of V_β chains of T-cell receptors with MHC class II antigens, which leads to continuous proliferation of T lymphocytes. They secrete increased amounts of various cytokines. This leads to a functional inactivation (anergy) of the respective T-cell clones. A part of the immunological defence reaction can be turned off by this N-protein-mediated mechanism, and as a result, the virus can spread more easily.

Immune Response and Diagnosis

No antibodies are detectable during the sometimes very long incubation period and the early stage of the disease. This can probably be attributed to the fact that the virus infects nerve cells very quickly; these are immune-privileged, do not possess MHC molecules on the surface and thus are not able to present foreign peptide epitopes. In addition, the virus moves along the axons in the form of nucleocapsids. Larger quantities of complete infectious virus particles are produced only at the end of the disease, so neutralizing antibodies against the G protein arise late. During the early phase of infection, natural killer cells do not seem to play an important role in the control of dissemination of the virus in the body. They recognize virus-infected cells by an MHC-independent mechanism. High concentrations of IFN-α and IL-2 can be detected in the organism at an early stage. Both cytokines can induce the activity of natural killer cells. Therefore, they play an important role in the context of non-specific defence reactions in the fight against virus-infected cells.

If G-protein-specific neutralizing antibodies are present in the late phase of infection or after vaccination, they can induce the antibody- or complement-mediated lysis of infected cells. Antibodies are also produced against nucleocapsid components. Monoclonal antibodies against specific epitopes of the N protein can provide protection from the disease for a long time after experimental infection, even if the virus is already present in the spinal cord. It is believed that N-protein-specific and G-protein-specific antibodies inhibit the transport nucleocapsids between neurons at the synapses. Cytotoxic T lymphocytes are directed primarily against epitopes of the G, N and P proteins. However, they are not sufficient to inhibit spread of the virus in the body. In this context, the activity of $CD4^+$ T_H cells is crucial.

Since the introduction of RT-PCR, diagnosis has been performed by detecting viral nucleic acids in cerebrospinal fluid, saliva and lacrimal fluid of infected people. Previously, laboratory diagnosis in humans was done by the histological evidence of Negri inclusion bodies (▶ Chap. 5, ▶ Fig. 5.3c) in touch preparations of the cornea and in skin biopsies from the neck. These are eosinophilic, cytoplasmic deposits of viral nucleoproteins present in rabies-virus-infected organisms and cells which were first described by Aldechi Negri in 1903. Negri inclusion bodies can be found in Amonn's horn of almost all patients who died of rabies.

In dead animals, diagnosis is performed by immunohistochemical or PCR detection methods. After an initial suspicion because of the conspicuous behaviour of the animal, diagnostic killing is prescribed by the official veterinarian for examination of the brain of the animal. Viral proteins can be detected in tissues by immunofluorescence tests using appropriate monoclonal antibodies. Killing of an infected or suspected animal can only be omitted if it has bitten a human. The infection of the animal is demonstrated by allowing the full expression of the disease under isolation conditions. If the animal does not die within 7–10 days, the case is not considered as rabies exposure.

Therapy and Prophylaxis

Pre-exposure vaccination of humans is done by a triple dose of killed rabies viruses, which were cultivated in human diploid cell lines (human diploid cell vaccine) or purified chick embryo cells (purified chick embryo cell vaccine). The vaccination is complemented after 1 year and boosted at regular intervals. When an unvaccinated person is bitten by a potentially rabid animal, postexposure prophylaxis must be carried out (postexposure vaccination). Every year, approximately ten million postexposure treatments are performed worldwide. They include regular wound treatment, passive immunization with locally and systemically administered immunoglobulin preparations which contain high concentrations of neutralizing antibodies against the G protein, and active immunization with inactivated vaccines. The latter are administered in six individual doses at short intervals. Owing to the long incubation period, this type of postexposure treatment is almost always successful, because it allows the development of a protective immune response before the possible onset of symptoms. Rabies is the only known viral disease in which an active vaccination can generate a lasting protection even for long time after exposure. The rabies vaccines available provide cross-immunity and protect against infections with the various rabies virus isolates from bats.However, there are only a few studies concerning the degree of protection.

Apart from the vaccination of humans, rabies is also effectively controlled by vaccination of domestic animals. Very effective killed vaccines are available against rabies of domestic animals. In most countries vaccination is not compulsory, but vaccinated dogs and cats are in a more favourable position in accordance with the animal health law, since they are not killed after contact with a suspected rabid animal, but they are only placed under veterinary observation. Rabies has been eradicated from some European countries (e.g. France, Belgium, Switzerland, Germany). This disease status has been essentially achieved by the very successful vaccination of foxes with a bait vaccine that contains attenuated rabies viruses. Contact is not dangerous for humans. Nevertheless, it is recommended to carry out the above-mentioned postexposure prophylaxis after contact with a bait vaccine. In the USA and some parts of Europe, a recombinant vaccinia virus which expresses the G protein of rabies virus is used for vaccination of wild animals. Recently, a modified bait vaccine has been developed for dogs. Its marketing authorization and use is urgently desired in countries such as India with a large number of human rabies cases after dog bites.

15.1.6 Animal Pathogenic Rhabdoviruses

Rhabdoviruses cause some severe animal diseases. The most important rhabdovirus is rabies virus, which was extensively discussed in the previous sections. Additionally, vesicular stomatitis virus plays an important role as a pathogen for cattle and horses on the American continent. It also has great importance as a model virus for the elucidation of the molecular processes that underlie rhabdovirus infections, and thus will be discussed in more detail.

Furthermore, bovine ephemeral fever virus is important as an animal pathogenic agent. It is widespread in tropical regions, causing a mild and transient disease in cattle, which is known as 3-day fever. The virus is transmitted by mosquitoes of the species *Anopheles* and *Culicoides*. Inactivated vaccines are available against the infection.

With regard to animal health requirements, rhabdovirus infections of fishes are important. These include, most notably, the pathogens of viral haemorrhagic septicaemia of trout, infectious haematopoietic necrosis of salmonids and the worldwide spring viraemia of carp. Infections with the first two pathogens are notifiable in several countries. The viruses are usually transmitted by direct animal contact, and lifelong virus carriers are epidemiologically important for maintaining infection chains.

15.1.6.1 Vesicular Stomatitis Virus

Epidemiology and Transmission

Vesicular stomatitis virus exists only on the American continent, and infects a number of animal species, such as horses, cattle and swine, but also humans. There are two serotypes, New Jersey and Indiana, and they are spread throughout the continent. Their infections do not elicit any cross-immunity. The virus is transmitted by a broad spectrum of insects, including mosquitoes, sandflies, blackflies, and other fly species. In addition to the insect-borne transmission, a direct transmission through animal contact or by indirect dissemination, e.g. by milking machines, is possible as well. The infection is important because of the economic consequences associated with the disease, which is especially noticeable in a decrease in milk yield in cattle.

Clinical Features

Vesicular stomatitis is associated with fever in cattle, swine and horses. The animals salivate and become lame. Intact or mostly burst vesicles (blisters) are found in the mouth, on the teats and on the coronary band, in swine often on the planum rostrale of the snout. In pigs and horses, infections with vesicular stomatitis virus can cause severe lameness. In humans, a transient, mild systemic infection with flu-like symptoms has been described.

Pathogenesis

The virus enters the skin through small injuries or arthropod bites and proliferates in the stratum spinosum. Spread occurs from cell to cell by direct infection of neighbouring cells. In contrast to the situation with foot-and-mouth disease (► Sect. 14.1.6), in such cases lesions are always primary aphthae.

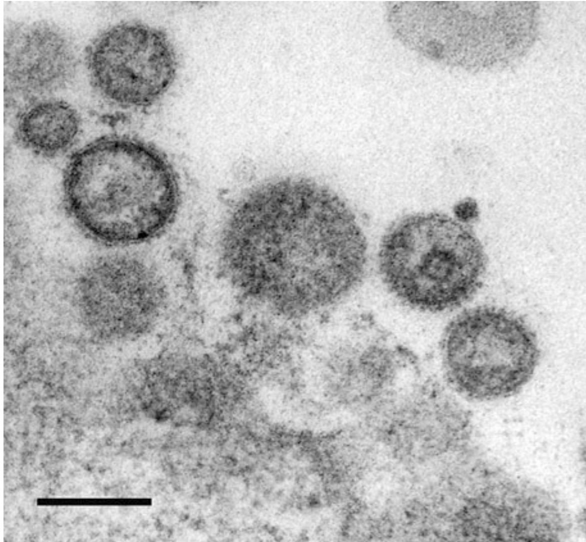
15.1.6.2 Immune Response and Diagnosis

Infections in cattle have to be clarified by differential diagnosis because of the similarity to foot-and-mouth disease. Diagnosis can be made by isolation of the virus from throat swabs, or better from the blister fluid followed by cultivation in Vero cells or by PCR detection.

15.1.6.3 Control and Prophylaxis

Vaccines are not available. Vesicular stomatitis is listed by the World Organisation for Animal Health as a notifiable disease.

15.2 Bornaviruses



Borna disease of horses was described in the village of Borna near Leipzig for the first time in 1894. In recent years, it has been becoming clear that, in addition to horses, also sheep and some other mammals can be infected with bornaviruses. Endemic regions with regionally limited occurrence of various virus strains are found primarily in Germany, Austria, Switzerland and Liechtenstein. The molecular characterization and genome sequencing was done by the groups of Juan Carlos de la Torre and W. Ian Lipkin nearly simultaneously in 1994. As a result, bornaviruses have been classified into the order *Mononegavirales*, as they have a non-segmented single-stranded RNA genome of negative polarity. However, they exhibit so many differences in genome organization that they cannot be assigned to the families *Rhabdoviridae*, *Paramyxoviridae or Filoviridae*. Their replication strategy also differs from that of other members of the order *Mononegavirales*: the genome replication of bornaviruses occurs within the nucleus and not in the cytoplasm of infected cells, as usually found in members of the order *Mononegavirales*. Consequently, bornaviruses have been classified into their own family, namely the family *Bornaviridae*.

Table 15.3 Characteristic prototypes of bornaviruses

Genus	Animal virus
Bornavirus	Borna disease virus (horses, sheep) (strains He-80, V, H24, H1766, etc.)
–[a]	Avian bornaviruses

[a] Proposed as a separate genus, but not yet known

15.2.1 Classification and Characteristic Prototypes

Bornaviruses infect mainly horses and sheep (Table 15.3). Nevertheless, they can be experimentally transmitted to a number of other hosts, such as rodents, cattle, cats, non-human primates and birds.

The creation a new genus has been proposed for recently described types of avian bornaviruses, but this has not yet been formally established and assigned a name.

15.2.2 Structure

15.2.2.1 Virus Particle

The spherical particles of bornaviruses are surrounded by a membrane envelope and have a diameter of 90–130 nm (Fig. 15.4). The viral glycoprotein GP is embedded in the envelope membrane (in older literature also known as G protein or gp84); after its synthesis, the GP protein is proteolytically cleaved into an amino-terminal fragment (gp56) and a carboxy-terminal moiety (gp43). The non-glycosylated matrix protein M (p16 or ORF3) is non-covalently associated with both the inside of the envelope and the P proteins of the helical ribonucleoprotein complex in the interior of the particle. It is constituted of N (p39 or ORF1, 39 kDa) and P (p24 or ORF2, 24 kDa) proteins, the RNA-dependent RNA polymerase (L protein, p190 or ORF5, estimated molecular mass 190 kDa) and the viral RNA genome. The N, P, M and GP proteins have been detected as gene products. The occurrence of p190 (L protein) is inferred from the size of the open reading frame and by analogy with other negative-sense RNA viruses.

15.2.2.2 Genome Organization and Structure

The genome of bornaviruses consists of single-stranded, negative-sense RNA and has a length of 8,910 nucleotides. The ends are complementary and can form panhandle-like double-stranded regions, with a length of 20 base pairs, and four terminal nucleotides are lacking at the 5′ terminus for complete hybridization to a double strand. These inverted terminal repeats are the promoters which induce the initiation of the synthesis of the complementary plus and minus strands during genome replication. The genome has six open reading frames (Fig. 15.5), which are

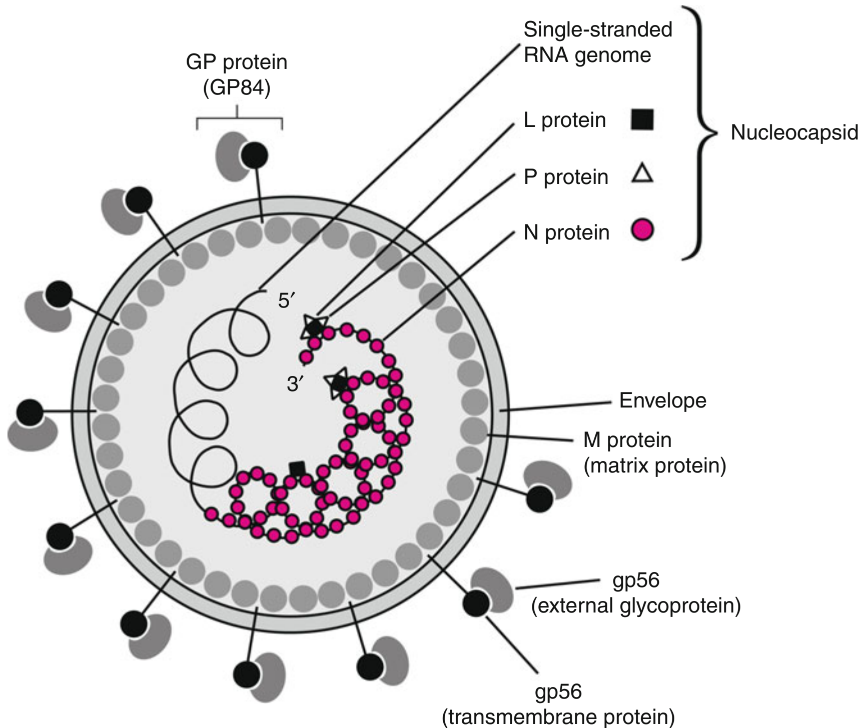


Fig. 15.4 Structure of a bornavirus particle

arranged in the order 3′-N-X-P-M-GP-L-5′. The reading frame for the X protein begins before that for the P protein and overlaps with it, but uses a different reading frame. An amino-terminal truncated variant of the P protein (P′) is generated by use of an alternative start codon. A sequence 43 nucleotides in length has been identified at the 3′ terminus of the genome; it does not code for proteins and corresponds to the leader region of other negative-sense RNA viruses. A trailer region of 55 nucleotides at the 5′ terminus is neither transcribed nor translated. Unlike the other members of the order *Mononegavirales*, bornaviruses do not exhibit the characteristic consensus sequences for termination and reinitiation of transcription between the open reading frames, which are separated by intergenic nucleotides (Sects. 15.1, 15.3 and 15.4). Transcription start signals are found only at four positions, namely at the 3′ end of the genome and upstream of the genes that encode proteins N, P and M. They are characterized by an uridine-rich sequence. Two introns were found in the genome, and are removed from the primary transcripts by alternative splicing. Intron I is located within the M reading frame, and is removed by alternative splicing during the synthesis of the mRNA species of the GP and L genes from a common larger precursor product, which is started at the

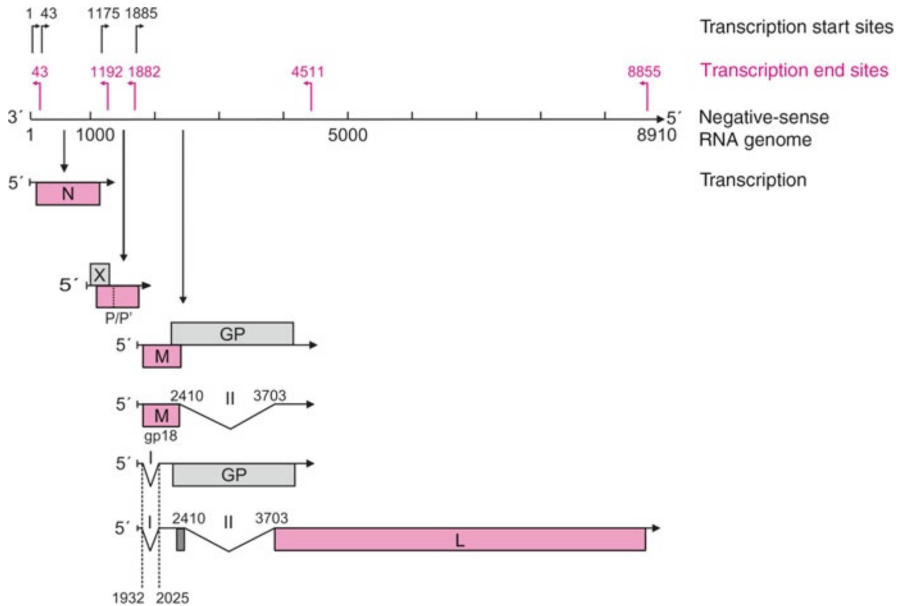


Fig. 15.5 Genome organization of bornaviruses. The genome is composed of a single-stranded, negative-sense RNA molecule. There are four transcriptional start sites (*right-directed arrows* above the genome *line*) and five transcriptional stop sites (*left-directed*, *red arrows*). Transcripts and splicing processes as well as the proteins that are translated from the different mRNAs are shown at the *bottom*. The different colour of proteins indicates that different reading frames are used for their synthesis. *I* and *II* represent the different introns that are excised from the transcripts by using alternative splice donor and acceptor sites

initiation sequence in front of the M gene. The excision of introns I and II is necessary for generation of the mature transcripts that serve for translation of the L protein (p190). If only intron II is excised, a mRNA arises that is used for translating the M protein. An unspliced, bicistronic RNA starts at position 1 of the genome and includes the N and P genes; it is an about 1,900-nucleotide-long transcript which is synthesized in two versions: a modified mRNA provided with a 5′-cap structure and a poly(A) tail, and an uncapped molecule that is not polyadenylated. The function of the non-modified version is unclear. The synthesis of the different mRNA species is stopped at four positions in the genome. These are characterized by the sequence $AU_{6\text{-}7}$, which is also the polyadenylation signal. They are situated immediately after the ends of the reading frames for proteins N, P, GP and L.

Avian Bornaviruses

Recently, it has been reported that bornaviruses also infect birds. Bornavirus-specific RNA sequences were detected in parrots with an inflammatory disease of the central, peripheral and autonomic nervous system.

This proventricular dilatation syndrome, also known as macaw wasting disease, is manifested primarily by gastrointestinal disorders, but also by ataxia and convulsions. Viral sequences are found not only in the nervous system of infected birds, but also in many other organs; infected birds excrete the virus in the faeces. Sequence analysis revealed that the pathogen detected is a classic bornavirus, although the sequence homology between these avian bornaviruses and equine isolates of Borna disease virus were less than 70 % at the RNA level and under 89 % at the protein level. Reports of experimental infections of susceptible parrots have not yet been published, so the aetiological role of these viruses in the clinical picture is still uncertain.

15.2.3 Viral Proteins

15.2.3.1 Ribonucleocapsid

According to its features, the nucleic acid binding N protein (39 kDa) corresponds to the N or NP proteins of other negative-sense RNA viruses and is associated with the genome. By using an alternative start codon in the same reading frame, an amino-terminal truncated version of the N protein (38 kD) is generated which lacks 13 amino acids and the nuclear localization signal present on this segment. The P protein is phosphorylated and also a component of the nucleocapsid; it acts as a cofactor for the activity of the L protein. The P protein is also synthesized in an amino-terminal truncated form (P′), which lacks the 55 amino-terminal residues. The functions of the truncated versions of the N and P proteins remain to be elucidated. The L protein corresponds to the RNA-dependent RNA polymerase of other negative-sense RNA viruses and is present in phosphorylated form. The X protein comprises 87 amino acids. It interacts with the cellular chaperone Hsc70: this interaction seems to bring about the transport of the X protein into the nucleus. Within the nucleus of infected cells, the X protein interacts with P proteins, which compete with the Hsc70 chaperones for the same binding site. Binding of X proteins to P proteins inhibits the RNA-dependent RNA polymerase activity of the L protein. How this affects the replication cycle in detail is unknown.

15.2.3.2 Envelope Proteins

The M protein is the matrix protein of bornaviruses. It assembles into homotetramers, and is essential for the formation of the particle structures. The GP protein has an amino acid sequence that exhibits the features of a type I membrane protein (glycosylation, amino-terminal signal peptide, carboxy-terminal localized, hydrophobic anchor region). In its glycosylated form it has a molecular mass of 84 kDa (gp84). After its synthesis in the Golgi vesicles, it is cleaved by the cellular protease furin into an external subunit (gp56) and

Table 15.4 Properties and functions of birnavirus proteins

Protein	Molecular mass (kDa)	Modification	Function
N	38/39		N protein; component of the nucleocapsid
P/P′	24/19	Phosphorylated	P protein; component of the nucleocapsid
M	16		M protein; matrix protein; tetramer
GP	84–94	Glycosylated	Envelope-anchored protein, cleavage by the protease furin into gp43 (carboxy-terminal domain, membrane fusion) and gp56 (amino-terminal domain, receptor binding)
L	190		RNA-dependent RNA polymerase; component of the nucleocapsid
X	10		Interaction with P protein and Hsc70

a membrane-anchored protein (gp43). Antibodies against the 244 amino acid residues of gp56 neutralize the virus. This also suggests the involvement of these protein domains in receptor recognition. The transmembrane protein gp43 has membrane fusion activity. Table 15.4 gives an overview of the characteristics of the bornavirus-specific proteins.

15.2.4 Replication

Many of the molecular details of bornavirus replication still remain uncertain; for example the cellular receptor has not been identified. Attachment of the virus is mediated by the protein gp56 on the surface of virions. After the virus has bound to the cell surface, penetration of the particles occurs by receptor-mediated, clathrin-dependent endocytosis. The cellular GTPase Rab5 seems to be involved in this process. The finding that bornaviruses cannot penetrate cells with reduced cholesterol content suggests the involvement of lipid rafts, which can only be formed under adequate conditions of high cholesterol levels. Transcription and genome replication occur not in the cytoplasm of the cell, but in the nucleus, a unique feature among the members of the order *Mononegavirales*. A nuclear localization signal is located in the amino-terminal domain of the N protein, and is responsible for the transport of the nucleoprotein complex to the nuclear pores and into the nucleus. The cell nucleus, as the site of the viral replication cycle, provides access to the cellular RNA splicing machinery. Thus, the M, GP and L proteins are translated from mRNA species that are generated by alternative splicing of a common precursor RNA (Fig. 15.5). A relatively long non-coding region is situated between the coding sequences of the genes N and P/X. The nucleotide sequences adjacent to the transcription termination signal at the end of the N gene determine the frequency of extended bicistronic transcripts with a length of 1,900 nucleotides, which span the genes N and P/X. This seems to be the mechanism that regulates the quantities of P and X proteins.

The processes that regulate the switch from the transcription to the replication mode during the bornavirus life cycle have not been examined in detail; however, the concentration of newly synthesized N proteins is presumably crucial, like in rhabdoviruses and paramyxoviruses. The complementary positive-sense RNA molecules (antigenomes) that are synthesized during viral genome replication exhibit (like the actual viral genomes) four overhanging nucleotides at the 3′ end. It has been postulated that strand synthesis is initiated at a cytosine residue located four nucleotides upstream from the 3′ terminus. It is also discussed that the synthesis of incomplete RNA strands limits viral replication in neuronal tissue, whilst maintaining gene expression. Furthermore, there are few data concerning the way in which bornaviruses are released from infected cells. This happens probably by budding from the cell surface.

15.2.5 Animal Pathogenic Bornaviruses

15.2.5.1 Borna Disease Virus

Epidemiology and Transmission

Borna disease, a sudden encephalomyelitis of horses and sheep, has been observed for 250 years and was first described in 1813. The name originates from an outbreak of the disease in a cavalry regiment in Borna (Saxony, Germany) in 1894. In addition, the disease has frequently been observed in certain regions of southern Germany and in Switzerland. In 1909, Ernst Joest and Kurt Degen discovered the inclusion bodies which were named after them in the nucleus of infected cells. In 1935, the pathogen was identified as a filterable agent and so as a virus.

It is believed that bornaviruses are spread worldwide in horses, sheep and goats; however, the pathogen has only been isolated from infected animals in Europe to date. Many different vertebrates, such as rabbits, rats, guinea pigs, mice, rhesus monkeys and birds, can be experimentally infected with bornaviruses, and establish persistent infections. Animals can be inoculated with the virus intracerebrally, intraperitoneally and intranasally. Since the virus persists in rats, and is excreted in large quantities in the urine following experimental exposure, it is believed that rodents are the natural reservoir of bornavirus. It has been discussed whether the staggering disease of cats is caused by a bornavirus infection. However, this association is still unresolved, and remains controversial because similar signs of disease can be found in cats which are not infected with bornaviruses.

It is believed that the transmission occurs naturally by droplet infection. It is not known how and in which phase of the disease infected animals excrete the virus. Studies of horses in central Europe revealed that about 12 % of horses have antibodies in the serum, but few of them develop symptoms of Borna disease. The virus is generally considered genetically stable. A novel subtype (No/98) was isolated from an infected horse; its nucleotide sequence differs from that of the reference strain by 15 %.

Clinical Features

After an incubation period of several weeks to several months, the first behavioural and gait abnormalities appear in infected animals. Later, hypoesthesia (decreased sensitivity to sensory stimuli), lethargy and paralysis are observable. The symptoms include disturbances of sensation, mobility and behaviour as well as somnolence, obesity, fever, and blindness, culminating in paralysis, nystagmus and coma. After experimental infection, chimpanzees develop behavioural disorders, and 90 % of the affected animals die within 1–3 weeks.

Bornavirus Infections in Humans: Fact or Fiction?

A great deal of interest has been generated by the finding that people with mental disorders (manic-depressive syndromes, schizophrenia and epilepsy) exhibit more often antibodies against bornavirus proteins N and P in comparison with the healthy human population; furthermore, bornavirus-infected monocytes have occasionally been detected in the peripheral blood of such patients. Whether bornavirus infection can actually cause psychiatric symptoms in humans has not yet been conclusively resolved. However, there are indications that the detection of bornavirus genomes in human cells is probably based mainly on laboratory contaminations. Bornavirus-specific antibodies that are detected in humans exhibit frequently, in contrast to immunoglobulins from infected animals, a relatively low affinity, which does not change over long observation periods. Therefore, it is assumed that the antibodies detected in humans are not directed against the bornavirus itself, but they may be directed against a related but not yet identified virus. Similarly, findings suggesting that bornavirus infections may trigger the development of obesity in humans are also unresolved.

Pathogenesis

In its natural transmission, bornavirus enters the nasal mucosa of animals. It has a strong neurotropism and ultimately infects the whole central and peripheral nervous system. The virus reaches the central nervous system probably by proliferating in the olfactory bulb. From there, it spreads predominantly in the form of nucleoprotein complexes intra-axonally into the brain and causes encephalomyelitis. Tissue alterations are limited to the grey matter, i.e. mainly cell-containing regions. They occur preponderantly in the limbic system through perivenous infiltration of macrophages as well as $CD4^{+}$ and $CD8^{+}$ lymphocytes. Viral proteins can be detected as Joest–Degen inclusion bodies in the nuclei of infected neurons and astrocytes.

Natural infections are usually subclinical in sheep, but the virus can be detected in such animals for months or years. It is not clear whether horses can

establish clinically inapparent, persistent infections. Experimentally infected rats and mice develop persistent infections. The X protein seems to play an important role in the induction of viral persistence because it inhibits the viral RNA polymerase by interaction with the P protein; it is also transported into the mitochondria of infected cells, and thereby prevents the induction of apoptotic processes. In addition, a restricted immune response can be detected in infected brains of persistently infected animals: possibly, the four overhanging nucleotides at the 3′ ends of the RNA genome and antigenome strands prevent the associated formation of an incomplete RNA duplex and, in the course of this, the activation of the RNA helicase retinoic acid inducible gene I (RIG-I). This enzyme usually recognizes double-stranded RNA or triphosphorylated 5′ ends of RNA molecules, and activates the non-specific immune system and the synthesis of class I interferons (► Chaps. 7 and ► 8).

Since bornaviruses do not cause a cytopathic effect in vitro, the pathogenesis of the disease is closely correlated to the immune response of the host. If adult Lewis rats – an important model system to study the pathogenesis of bornavirus – are infected, they become ill and die after a few weeks. Infected newborn or immunocompromised, athymic mice do not develop disease symptoms, and infectious viruses are detectable in the central nervous system lifelong. This persistent infection is characterized by the absence of inflammatory infiltrates. If rats with asymptomatic, persistent infection are specifically transfused with sensitized lymphocytes, then they will develop symptoms. In addition to these immunopathogenic mechanisms, some viral proteins seem to be directly involved in the development of symptoms. For example, the P protein interacts with γ-aminobutyric acid receptor associated protein in infected cells, thereby inhibiting the transport of the γ-aminobutyric acid receptor to the cytoplasmic membrane. Since γ-aminobutyric acid is an inhibitory neurotransmitter, it is presumed that the reduced γ-aminobutyric acid receptor concentration can cause neurological changes in infected animals.

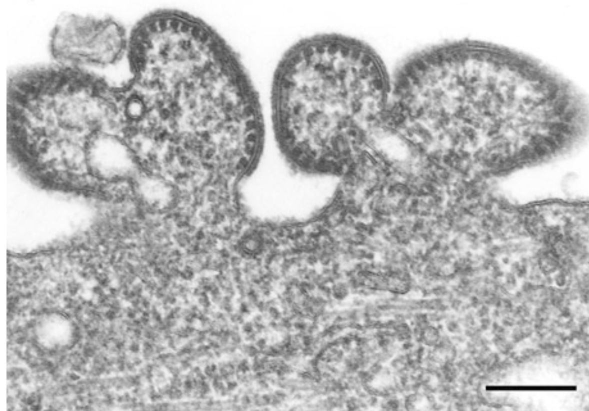
Immune Response and Diagnosis

Cytotoxic T cells and antibodies are primarily directed against N and P proteins, and immunoglobulins have a neutralizing effect against the external gp56 subunit of the GP protein. The detection of specific antibodies in serum or cerebrospinal fluid is performed using immunofluorescence tests, Western blotting or ELISA. The occurrence of viral RNA in blood and tissue cells can be detected by PCR.

Therapy and Prophylaxis

There is no therapy for Borna disease. In the former East Germany, a live vaccine was approved on the basis of attenuated bornaviruses. However, little is known regarding its efficacy and safety.

15.3 Paramyxoviruses



The family *Paramyxoviridae* exhibits great similarity to the families *Rhabdoviridae* and *Filoviridae* with respect to replication mechanisms and strategies. However, there are no sequence homologies at the nucleic acid and amino acid levels. Paramyxoviruses also have a non-segmented, continuous RNA genome with negative-sense polarity. They are widespread in humans and animals, and cause, to some extent, severe diseases. Examples of diseases caused by pathogenic paramyxoviruses are mumps and measles and their associated complications as well as the severe infections of the respiratory tract by respiratory syncytial virus. Measles virus rarely provokes persistent infections, where the virus remains in the organism for years. Severe animal diseases such as canine distemper and rinderpest are caused by pathogens that are closely related to measles virus. For many years, Sendai virus was regarded as a well-studied prototype of paramyxoviruses; it was discovered by Masahiko Kuroya and co-workers in the city of Sendai in Japan in 1952 as they infected mice with tissue samples from a child who had died of pneumonia. Therefore, the virus was initially considered to be a human pathogen. However, this pathogen is actually murine parainfluenza virus 1, which is widespread in mice, and rarely causes diseases in humans. Some years later (1956–1960), human parainfluenza viruses were discovered as the cause of diseases particularly of the respiratory tract of children. Parainfluenza virus 2 (croup-associated virus), which causes pseudocroup and was isolated from patients in 1955, is particularly of great importance among them.

In 1994, deaths of horses and horse owners rapidly gained international interest, although they occurred only in a small area in Hendra, a suburb of Brisbane (Australia). A paramyxovirus was identified as the causative agent, which was initially denominated "Australian equine morbillivirus". However, it was neither an Australian virus nor a horse virus or a morbillivirus, and thus it has been

Table 15.5 Characteristic prototypes of paramyxoviruses

Subfamily	Genus	Human virus	Animal virus
Paramyxovirinae	*Respirovirus*	Human parainfluenza viruses 1 and 3	Bovine parainfluenza virus 3 Sendai virus (murine parainfluenza virus 1) Simian virus 10
	Rubulavirus	Mumps virus Human parainfluenza viruses 2, 4a and 4b	Canine parainfluenza virus 2 Simian virus 5 Simian virus 41
	Avulavirus		Newcastle disease virus (avian paramyxovirus 1) Avian paramyxoviruses 2–9
	Morbillivirus	Measles virus	Canine distemper virus Rinderpest virus Peste-des-petits-ruminants virus Phocine distemper virus (distemper of seals) Whale morbillivirus Dolphin distemper virus
	Henipavirus	Hendra virus Nipah virus	Hendra virus (horses) Nipah virus (swine, dogs)
	Aquaparamyxovirus		Atlantic salmon paramyxovirus
	Ferlavirus		Fer-de-lance paramyxovirus
Pneumovirinae	*Pneumovirus*	Respiratory syncytial virus	Bovine respiratory syncytial virus
	Metapneumovirus	Human metapneumovirus	Avian metapneumovirus

designated as Hendra virus. This virus is endemic in fruit bats (*Pteropodidae*), which are native not only to the Australian continent, but also to New Guinea and many parts of Malaysia. In 1997 and 1998, another paramyxovirus was isolated from fruit bats, Nipah virus, which also causes severe diseases in humans and animals. In recent years, it was repeatedly associated with regional epidemics in Malaysia, India and Bangladesh.

15.3.1 Classification and Characteristic Prototypes

Paramyxoviruses are classified into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*. The former are classified into seven genera (Table 15.5). Paramyxoviruses are further grouped according to the function of their envelope proteins: respiroviruses, rubulaviruses, avulaviruses, ferlaviruses and aquaparamyxoviruses have a neuraminidase and can agglutinate erythrocytes; thus, they have haemagglutination activity. Both functions are combined in the viral envelope protein HN (haemagglutinin–neuraminidase). Rubulaviruses, which include

mumps virus, additionally encode a small hydrophobic SH protein that is associated with the envelope. With respect to ferlaviruses and aquaparamyxoviruses, there are only few data: fer-de-lance paramyxovirus was isolated from snakes, whereas Atlantic salmon paramyxovirus is an important cause of loss in seawater-farmed Atlantic salmon. Morbilliviruses, which include measles virus, exhibit haemagglutination properties, although they do not possess a neuraminidase. Hendra virus and Nipah virus from the subfamily *Paramyxovirinae* are taxonomically classified into the genus *Henipavirus*. The members of the subfamily *Pneumovirinae* constitute the two genera of pneumoviruses. These include respiratory syncytial virus and metapneumovirus. Both genera have neither haemagglutination nor neuraminidase activity. Human metapneumovirus, which was isolated in the Netherlands from children with respiratory infections in June 2001, and avian metapneumovirus, also known as turkey rhinotracheitis virus, are currently the only two known metapneumoviruses. They differ from pneumoviruses in the gene sequence in the genome, but have the same particle structure.

15.3.2 Structure

15.3.2.1 Virus Particle

Infectious virus particles have a diameter of 150–250 nm. They are primarily spherical, but filamentous forms can also be found occasionally. The nucleocapsid consists of P, L and N/NP proteins, which are associated with the single-stranded, negative-sense RNA genome, forming a helical nucleoprotein complex. It is surrounded by an envelope (Fig. 15.6). The L protein ("L" for "large", molecular mass above 200 kDa), possesses RNA-dependent RNA polymerase activity and is associated with the P protein ("P" for "phosphoprotein"). The N or NP protein ("N" for "nucleocapsid") is the main protein component of the nucleocapsid.

Surface proteins are embedded in the viral envelope: the F protein ("F" for "fusion") is a heterodimer composed of F_1 and F_2 subunits. It is glycosylated and modified with fatty acids and has a molecular mass of about 60–65 kDa. It mediates the activity of the virions to induce membrane fusions. It can be found in all paramyxoviruses.

The HN protein ("HN" for "haemagglutinin–neuraminidase", molecular mass 60–70 kDa) of respiroviruses, avulaviruses, rubulaviruses, aquaparamyxoviruses and ferlaviruses possesses neuraminidase activity and exerts a haemagglutination effect. It is also responsible for the attachment of virus particles to the cells. In addition to modifications by sugar groups, even aliphatic acid molecules have been found to be attached to the protein in some virus types. It is an oligomeric protein, in which the individual components are connected by disulphide bonds. Most neutralizing antibodies are directed against this protein. In morbilliviruses, the H protein (79 kDa in measles virus) exerts the functions for attachment and haemagglutination; neuraminidase activity is lacking. The henipaviruses, pneumoviruses and metapneumoviruses lack both haemagglutination and neuraminidase activity. The envelope protein that mediates attachment of the

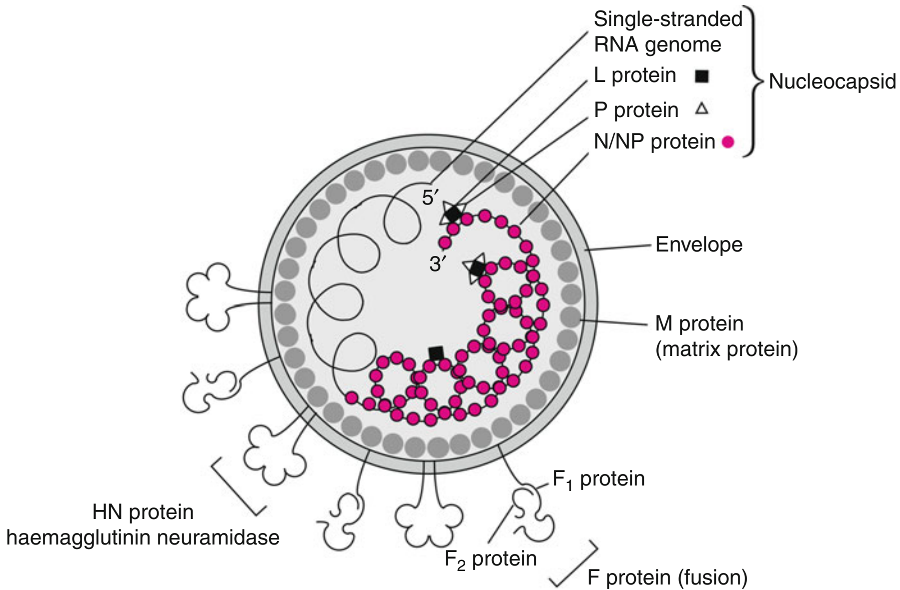


Fig. 15.6 Structure of a paramyxovirus particle using respirovirus as an example. The genome is composed of a single-stranded RNA, which interacts with the N, P and L proteins to form a helical nucleocapsid. The nucleocapsid is surrounded by an envelope containing HN (tetramers) and F (heterodimers composed of F_1 and F_2 chains) proteins. The M protein is associated with the inner side of the envelope and simultaneously interacts with the components of the nucleocapsid

virus to the cells is denominated G protein ("G" for "glycoprotein"). It has a molecular mass of 84–90 kDa, approximately two thirds of which is due to carbohydrate groups.

Another viral protein, M protein ("M" for "matrix"), is associated with the envelope membrane, but is not exposed on the surface of the particle. It lines the inside of the envelope and interacts with the N proteins of the nucleocapsid. It has a molecular mass of approximately 29 kDa (pneumoviruses) to 40 kDa (parainfluenza viruses) and is characterized by a high proportion of basic amino acids.

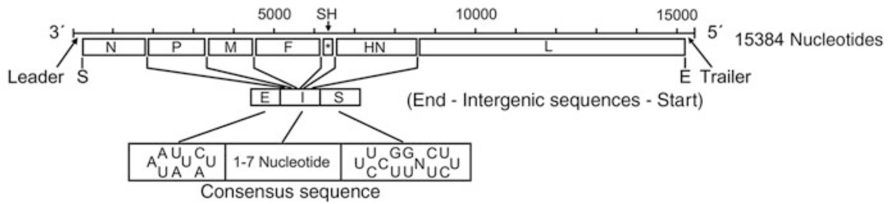
15.3.2.2 Genome Organization and Structure

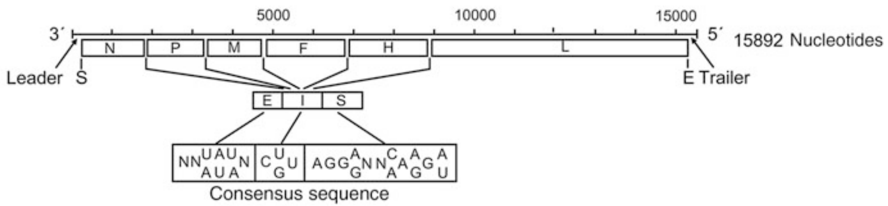
The genome of paramyxoviruses consists of single-stranded, negative-sense RNA; thus, it does not function as a mRNA, and is not infectious. The genome has a length of 15,384 nucleotides in mumps virus and Sendai virus, 15,463 nucleotides in parainfluenza virus 3, 15,892 nucleotides in measles virus and 15,222 nucleotides in respiratory syncytial virus. The genome of the newly isolated human metapneumovirus has a length of 13,373 nucleotides. Among animal pathogenic morbilliviruses, canine distemper virus and rinderpest virus have 15,690 and 15,882 nucleotides, respectively. The genomes of Hendra virus and Nipah virus are considerably longer: they comprise 18,234 (Hendra virus), 18,246 (Nipah virus,

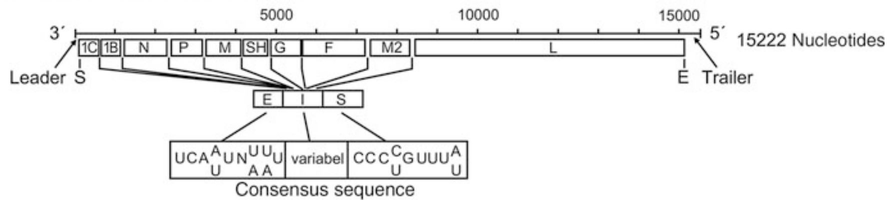
Malaysia strain) and 18,252 (Nipah virus, Bangladesh strain) nucleotides. The genome is complexed with proteins, forming a nucleocapsid. This complex has a left-handed helix structure with a diameter of 14–17 nm. A hollow cylinder of about 5 nm in diameter is formed inside the helix. Approximately 2,400–2,800 N protein molecules are associated with the RNA (11–13 molecules per helical turn). Accordingly, one N protein binds to about six nucleotides. The high protein loading protects the genome from destruction by nucleases, and confers on it the required flexibility for packaging into virus particles. In addition, the N protein together with the P and L proteins is necessary for transcription of the genome. The latter are also a part of the nucleocapsid: per particle, there are about 300 P protein molecules and 30 L protein molecules. These are associated by non-covalent interactions with each other and with the N proteins and can be experimentally detached from the nucleocapsid.

A short leader sequence of 52–54 nucleotides is located at the 3′ end of the genome, which is indeed transcribed, but not translated (Fig. 15.7). At the 5′ terminus of the genome, there is a non-transcribed region of 40–44 nucleotides, which is referred to as a trailer. In this region there are *cis*-active initiation signals, which promote the start of the polymerization reaction that leads to the synthesis of new genomic RNA molecules and their packaging into virus particles during assembly. The leader and trailer regions contain sequences which form secondary structures in the form of hairpin loops. However, these structures are not relevant for the initiation of transcription and replication; these processes are primarily dependent on the nucleotide sequence in the leader and trailer regions. Short non-transcribed sequences are also located between the various genes (intergenic nucleotides). They have the following sequences: CUU or CGU in measles virus, GAA or GGG in Sendai virus and GAA in parainfluenza viruses. In mumps virus, their length ranges between one and seven nucleotides. Conserved sequences are situated at the start and end sites of the genes (S and E consensus sequences). They flank the intergenic regions and ensure the orderly transcription of the genome segments.

Among the members of the subfamily *Paramyxovirinae* the arrangement of the genes on the nucleic acid molecule is very similar. In the 3′–5′ direction, the gene order is N-P-M-F-H/HN-L (Fig. 15.7). The order is different in the genome of pneumoviruses, which are characterized by the lack of the sequences encoding the HN and H proteins. The pertinent region comprises the genetic information for a gene product of approximately 22 kDa, which is denominated M2-1 protein and can be detected in irregular quantities in the matrix protein layer of virus particles. A short open reading frame (M2-2), which overlaps with the end of the M2-1 open reading frame, encodes a 90 amino acid protein of unknown function. The region of the genome that encodes the G protein is located between the genes for the M and F proteins. In addition, two shorter reading frames (1B or NS2 and 1C or NS1) are located in the 3′ region of the pneumovirus genome; their functions are also unknown. A 1A or SH gene is present in pneumoviruses and mumps virus. In mumps virus, it encodes a short, hydrophobic peptide of about 5 kDa, whereas SH proteins of 13–40 kDa can be found in pneumoviruses. On the other hand,







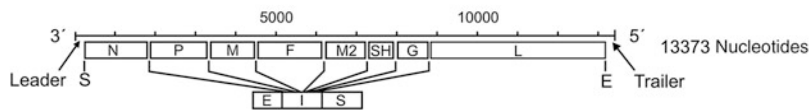


Fig. 15.7 Genome organization of paramyxoviruses. (**a**) Mumps virus. (**b**) Measles virus. (**c**) Respiratory syncytial virus. (**d**) Human metapneumovirus. The genome consists of a single-stranded, negative-sense RNA molecule. Beneath the line that represents the RNA, the position and length of the different virus-encoded genes are indicated; these are transcribed into mRNA during the replication process. Regulatory sequences are located at the ends and between the genes, and are designated E, I and S. The E sequences serve to stop the transcription, the I sequences (intergenic) are skipped and the S sequences control restarting of mRNA synthesis. The specific consensus sequences for the respective viruses at the gene transition sites are indicated; those of human metapneumovirus have not yet been conclusively determined. 1C (NS1) and 1B (NS2) are the genes encoding the non-structural proteins, which are found in respiratory syncytial virus. The SH gene codes for a small, highly hydrophobic membrane protein. It is found in the genomes of pneumoviruses and metapneumoviruses, as well as in mumps virus, but not in all members of the genus *Rubulavirus*. The 3′ terminus of the M2 transcript overlaps with the 5′ end of the mRNA for the L protein at the transition site between the M2 and L genes of respiratory syncytial virus and human metapneumovirus

the arrangement of genes in the RNA genome of metapneumoviruses is different from all others. They also encode only one glycoprotein, the G protein. Its gene is located directly before of the reading frame encoding the L protein. The reading frame for the G protein is preceded by the M2 and SH genes, whereas the reading frame for the F protein follows the sequences encoding the N, P and M proteins (Fig. 15.7d), as similarly found in respiroviruses, rubulaviruses and morbilliviruses.

In comparison with other paramyxoviruses, Nipah virus and Hendra virus have an extended reading frame encoding the P gene.

The genome of paramyxoviruses contains, in addition to these relatively easily identifiable genes, further encoding capacity: in the coding region for the P protein of measles virus as well as respiroviruses and rubulaviruses, a second reading frame is located which uses a different reading frame and encodes two forms of a non-structural protein (C and C′, "C" for "cellular") and the polypeptides Y1 and Y2, which use a different starting point. The X protein is an amino-terminal truncated version of the P protein. Another variation in respiroviruses and morbilliviruses is that mRNA molecules of the P gene region are modified by targeted insertion of guanosine residues during the process of RNA editing in such a way that new reading frames arise (for proteins V and W). By contrast, in rubulaviruses, the V protein is translated from an mRNA that is collinear to the genome sequence. On the other hand, the synthesis of the P protein is dependent on the insertion of two guanosine residues into the transcripts. The occasional synthesis of the D and I proteins has been found in some respiroviruses by combination of the different variation possibilities.

15.3.3 Viral Proteins

15.3.3.1 Structural Proteins

Envelope Proteins

The HN, H or G proteins are anchored in the envelope of the various paramyxoviruses and are responsible for the specific attachment of the virus particles to the cell surface. These are type II membrane proteins, i.e. they have no signal peptide at the amino terminus, but have a hydrophobic sequence of 25–30 amino acids in the vicinity of the amino terminus. It acts as a signal for the transport of the translation complex to the endoplasmic reticulum membrane, enables the protein chain to pass into the lumen and serves as a transmembrane region to anchor the polypeptide. In this case, a signalase does not cleave. Consequently, approximately 30–40 amino-terminal amino acids are located in the cytoplasm, and the sequences that follow the hydrophobic domain are oriented into the lumen of the endoplasmic reticulum. There or on the surface of the viral envelope, they are modified by addition of sugar groups. The proteins are present as oligomers, which are linked by disulphide bonds. Presumably, these are tetramers.

The HN protein of respiroviruses and rubulaviruses has a domain with strong homology to the neuraminidase of influenza viruses (▶ Sect. 16.3). It cleaves terminal *N*-acetylneuraminic acid (sialic acid) residues from complex carbohydrate groups. *N*-Acetylneuraminic acid residues are used by many members of paramyxoviruses as cellular receptors. They are modifications of lipid and protein components on cell surfaces. The attachment to them is mediated by the amino acids surrounding position 400 of the HN protein. The neuraminidase activity removes these receptor groups. Probably, this ensures the efficient spread of a virus population: On the one hand, the removal of these groups which act as a receptor renders the infection of the same cell with other virus particles more difficult. On the other hand, it prevents viruses released during the later stages of the replication cycle from reacting immediately with receptors of the infected and destroyed cell, and thus not being available for the next infection cycles.

The H proteins of morbilliviruses do not have neuraminidase activity, but they bind to *N*-acetylneuraminic acid residues and exhibit a significant homology to HN proteins of the other gener*a* of the subfamily Therefore, the structure of these proteins and their oligomerization might be very similar, especially because the positions of cysteine residues are highly conserved. This also suggests a close relationship between the HN and H proteins. With 298 amino acids, the functionally clearly different G proteins of pneumoviruses are only half the size of HN and H proteins, which are nearly 600 residues long. They exhibit, in contrast to the latter, a very high content of sugar modifications, which are linked O-glycosidically to serine or threonine residues. Therefore, the G proteins of pneumoviruses and metapneumoviruses have molecular masses of 90 or 80 kDa.

F proteins possess highly conserved sequences, and are anchored in the envelope. The heterodimeric complex of F_1 and F_2 units assemble into trimers and induces the fusion of the viral envelope with the cellular membrane after attachment of the particles to a cell. The F protein is synthesized as precursor polypeptide F_0. It possesses a canonical signal peptide at its amino terminus; this is required for transport of the translation complex into the endoplasmic reticulum membrane. After translocation of the amino acid chain through the membrane, a hydrophobic sequence at the carboxy terminus causes anchoring of the F_0 protein in the membrane, and the signal peptide is cleaved. The F_0 protein is glycosylated in the Golgi apparatus during its transport into the cell surface. The processing of the F_0 protein into the amino-terminal F_2 subunit (approximately 10–20 kDa in the different viruses; Table 15.6) and the F_1 protein is performed by a protease that is localized in the Golgi region. The cleavage site lies between a region of basic amino acids and a hydrophobic domain. After the cleavage, the latter constitutes the amino-terminal end of the membrane-anchored F_1 protein. The F_2 subunit remains covalently linked with the F_1 protein by a disulphide bridge (Fig. 15.8). As a result of proteolysis of the F_0 protein, a strongly hydrophobic region of about 25 amino acids is exposed at the amino terminus of the F_1 subunit, which constitutes the fusogenic domain of the F protein complex. The conformation of F proteins changes during the attachment process: sequence repeats consisting of two to

Table 15.6 Properties and functions of different paramyxovirus proteins

Protein	Parainfluenza virus 1	Mumps virus	Measles virus	Respiratory syncytial virus	Function
Structural proteins					
F_0	63 kDa	59 kDa	55–60 kDa	68–70 kDa	Precursor protein; envelope protein; it is processed into F_2 and F_1
F_2	10–12 kDa, glycosylated	18–20 kDa, glycosylated	18–20 kDa, glycosylated	20 kDa, glycosylated	Amino-terminal region of F_0; signal peptide at the amino terminus
F_1	50–55 kDa, glycosylated	46–48 kDa, glycosylated	41 kDa, glycosylated and acylated	48 kDa, glycosylated and acylated	Carboxy-terminal region of F_0; fusion peptide at the amino terminus, transmembrane region (type I membrane protein), neutralizing antibodies, forms with F_2 a fusion-active heterodimer (F protein)
HN	69–72 kDa, glycosylated and acylated	64 kDa, glycosylated and acylated	–	–	Binding to sialic acid, neuraminidase, haemagglutination; attachment; neutralizing antibodies; oligomer (dimer and tetramer), transmembrane region at the amino terminus (type II membrane protein)
H	–	–	79 kDa, glycosylated and acylated	–	No neuraminidase activity; other functions as for HN protein
G	–	–	–	84–90 kDa, O-glycosylated	No neuraminidase activity; no haemagglutinin; other functions as for HN protein
M	39.5 kDa	42 kDa	36 kDa	29 kDa	Matrix protein; forms a protein layer on the inner side of the envelope
N/NP	58 kDa	61 kDa	60 kDa	43 kDa	Nucleocapsid protein; interacts with the RNA genome
P	67 kDa, phosphorylated	42 kDa, phosphorylated	72 kDa, phosphorylated	27 kDa, phosphorylated	Complements the polymerase activity of L protein; binds to L and N proteins
L	256 kDa	257 kDa	200 kDa	250 kDa	RNA-dependent RNA polymerase; binds to N and P proteins; nucleocapsid component
M2-1	–	–	–	22 kDa, phosphorylated	Zn^{2+} binding, present in virions in small amounts, regulates transcription and replication

(*continued*)

Table 15.6 (continued)

Protein	Parainfluenza virus 1	Mumps virus	Measles virus	Respiratory syncytial virus	Function
Non-structural proteins					
1A/SH	–	6.7 kDa (57 amino acids)	–	64 amino acids	Membrane protein, hydrophobic, partially glycosylated.
1C/ NS1	–	–	–	14 kDa (139 amino acids)	Causes ubiquitination and proteolytic degradation of STAT2
1B/ NS2	–	–	–	15 kDa (124 amino acids)	Inhibits RIG-I-dependent expression of class I interferons
C	23.3 kDa (199 aa)	–	20 kDa	–	Inhibition of genome transcription
D	About 20 kDa	–	?	–	Amino-terminal truncated C protein
V	22–28 kDa, phosphorylated	24 kDa, phosphorylated	46 kDa, phosphorylated	–	Insertion of G residues by RNA editing; cysteine-rich

RIG-I retinoic acid inducible gene I

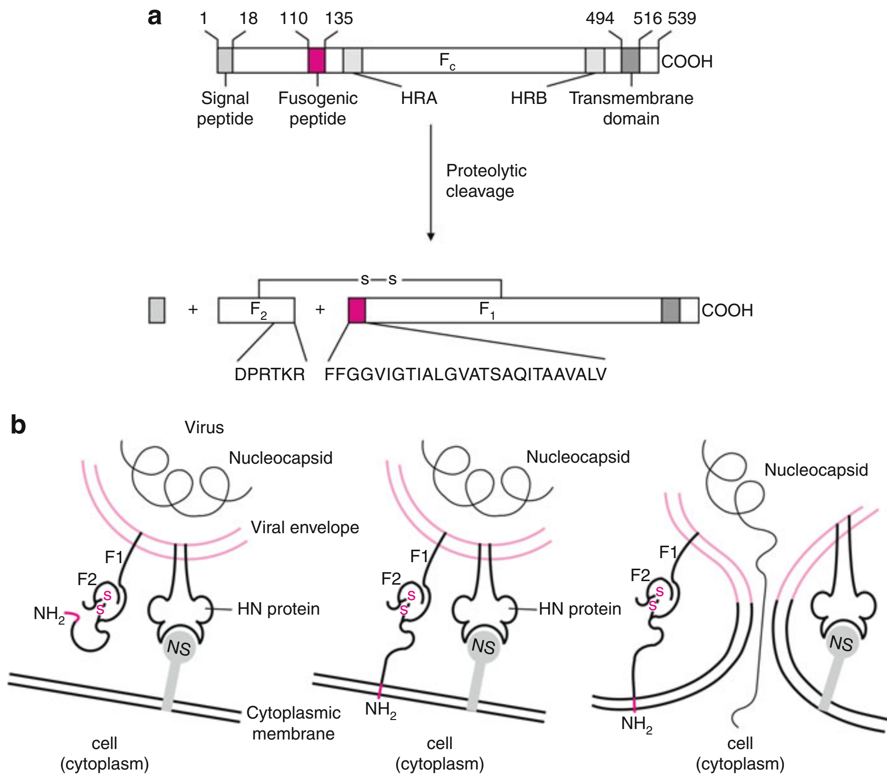


Fig. 15.8 The F protein of paramyxoviruses. (**a**) Localization of functionally active domains within the F protein of human parainfluenza virus 3 (signal peptide; fusion peptide; heptad repeat A, *HRA*; heptad repeat B, *HRB*; transmembrane region). The protein is cleaved by a trypsin-like protease into an amino-terminal F_2 subunit and an envelope-anchored F_1 subunit. Both subunits are mutually linked by an intermolecular disulphide bridge. (**b**) Postulated molecular mechanism of F-protein-mediated membrane fusion. The virus particle binds via the HN protein to a terminal *N*-acetylneuraminic acid residue, which is located on proteins or lipids on the cell surface. The hydrophobic amino acids at the amino terminus of the F_1 protein, which is embedded in the envelope of the virus, come close to the cytoplasmic membrane and attach to it. In this way, a close linkage between the viral envelope and the cell membrane is created, which promotes the fusion of both membranes, and the penetration of the viral nucleocapsid into the cell

seven amino acids, which are known as heptad repeats A and B (HRA, HRB), are located adjacent to the fusogenic region and the transmembrane domain. The conformational change induces a bundle structure composed of six α-helices, that are formed by the HRA and HRB regions of the trimeric F protein complexes. This leads to a rearrangement of the fusogenic domain, which makes contact with the cytoplasmic membrane, and mediates the fusion with the viral envelope (Fig. 15.8b). Antibodies directed against this fusion peptide of the F_1 protein, have a neutralizing effect and prevent the virus from entering the cell.

The M protein is a relatively small non-glycosylated protein which is associated with the viral envelope. Neither a signal peptide nor a transmembrane domain can be identified in the sequence of the M protein. It is able to interact with the domains of the two other viral glycoproteins that are localized in the cytoplasm and assembles in such a way that it forms a matrix layer on the inside of the envelope. The M protein is also capable of interacting with the nucleocapsid, especially with the N protein. It is very important for the formation of virus particles, packaging of viral RNA, and virus assembly during morphogenesis. In genetic engineering systems, virus-like particles can only be produced if the viral glycoproteins are generated along with M proteins.

Small amounts of the M2-1 protein can be found in respiratory syncytial virus particles. It is associated with amino acids in the amino-terminal domain of M proteins; it is phosphorylated and has in its sequence a Zn^{2+}-binding motif. It is also associated with ribonucleoprotein complexes in the cytoplasm of infected cells. It binds to the leader sequences on the viral genome and regulates the processes for switching between transcription and replication. Furthermore, it acts as an antiterminator promoting the synthesis of continuous positive-sense RNA strands. Moreover, the M2-1 protein promotes the interaction of M proteins with the nucleocapsid, which initiates the late steps of morphogenesis.

Nucleocapsid Proteins

The nucleocapsid consists of three different viral proteins which form a complex with the RNA genome. The main component is the N protein, which is tightly associated with the RNA. The amino-terminal domains of the protein are responsible for binding to the nucleic acid, whereas the carboxy-terminal third is exposed on the surface of the ribonucleoprotein complex, and interacts with P and L proteins. The complex is composed of N, P and L proteins, and is involved in its entirety in the transcription of the RNA genome. Although the N protein is enzymatically inactive, its involvement in the process was, however, demonstrated by the finding that "naked" RNA cannot be transcribed by P and L proteins. The N protein additionally interacts with M proteins, which are associated with the cytoplasmic membrane of the infected cell and with the viral envelope. This binding is essential for the proper folding and packaging of nucleocapsids into the developing particles.

The L protein sequences are conserved in all paramyxoviruses. Only a few molecules associate with the nucleocapsid in the virus particle and in infected cells. L proteins are not uniformly scattered throughout the entire length of the nucleocapsid, but they are rather frequent in transcriptionally active regions with which they are non-covalently linked. The L protein constitutes the RNA-dependent RNA polymerase; it is not able to function alone, but solely in a complex along with the N and P proteins. In addition, it is also probably involved in the phosphorylation of P and N proteins.

The L protein is associated with the P protein, the third component of the nucleocapsid, which is also present in clusters owing to that interaction. It is a phosphorylated, probably trimeric polypeptide. The phosphate groups

are predominantly located in the amino-terminal region. During the transcription process, the P protein can change its localization in the nucleocapsid: it moves along the nucleocapsid, and thus is considered as a "mobile" protein. The complex of L and P proteins is not only responsible for the synthesis of the various RNA products, but also for the capping and methylation reactions at the 5′ ends of mRNA molecules, as well as for 3′-terminal polyadenylation.

Model of F-Protein-Mediated Membrane Fusion

The following model has been developed to explain the processes of attachment, membrane fusion and infection in paramyxovirus. In the first step, the virus particles attach to receptors on the cell surface by a specific interaction of the HN protein or the H protein. As a result, the surface of the viral envelope and the cytoplasmic membrane come into very close spatial proximity. In the second step, structural rearrangements within the F_1 protein lead to the formation of an α-helical bundle structure by the HRA and HRB sequence repeats. This enables the highly hydrophobic amino terminus of the F_1 proteins, which prefer the hydrophobic environment in the lipid layer, to interact with the cytoplasmic membrane, thus creating an additional direct linkage between the virus and the cell: the F_1 protein is now anchored in the viral envelope by its carboxy terminus, and it is simultaneously linked with the cytoplasmic membrane through its amino terminus. The two membrane compartments now come into contact with each other, and the fluid nature of lipid bilayers promotes their fusion. The viral envelope and the cell membrane merge into each other, and the nucleocapsid of the virion is released into the interior of the cell.

15.3.3.2 Non-Structural Proteins

Some paramyxoviruses, such as mumps virus, along with pneumoviruses and metapneumoviruses (Fig. 15.7a, d) have a short open reading frame in their genomes, which encodes a generally small hydrophobic SH protein ("SH" for "strongly hydrophobic"), which exhibits the characteristics of a membrane-anchored protein (type II). Its function is largely unknown, and it has not yet been proven unambiguously as a part of the virion. It is also uncertain whether it is expressed in infected cells. If the SH gene is destroyed by mutation, then SH-deleted viruses exhibit unaltered replication properties in cell culture and unchanged pathogenicity in animal models. There is evidence that the SH proteins of mumps virus and respiratory syncytial virus prevent the TNF-mediated activation of nuclear factor κB.

Two additional non-structural proteins can be inferred from the genome sequence of pneumoviruses. The proteins NS1 and NS2 have been assigned to reading frames 1C and 1B, respectively. The NS1 and NS2 proteins of respiratory syncytial virus block the interferon-mediated defence responses by initiating the

ubiquitination and degradation of STAT2 proteins by the proteasomes. Additionally, NS2 proteins prevent the activation interferon-β (IFN-β) gene expression. In the course of this process, NS2 proteins bind to the amino-terminal caspase activation and recruitment domain (CARD) of RIG-I, a cytoplasmic RNA helicase, which induces the synthesis of class I interferons as part of the innate immune system (▶ Chap. 8).

Non-structural proteins are also present in other paramyxoviruses. Their functions are in most cases unclear. Sequences within the P gene are responsible for their synthesis. In Sendai virus, seven to eight different proteins can be synthesized originating from the P gene:

1. The P protein, which is collinear to the genome sequence.
2. The X protein, which is translated from the same RNA using the same reading frame as for the P protein, but starting from a downstream AUG start codon, thus representing an amino-terminal truncated version of P protein.
3. The C protein family, which is translated from alternative reading frames by using different start codons, resulting in proteins of approximately 20 kDa, which differ with regard to their amino termini. There are indications that the various C proteins inhibit the transcription of the viral genome.
4. The V and W proteins, whose synthesis requires, in respiroviruses and morbilliviruses, sequence modifications by RNA editing of the mRNA that encodes the P protein. In this process, one or two guanosine residues are post-transcriptionally inserted into the sequence, leading to a reading frame shift. In contrast, the synthesis of the rubulavirus P protein is dependent on the insertion of two guanosine residues into the transcripts. In any case, the V and P proteins, which are translated from this RNA species, share identical amino-terminal regions. The sequence changes only from the editing site because of the shifted reading frame. Similar products have been found in most other paramyxoviruses with respect to the sequence, and thus possibly also concerning the function. The C protein of human parainfluenza viruses and measles virus is translated using an alternative start codon in a different reading frame. The D protein of parainfluenza viruses is a truncated version of the C protein. On the other hand, mumps virus does not have a corresponding protein version; in this case, the synthesis of the P protein is dependent on the post-transcriptional insertion of one nucleotide, whereas the V protein is translated from the unmodified RNA.

In the carboxy-terminal domain of the V protein, which is conserved among all paramyxoviruses, there are seven cysteine residues that are arranged resembling a zinc finger domain. In some paramyxoviruses, such as simian virus 5, the V proteins are components of the virus particle, and are associated with the nucleocapsid. The V proteins have been associated with the ability of the virus to evade the interferon-mediated defence mechanisms. They exert that function in different ways: They inhibit both interferon synthesis and the interferon-dependent signalling cascades, they inhibit the defence strategies that depend on double-stranded RNA molecules, they prevent the transition of infected cells into apoptosis and they alter cell proliferation by slowing down the transition of the cells from the

G1 phase to the S phase, and by retarding the course of the S phase as well. The V protein of simian virus 5 interacts with damaged DNA-binding protein 1 (DDB1). Possibly, this prevents the activation of the cellular E2F factors by DDB1. During the G1 phase, the E2F factors are present in complex with the retinoblastoma protein RB105. The cell-cycle-dependent phosphorylation of RB105 at the transition of the G1 to the S phase of the cell cycle leads to the release of E2F factors, which then can develop their function as transcription factors. This process is inhibited by DDB1 by retarding a rapid entry into the S phase (▶ Chap. 6, ▶ Sects. 19.2–19.4). The binding of V proteins to DDB1 may contribute to maintaining its function in delaying the cell cycle, and to preventing the E2F activity.

Paramyxoviruses Capable of Evading Interferon-Mediated Defence Responses by Functions of their Non-Structural Proteins

Human parainfluenza viruses have developed mechanisms that allow them to evade the interferon-mediated immune response. Although this property is probably shared by all paramyxoviruses, the various viruses use different ways to achieve this goal.

In rubulaviruses, V proteins cause ubiquitination that results in proteasome-mediated degradation of STAT1 proteins (simian virus 5), STAT2 proteins (human parainfluenza virus 2) or STAT1 and STAT3 proteins (mumps virus). STAT proteins are components of the signalling cascade that is triggered by binding of class I interferons to their receptor, inducing the expression of interferon-dependent genes (▶ Chap. 8). The V proteins of Newcastle disease virus, a representative of the genus *Avulavirus*, cause a similar effect.

The V proteins of henipaviruses (Hendra virus and Nipah virus) differ completely from the V protein variants of rubulaviruses with regard to the amino acid sequences of the amino-terminal domain, but they also influence STAT-dependent signal transduction. They do not induce the proteolytic degradation of STAT proteins, but they give rise to the assembly of STAT1 and STAT2 molecules into large protein aggregates in the cytoplasm. This complex formation prevents the interferon-induced phosphorylation of tyrosine residues in STAT proteins. This effect interrupts the interferon-induced signal transduction.

The V proteins of morbilliviruses (measles virus, canine distemper virus) prevent the transport of phosphorylated STAT proteins into the cell nucleus, and thus impede the interferon-mediated signal transduction. In respiroviruses, the amount of phosphorylated STAT1 factors is reduced in cells infected with human parainfluenza virus 3, thus interrupting the signalling cascade that is induced by interferon receptor binding. The V proteins are not responsible for this effect, but the members of the C protein family, C, C′, Y1 and Y2, are: different mechanisms have been found in this case, and range from induction of the proteolytic degradation to the C-protein-mediated

aggregation of STAT proteins. It is unclear what functions emanate from the V proteins of these virus types.

In contrast, respiratory syncytial virus does not influence the amount of STAT1 proteins, but it is, nevertheless, able to evade the interferon-mediated immune response. Its NS1 and NS2 proteins block the interferon-mediated defence reactions by inducing the degradation of STAT2 via proteasomes. In addition, NS2 inhibits the expression of INF-β-regulated genes by binding to RIG-I. Finally, both non-structural proteins can suppress the maturation of dendritic cells.

15.3.4 Replication

Paramyxoviruses attach to the surface components of target cells by using their HN, H or G proteins. Respiroviruses, rubulaviruses and avulaviruses bind through their HN proteins to sialic acid (*N*-acetylneuraminic acid) residues, which exist as terminal sugar units of N-glycosidically bound carbohydrate modifications on cell surface structures. In Sendai virus and Newcastle disease virus, they are glycolipids with terminal *N*-acetylneuraminic acid residues of neolactogangliosides. However, the binding specificity is determined not solely by the terminal sugar residue, but also by the core unit of gangliosides. Human parainfluenza viruses 1 and 3 bind to terminal sialic acid residues of oligosaccharides with branched *N*-acetyllactosaminoglycans. These viruses are also able to cleave the sialic acid residues by the neuraminidase activity of their HN proteins; therefore, like influenza viruses (► Sect. 16.3), they have receptor-destroying activity. Signalling lymphocytic activation molecule (SLAM; also known as CDw150) has been identified as a cellular receptor of measles virus. By contrast, attenuated measles vaccine viruses use the protein CD46 as a receptor. An interaction with heparan sulphate has been described for the G protein of respiratory syncytial virus. On the other hand, the G proteins of Nipah virus bind to ephrin-B2 and ephrin-B3. These transmembrane proteins are located on the surface of endothelial cells.

In the following step, the fusion-active F protein mediates the direct fusion of the viral envelope with the cytoplasmic membrane (Sect. 15.3.3), a process that leads to the penetration of the nucleocapsid into the cell. All subsequent steps occur within the cytoplasm of the infected cell. The F protein of human metapneumovirus seems not only to be responsible for the fusion between the viral envelope and the cell membrane, it also binds to $\alpha_v\beta_1$ integrins by an RGD (arginine–glycine–aspartic acid) sequence motif, thus also contributing to the attachment process.

The RNA genome of paramyxoviruses cannot be directly translated into proteins. The protein synthesis and all further replication steps are dependent on the transcription of the genome into suitable mRNA species. Hence, an RNA-dependent RNA polymerase is associated with the nucleocapsid, and is introduced along with it into the cell. The three proteins that associate with the nucleic acid are necessary for the transcription process; the L protein constitutes the

actual RNA polymerase. The primary transcription is initiated in the leader sequence region at the 3′ end of the RNA, which is located upstream of the first gene. A short RNA is synthesized which is complementary to the leader region and ends at the beginning of the N gene. The polymerase complex remains associated with the viral genome, it glides along the template for a few nucleotides and resumes transcription at the start signal of the N gene. RNA synthesis is stopped in the E consensus sequence at the end of the gene. The intergenic nucleotides are skipped, and the polymerization reaction starts again at the S consensus sequence of the neighbouring P gene. These successive processes – start of mRNA synthesis, polymerization, termination of mRNA synthesis, skipping the intergenic nucleotides – are repeated for each gene region, so finally six different mRNA species are present, which correspond to the genes N, P, M, F, HN/H/G and L (Fig. 15.9). All RNA molecules are capped at the 5′ end and polyadenylated at the 3′ terminus. The polyadenylation signal is probably constituted by the uracil-rich sequence that is located within the E consensus sequences of all paramyxoviruses. The 5′-terminal modification of RNA molecules with cap structures is performed by the nucleocapsid protein P.

The multiply occurring process of stop/skip/restart at the E/I/S consensus sequences, which is repeated many times during course of mRNA synthesis, is

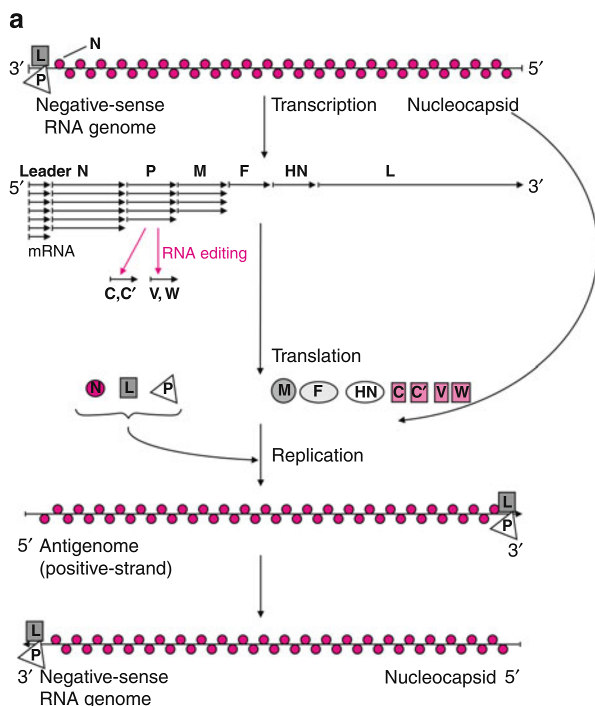


Fig. 15.9 (continued)

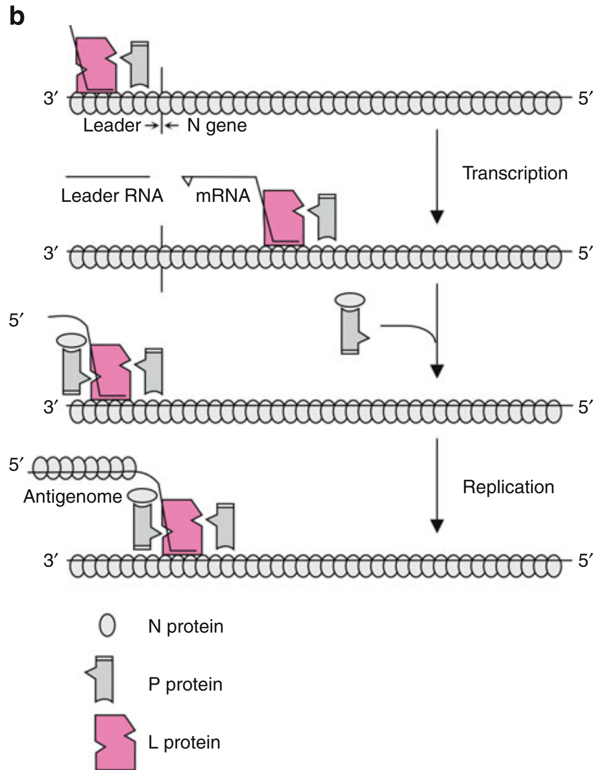


Fig. 15.9 (**a**) Genome replication in paramyxoviruses. The negative-sense RNA genome is present in the cytoplasm of infected cells in complex with viral proteins N, P and L. The first step is the synthesis of mRNAs, which is catalysed by the RNA-dependent RNA polymerase activity of the L protein; subsequently, the mRNAs are translated into the respective proteins. The short leader RNA does not code for proteins and probably has regulatory functions. The transcription stops at regulatory sequences between the individual genes, skips the intergenic regions and reinitiates mRNA synthesis. This process is not always successful. This leads to the formation of a concentration gradient of transcripts, which steadily decreases with advancing direction of transcription. The mRNA of the P gene is modified by post-transcriptional editing processes, which lead to the emergence of the V and W proteins. The use of an alternative start leads to the synthesis of the C and C′ proteins. If sufficient amounts of newly synthesized N proteins are present in the cell, then they will cause along with P and L proteins regulatory elements to be skipped at the intergenic transition sites. The result is a continuous positive-sense RNA molecule that is associated with N proteins throughout its entire length. It serves as an antigenome, and thus as the template for the synthesis of genomic negative-sense RNA molecules. (**b**) Model of the processes and protein interactions during mRNA synthesis and genome replication (following a proposal by Wolfgang Neubert, Max Planck Institute for Biochemistry, Martinsried, Germany). The RNA-dependent RNA polymerase initiates transcription of the negative-sense RNA genome at the 3′ terminus. The stop signal at the end of the leader sequence terminates this process, the intergenic nucleotides are skipped and RNA synthesis resumes at the next start signal. If newly synthesized N and P proteins are present in the cell after translation of mRNAs, they aggregate as a complex with the L protein at the 3′ end of the genomes. Free N proteins aggregate with the transcript, preventing the synthesis from being terminated at the intergenic regulatory sequences. In this way, continuous RNA strands arise, which acting as antigenomes serve as a template for the production of new viral genomes

not always successful. Occasionally, the enzyme complex falls from the RNA template during skipping of intergenic sequences. As a result, an RNA concentration gradient is formed in the direction of transcription. The N gene, which is located closest to the 3′ end of the genome, is the most transcribed gene, whereas the mRNA for the L protein is present in the lowest concentration. The proportions of the respective proteins are similar. The first viral proteins can be detected in the cell approximately 2–4 h after infection. The function of the short, uncapped leader RNA is uncertain. It is possibly important for the correct binding of the L protein and for the precise start of transcription. Whether the leader RNA of paramyxoviruses is able switch off cellular metabolic processes, like that of vesicular stomatitis virus, is not known (Sect. 15.1.4).

RNA editing processes are necessary for the synthesis of the non-structural proteins C, C′, Y1, Y2, V und W. RNA editing occurs in all human paramyxoviruses. This process, in which the insertion of one or two guanosine residues at a defined position of the mRNA – often in a poly(U)-rich region, similar to the polyadenylation signals – alters the reading frame, and leads to the synthesis of V proteins in respiroviruses and morbilliviruses or P proteins in rubulaviruses. In their amino-terminal region, the sequence of the non-structural proteins corresponds to that of the P protein, but otherwise they exhibit a highly cysteine rich amino acid sequence. RNA editing does not occur solely in the mRNA encoding the P protein. In some viruses, similar processes in the mRNA of the C protein lead to another protein variant, the W protein.

The synthesis of a continuous RNA molecule with positive polarity is a prerequisite for the generation of new viral genomes. This means that in addition to the various mRNA species that cover the regions of individual genes, full-length RNA molecules must also be synthesized that are not terminated at the E/I/S consensus sequences, but that are generated by read-through transcription. Only such a continuous RNA product can serve as a template for the synthesis of new RNA genomes.

The switching from the transcription of individual mRNA species to the replication mode, which generates continuous RNA strands with positive polarity, is dependent on the amount of N protein, which is – as already mentioned – the most abundant protein. If a sufficient quantity of N proteins is present in the cytoplasm, they interact with the leader RNA and prevent the stopping of transcription at the E consensus sequences, which in turn are read through. This mechanism of antitermination generates a continuous RNA molecule (antigenome), which is complexed with N proteins throughout its entire length (Fig. 15.9b). At the same time, a complex between N and P proteins seems to be formed, and this interacts with the L protein and alters its activity, promoting the synthesis of continuous antigenomes. The regulation of the switching mechanism between genome transcription and genome replication by the quantity of newly synthesized N proteins is simple and rational: a switch can only occur when sufficient protein has been synthesized. The M2-1 protein of pneumoviruses seems to be involved in the antitermination process as an additional regulatory factor.

The antigenomes present in the cytoplasm are used as templates for the production of new viral genomes. N proteins also bind to them immediately. Simultaneously, the translation of the other viral proteins occurs. The envelope proteins HN/H and G as well as the F_0 protein are modified in the Golgi apparatus; the latter is cleaved into the F_1 and F_2 subunits, and is transported to the cell surface. The M proteins accumulate on the inside of the cytoplasmic membrane and form a matrix protein layer by interacting with the F and HN, H or G proteins that are associated with lipid rafts. These proteins, in turn, interact with the ribonucleoprotein complexes composed of the RNA genome and N, P and L proteins, the membrane invaginates at these sites and the resulting new virus particles are released by budding on the cell surface. Although in measles virus infections the viral surface proteins H and F are present on the entire cell surface, the release of virus particles in polarized cells occurs exclusively at the apical cell side.

15.3.5 Human Pathogenic Paramyxoviruses

15.3.5.1 Human Parainfluenza Viruses

Epidemiology and Transmission

Four types of human parainfluenza viruses are known today. They are disseminated worldwide and cause respiratory diseases in infants and children. Infections with parainfluenza viruses 1, 2 and 3 are primarily expressed as disorders of the upper respiratory tract, and less frequently as bronchitis and bronchiolitis, and they are the main cause of croup. Frequently, the infections are asymptomatic. At the age of 4–5 years, most children have been infected with these viruses; reinfections with moderate courses occur in adulthood. Severe infection courses with parainfluenza virus 3 are observed in infants during the first few months of life, and with parainfluenza viruses 1 and 2 only later. Parainfluenza virus 4 usually causes harmless infections of the upper respiratory tract in children and young adults. All these viruses are transmitted by direct contact with infected people and by droplet infection. Viruses can be excreted up to several months after infection.

Clinical Features

After infection, the symptoms in children are cough, hoarseness and temperature above 38 °C. These symptoms can develop into rhinitis, pharyngitis and bronchitis. Fever and clinical signs of the disease usually disappear after 5–7 days. When croup has developed, the symptoms persist; coughing increases, and has a tinny, barking sound. This is associated with very severe respiratory distress, which the children try to compensate through increased use of the respiratory muscles. Nonetheless, the children usually recover quickly, but some children contract asthma later. Clinically severe infections by parainfluenza viruses are occasionally found in elderly patients. Patient-specific factors such as susceptibility to allergic reactions and psychosocial parameters can also contribute to the gravity of the disease.

Pathogenesis

The virus infects cells of the mucous membranes of the nose and throat. From there, it spreads out through the larynx into the bronchi and lower respiratory tract, infecting the ciliated epithelium. Mononuclear cells, lymphocytes, dendritic cells and macrophages are found in the submucosa and in the peribronchial tissue. The pertinent tissues are oedematous and show increased mucus production. Interferons and interleukins (IFN-γ, TNF-α, IL-2, IL-6, and IL-10) are locally detectable 3–6 h after infection. They indicate that an inflammatory process has occurred. It is believed that in the development of pneumonia, in addition to the direct cellular destruction of the alveolar epithelium, it is secondarily damaged by immunological processes. Besides interferons and interleukins, cytotoxic T cells, IgE antibodies and antigen–antibody complexes are presumably involved. The symptoms of croup, which are occasionally observed mainly in 1–2-year-old children, are caused by strong swelling of the mucosa of the larynx, which severely impairs both inhalation and exhalation. Locally increased concentrations of IgE antibodies and histamine can be found in patients, and may be associated with the relatively frequent development of asthmatic conditions in later years. Even cytotoxic T lymphocytes and immunopathological damage of the epithelium, which is induced by them, may possibly be involved in this process.

It is unclear what determines the virulence of the infection. There is evidence that it is associated with the extent of cleavage of the F_0 protein: if infected individuals whose mucous epithelial cells have an appropriate protease or if the infecting virus has an easily recognizable cleavage site for cellular proteases, a very rapid infection with spread of the virus can occur by cell fusion. The macrophages that are present in the mucosa exhibit reduced phagocytosis activity for staphylococci, whereby a bacterial superinfection can easily emerge, which is exacerbated by the infection-associated damage of the mucous membrane. This in turn can facilitate spread of the virus into the epithelial cells of the lung, as staphylococci and streptococci have proteases that cleave the F_0 protein. In this way, more infectious particles arise. Furthermore, it has been shown that specifically introduced mutations in the genome of human parainfluenza virus 3 which cause a defective synthesis of C, D and V proteins mitigate the infection in vitro and in animal models (hamsters and vervet monkeys).

Immune Response and Diagnosis

IgM, IgA and IgG antibodies against structural proteins are produced during the course of infection. After infection, IgA and IgG antibodies against viral HN and F surface proteins are present. Especially, neutralizing IgA antibodies in the nasal mucosa in adults are considered as a sign of protection against reinfections. At the level of T_H lymphocytes, epitopes of the F and N proteins preferably seem to stimulate the population of T_H1 cells, whereas those of the G protein activate rather T_H2 cells.

The infection with parainfluenza viruses has to be distinguished from other diseases with similar symptoms (influenza A virus, influenza B virus, respiratory

syncytial virus, metapneumovirus, adenovirus, *Haemophilus influenzae*). The diagnosis of fresh infections is performed by isolation of the virus from sputum or pharyngeal lavage and subsequent cultivation (all four virus types can be well propagated) in, for example, monkey kidney cell lines (LLCMK2). A faster diagnosis is achieved by direct detection of viral proteins in the antigen rapid test, or by the detection of viral genomes by reverse transcription PCR (RT-PCR) from respiratory materials. Antibodies in the serum can be detected by ELISAs, but depending on the test, usually only 8–12 days after onset of the disease, when the symptoms have already subsided. Therefore, the evidence of fresh infections is a domain of direct virus detection methods.

Therapy and Prophylaxis

There is neither a vaccine nor an antiviral therapy.

15.3.5.2 Mumps Virus

Epidemiology and Transmission

Mumps virus has a global distribution, and exists only in humans. There is only one serotype; however, 12 genotypes have been characterized, and they have a differential regional distribution: genotypes C, D, E, G and H are predominantly found in the Western Hemisphere, whereas genotypes B, F and I prevail in Asian countries. Mumps virus is transmitted by droplet infection, and is excreted by infected people up to 7 days before and 9 days after the onset of the disease. About 90 % of adults are seropositive; in young adults this is because of the vaccine that was introduced in the 1970s. Epidemic forms of mumps occur mainly in winter and spring in periods of 2–7 years. However, sporadic cases are also known. The virus can be transmitted experimentally to monkeys, cats and dogs; it can be adapted to embryonated chicken eggs and cultivated in cell culture.

Clinical Features

The disease breaks out about 2–3 weeks after contact with the virus; approximately one third of cases are asymptomatic. In 95 % of symptomatic infections, an inflammation of the salivary glands, particularly of the parotid gland, can be observed, and these strongly swell within 2 days. A moderate fever that lasts several days develops. Approximately 10 % of illnesses are associated with a mild meningoencephalitis. In 25 % of affected men, testicular inflammation can additionally be observed, which can lead to sterility in cases of bilateral infection. Roughly, 1–5 % of affected women develop inflammation of the ovaries, which, however, are not associated with further complications. The virus can infect other organs, such as the pancreas, thyroid glands and kidneys, and may lead to inflammation there. In some cases, post-infectious arthritis of the large and small joints has been observed. It is still unclear how they arise. Late complications of mumps are deafness due to infections of the inner ear, and possibly diabetes mellitus after pancreatitis. Whether mumps infections cause spontaneous abortion in pregnant women during the first trimester

of pregnancy has not been conclusively demonstrated. Older data suggest an increased rate of abortion; however, this has not been confirmed in recent studies.

Mumps Has Been Known for a Long Time

Hippocrates described mumps as swollen salivary glands and inflammation of the testes in the fifth century BC, and differentiated it from other infectious diseases. The striking symptoms have also been reflected in the fine arts: for example a “foolish virgin” exhibits the typical signs of the disease on the portal of Strasbourg Cathedral.

Pathogenesis

Mumps virus enters the mucosa of the throat, nose and pharynx by droplet infection and proliferates in the epithelial cells. Subsequently, lymphocytes are infected, and the virus spreads with them to the local lymph nodes, and then by the bloodstream throughout the body. Then, the mumps virus colonizes the salivary glands, the pancreas, the testes, the ovaries and other glands and gives rise to cell damage in these organs. A systemic infection arises, during which patients are viraemic and excrete the virus in their saliva and urine. The reaction of the immune system leads to inflammations associated with generalized exanthema, swelling of the organs and other cell damage. Testicular inflammation, in which not the sperm-forming epithelium, but the interstitial cells are infected, occurs only in cases of infection after puberty. This is ascribed to hormonal influences, or the appearance of new infectable cell types. During the viraemia, the virus can migrate into the meninges of the brain and spinal cord; it proliferates in these cells and cause meningoencephalitis or meningitis. There is evidence that the virus is able to proliferate even in the central nervous system of asymptomatic patients.

Immune Response and Diagnosis

Mumps infection confers lifelong immunity. Clinically apparent reinfections have not yet been observed. In the course of infection, IgM, IgA and IgG antibodies are produced, and can be detected by ELISAs, allowing serological diagnosis; in acute infections, the viral genomes can be detected by RT-PCR. Antibodies against the NP protein appear early during the course of infection. IgG antibodies against the viral F, HN and NP proteins indicate a past infection. Especially the antibodies against the HN protein exert a neutralizing effect. Little is known about the cellular immunity and immunopathological processes. However, as the virus proliferates in activated lymphocytes, a reduced reactivity of this cell population is found when the cells are incubated with antigens.

Therapy and Prophylaxis

Vaccination against mumps is performed with an attenuated virus strain. Several vaccine strains have been obtained by adaptation to embryonated chicken eggs and

subsequent cultivation in embryonic chicken cell cultures. The strain Jeryl Lynn is the standard vaccine virus strain in the USA and most European countries. The molecular basis of attenuation is not known. An antiviral chemotherapy does not exist.

15.3.5.3 Measles Virus

Epidemiology and Transmission

The first description of the disease was about 1,000 years ago. Since the virus is highly contagious and does not have a reservoir besides humans, the low human population density in earlier centuries was probably not sufficient to maintain the infection chain. The pathogens of rinderpest and canine distemper are closely related to measles virus (Sect. 15.3.6). There is evidence that at the time when the first humans settled down, i.e. approximately 8,000–10,000 years ago, a virus with similarity to rinderpest virus or canine distemper virus adapted to humans, and survived within the human population. Its infections caused measles. The first epidemics were described in the late Middle Ages, when the human population density increased; in addition, the development of trade relations between Europe and Asia facilitated the dissemination of the virus. During the discovery of the Americas, the European conquerors introduced measles to the continent, and the infection caused severe epidemics with fatal diseases among the native American human population. Similar occurrences have also been reported from island nations (Fiji, Faroe Islands) and in African natives from the sub-Saharan Sahel region, where measles virus was also introduced. An immune protection was not present in those populations. Therefore, and also owing to insufficient care and poor nutrition, the disease developed very severe courses there. In 1906, the Austrian paediatrician Clemens von Pirquet described the immunosuppressive effect of measles virus. He ascertained that an initially positive tuberculin reaction became a negative one during the course of the disease. Therefore, measles virus was the first virus in which an immunosuppressive effect was described. In 1911, it was transmitted to monkeys, and it was isolated from cell cultures for the first time in 1954.

Even today, measles virus is disseminated worldwide. Although there is only one serotype, more than 20 genotypes with different regional prevalences are known; these are divided into eight strains (clades A–H). The mortality rate is still very high in underdeveloped regions. Although the mortality rate has decreased in recent years from about 800,000 cases in year 2000, nevertheless, approximately 200,000 people died from the viral infection according to estimations by the World Health Organization in 2007, and most of these were children. The dissemination occurs by droplet infection via the upper respiratory tract, as well as through the conjunctiva or by direct contact. Very small amounts of virus are sufficient for a successful infection. The disease usually occurs in childhood and in early adolescence up to the age of 15 years.

Clinical Features

Measles is a severe disease that is associated with high temperature. Asymptomatic or subclinical courses occur very rarely. In adults the disease is particularly severe.

Table 15.7 Variants of measles encephalitis

	Acute parainfectious/ postinfectious encephalitis (autoimmune encephalitis)	Acute progressive infectious encephalitis	Subacute sclerosing panencephalitis
Incidence	About 1:1,000 in measles diseases of immunologically healthy individuals	In immunodeficiencies (HIV infection, transplant patients, treatment with cytostatic agents)	Approximately 1:25,000, particularly in male patients who were infected at the age of up to 2 years
Occurrence	Directly following measles	6–10 months after measles infection	5–16 years after measles infection
Detection of virus in the brain	No	Yes	Yes, many mutations
Viral replication	No	Yes	No, synthesis of ribonucleoprotein complexes
Inclusion bodies	No	Yes	Yes, a few
Cellular immunity	Lymphocytes against basic myelin	Impaired by immunodeficiency	Normal

The incubation period is about 9–11 days. The first symptoms include conjunctivitis, photophobia, cough, rhinitis and rapidly rising temperature. A certain sign of measles is the presence of so-called Koplik's spots in the oral mucosa. Fever subsides about 2–3 days after the first clinical signs. A skin rash appears only thereafter; it is initially manifested behind the ears and neck and subsequently in the face. The appearance of the rash is simultaneously accompanied with a further rise of temperature up to 40 °C. It spreads throughout the body within 2–3 days. The rash subsides after a further 2–3 days. The symptomatic phase of measles lasts about 10 days.

Complications include pneumonia, bronchopneumonia, otitis media, thrombocytopenia, encephalitis and parainfectious autoimmune encephalitis (probability 1:1,000; Table 15.7). EEG alterations are found in 50 % of cases, however without recognizable changes in the central nervous system. However, it is unclear whether they are caused by an infection of the brain or the vessel walls of the brain or by secondary cytokine effects. Liver inflammation occurs particularly in adults. It is primarily restricted to the epithelium of the bile ducts. An infection of the intestinal epithelium, starting from capillaries, is common and manifests itself as diarrhoea. In immunocompromised people, measles has a very severe course, in part causing giant cell pneumonia, which is characterized by the appearance of multinucleated cells in the bronchial tree and in the lung.

Pathogenesis

SLAM (also known as CDw150), the cellular receptor of measles virus, has been identified on lymphocytes and macrophages. Rinderpest virus and canine distemper

virus also use the analogous SLAM proteins of animals as a receptor; therefore, it can be presumed that this surface protein is generally responsible for the lymphotropism of morbilliviruses and their immunosuppressive effect (Sect. 15.3.6). The cell tropism of measles virus is additionally affected by interaction with other cell surface factors. Accordingly, the vaccine strains of measles virus bind to the amino acids of the first two short consensus repeat (SCR) domains of the protein CD46. CD46 is a member of the regulators of complement activity (RCA) family. It protects the cells from unspecific lysis by the complement. Amino acid residues 473–477 in H protein are probably directly involved in the interaction. The relatively low CD46-binding affinity of wild-type viruses is influenced by amino acid 481 of H protein. Attenuated measles viruses carry a tyrosine residue instead of an asparagine, and this mutation increases the binding affinity. CD46 is internalized by attachment and penetration of the vaccine viruses via F-protein-mediated membrane fusion. Infected cells, which become depleted of CD46, are lysed by the complement system and are eliminated from the organism, leading to a break in the infection chain. H proteins of virulent strains do not bind to CD46. As a result, the infected cells are not lysed early by the complement system and the virus can spread rapidly.

Measles virus initially infects mucosal cells of the upper respiratory tract. The consequences are sneezing, rhinitis and coughing. From the upper respiratory tract, the virus spreads further into the bronchial tree, and causes a peribronchial inflammation. During the further course of infection, the damage to the bronchial epithelium facilitates a superinfection with different bacterial pathogens so that in addition to the primary viral lung inflammation, also a secondary bacterial pneumonia can arise. The viruses that are released at the primary infection sites infect macrophages and dendritic cells there. These cells transport the virus to the local lymph nodes, where the virus encounters a variety of infectable monocytes, macrophages and B and T lymphocytes; the first viraemia originates from the lymph nodes. Other possible proliferation sites are endothelial cells and all lymph nodes, resulting in generalized lymph node swelling. The cells proliferate, but the disease remains as a clinically inapparent hyperplasia. During infection, very few free viruses are present in the blood; the pathogens are still predominantly cell-bound.

The characteristic Warthin–Finkeldey giant cells arise in the mucosa of the mouth and throat by F-protein-mediated membrane fusion of infected endothelial cells with neighbouring cells. Later, they can also be found in many other tissues. In contrast to influenza viruses and parainfluenza viruses, the viral envelope proteins H and F are localized throughout the entire surface of the polarized cell during a measles virus infection; however, the release of particles occurs exclusively at the apical side of the cell. The viral proteins at the basolateral side do not induce budding of progeny viruses, but induce the fusion of infected cells with neighbouring cells. In this way, the viral infection can spread to other tissues and induce inflammation processes, as well as overcome neuronal synapses. A tyrosine residue in the cytoplasmic domain of the H and F proteins is responsible for the basolateral protein transport.

During the second viraemia, which lasts approximately 3–5 days, the virus proliferates in many organs (kidney, liver, bladder, gastrointestinal tract, skin, thymus).

Immigrating monocytes produce IL-1. However, the synthesis of TNF-α und IL-12 is decreased. The capillary infections of the skin along with the immune complexes, which accumulate at the endothelium of the capillaries, are manifested as an exanthem. Accordingly, the appearance of the rash denotes the beginning of the immune response, which is absent in patients with a damaged immune system, who do not develop an exanthem. MHC class I restricted cytotoxic T lymphocytes migrate into the infected skin regions and lyse infected cells, thus eliminating the virus from the organism. There is evidence that measles virus can persist for long periods after infection, and viral proteins can be detected in the lymph nodes. Whether in these cases a genetic defect determines a deficiency in recognizing measles-virus-specific T-cell epitopes is unclear.

Today, three forms of encephalitis are known to be associated with measles virus infections (Table 15.7):

1. Acute parainfectious or postinfectious encephalitis or autoimmune encephalitis. This form of encephalitis is found in about 1 of 1,000–2,000 measles infections. It occurs primarily in children older than 2 years, and develops simultaneously with the exanthem or about 1 week after the appearance of the rash. Measles virus is not detectable in the brain. Postinfectious encephalitis is manifested by demyelination of the myelin sheaths in the brain due to autoimmune reactions. It is unclear triggers the immune system to attack the body's own tissue, and whether similarities between viral and cellular proteins (such as the basic myelin) may play a role. Ten to 20 % of postinfectious encephalitis cases are fatal. Pathohistologically, they are characterized by dilated vessels, haemorrhagies and perivascular infiltration with macrophages, lymphocytes and plasma cells. The disease is associated with dizziness, delirium, coma and paralysis. In animal experiments, it can be transmitted by lymphocytes as "allergic encephalitis".
2. Acute progressive infectious encephalitis or acute inclusion body encephalitis. This always fatal form of encephalitis follows the acute disease after a break of 6–10 months. It occurs only in patients with immunodeficiencies. In these cases, measles virus can be isolated from the brain. It behaves as an opportunistic pathogen. The disease spreads preferentially into the cell-containing regions of the brain. Many neurons and glial cells, in which the virus proliferates, exhibit inclusion bodies.
3. Subacute sclerosing panencephalitis (SSPE). The always fatal SSPE is a rare, late complication of measles virus infection. Possibly, in old dog encephalitis canine distemper virus causes a clinical picture similar to that of SSPE (Sect. 15.3.6). SSPE is based on a persistent measles infection of the brain. It appears 6–15 years after acute measles; it affects approximately one in every 10,000–25,000 children infected with measles virus. SSPE develops more frequently in boys than in girls, especially if the boys are infected before the age of 2 years and the disease is histopathologically manifested as nodular encephalitis. The first reference to this late effect of measles is very high antibody titre against the H and N proteins, both in the blood and in the cerebrospinal fluid. Presumably, the virus infects the endothelial cells of

the brain and migrates from there into the brain. Measles virus genome sequences and inclusion bodies are found in the cytoplasm and the nucleus of infected neurons; in the later stages of the disease, oligodendrocytes, astrocytes and endothelial cells are infected. However, no infectious measles viruses can be isolated from the brains of patients. The viral genomes exhibit various mutations, especially in the H, M and F genes, whereas the regions of the genome coding for the N, P and L proteins are largely unchanged. This enables the pathogens to transcribe and replicate their genomes notwithstanding the reduced production of infectious progeny viruses. Presumptively, the infection can spread by transmission of nucleoprotein complexes and through cell fusions. Inclusion bodies are rare in the cells. The cause of this process is probably a defect of viral replication in brain cells, which results in an abortive form of infection. It is not known at which level the infection cycle is perturbed. In a model system with transgenic mice which produce the human protein CD46, the viral C and V proteins emerged as virulence factors that influence the development of the disorders of the central nervous system which are caused by measles virus. The infection with viruses which do not synthesize V proteins spreads significantly more slowly in the mice, which also exhibit a lower mortality rate. When a mutant that does not produce C proteins was used, the survival rate was also relatively high, even though in this case the virus spread with similar efficiency in the mice and in the brain in comparison with the wild-type strain. This shows that both proteins regulate the virulence of measles virus in different ways.

Immune Response and Diagnosis

Along with the appearance of the exanthem, IgM and later IgG antibodies against the F, H and N proteins can be detected by immunofluorescence, haemagglutination-inhibition and complement-fixation tests and ELISAs. In the case of reinfection, IgG antibodies against the H and F proteins protect against new spread of measles virus in the organism; IgA is responsible for the mucosal immunity. The diagnosis of measles can be achieved clinically or by antibody detection. For instance, the virus can be isolated from nasopharyngeal smear, blood and bronchial lavage or can be detected by RT-PCR. It can also be cultivated in many human cell lines or in green monkey kidney cell lines (Vero cells), although this method does not play a diagnostic role any more. The F-protein-mediated cell fusion induces the generation of polynuclear giant cells in cultures.

In the course of the acute disease, cytotoxic T lymphocytes ensure the elimination of virus-infected cells from the various organs. The importance of the cellular immune system is additionally shown by the fact that the infection proceeds normally in patients with disorders of the humoral immune system (agammaglobulinaemia). Measles-virus-specific $CD4^+$ T_H cells, predominantly the T_H2 type, are activated at the beginning of skin eruption. They recognize epitopes on the H, N, M, F and P proteins and secrete cytokines, which activate

macrophages and other T lymphocytes and induce the proliferation of B cells. Subsequently, the latter produce antibodies against measles-virus-specific proteins. A fraction of the $CD4^+$ cells can also lyse infected cells that express MHC class II proteins. Already at this time, there are also large quantities of IFNγ in the blood. Increased levels of IL-2 and IL-4 can be detected at the same time and thereafter. On the other hand, there is no proliferation of long-term-activated T_H1 cells. Possibly, they are functionally directly impaired by the infection or after contact with viral proteins. For example, the signal transduction pathway for activating the expression of Akt kinase is interrupted in primary human T lymphocytes after contact with the viral F and H proteins. This phosphokinase is induced by interaction of the IL-2 receptor with IL-2, and causes the subsequent release and transport of the active nuclear factor κB transactivator into the nucleus. The IL-2-dependent induction of the Jak/STAT signalling pathway is blocked in the cells after contact with the H and F proteins. This shows that measles virus uses a special strategy to suppress the host immune response. Presumably, these processes are the reason for the immunosuppression that occurs and which is initially confined to the measles virus infection. However, during the further course of infection, it adopts a general character, and is also extended to other pathogens (including tuberculosis bacteria).

Therapy and Prophylaxis

The best prophylaxis is the active immunization with an attenuated live vaccine (Schwarz strain), which provides a long protective effect. The attenuated measles vaccine virus was developed by John Frankling Enders by adaptation to embryonated chicken eggs followed by repeated passaging in embryonal chicken fibroblasts. The vaccine has been available since 1963. If children who could not be vaccinated because of another serious disease are exposed to measles virus, a passive immunization should promptly occur by the administration of virus-specific immunoglobulins – i.e. within 96 h. In cases of severe pneumonia caused by measles virus, ribavirin is used experimentally.

15.3.5.4 Respiratory Syncytial Virus

Epidemiology and Transmission

The chimpanzee coryza agent was isolated from chimpanzees with rhinitis in 1956. It induced similar symptoms in zookeepers. In 1957, Robert M. Chanock and collaborators discovered a virus in children with severe diseases of the lower respiratory tract which has been identified as being identical to that of chimpanzees and was designated “respiratory syncytial virus”. This name refers to its property of causing diseases of the respiratory tract and of inducing syncytia formation in vitro. There are subtypes A and B, which differ particularly in their G protein sequences. Infections with respiratory syncytial virus are highly contagious – and there are up to 10^6 infectious virus particles in 1 mL of saliva. The virus is mainly transmitted by droplet infection and direct contact, e.g. with the bedclothes of infected people. Especially children become infected during the winter months. More than 80 %

of them possess antibodies against the virus by the age of 4 years. Respiratory syncytial virus is considered the principal infectiological problem of the first year of life. Infants aged between 6 weeks and 6 months are the most susceptible. Re-infections with mild disease forms are found in connection with the decline of antibody concentration in elderly people. Nosocomial infections are common in old people's homes, kindergartens and clinics. Infections of immunosuppressed individuals are especially feared, for instance after transplants.

Clinical Features

The incubation period lasts about 3–5 days. The disease is manifested by mild to life-threatening influenzal infection with fever and rhinitis. After 1–2 days, the disease frequently develops as inflammations of the throat (pharyngitis), the tracheal mucosa (tracheitis) and bronchi. Up to 40 % of infants and young children develop a bronchiolitis with cyanosis (blue discoloration of the skin) and lung inflammation, and up to 5 % develop croup. After alleviation of symptoms, excretion of the virus may last for a long time. Complications include middle-ear infections, which are likely augmented by bacterial superinfections. In immunosuppressed patients, pneumonia is a life-threatening complication.

Pathogenesis

Respiratory syncytial virus enters the upper respiratory tract via droplet infection. It proliferates in the cells of the mucosal epithelium and spreads from there into the lower respiratory tract, migrating from cell to cell within 1–2 days, especially in infants and young children. Animal experiments revealed that the virus is also transported to the alveoli via the ciliated epithelium and by aspirated secretions. The bronchial and alveolar epithelial cells are destroyed by syncytia formation. Cytoplasmic inclusion bodies are formed in the cells, which consist of accumulations of nucleocapsids. The infected epithelial cells swell, become necrotic and are released in the saliva and in the bronchial secretions. The submucosal and outer tissue layers are highly oedematous and the mucus secretion is increased. This results in the formation of clots, which obturate the alveoli and lead to a reduced CO_2/O_2 exchange that culminates in the development of cyanosis. Besides epithelial cells, the viruses can also be detected in granulocytes, monocytes and macrophages in the peripheral blood. However, these cells do not release viruses, so a viraemia does not arise.

In the bronchi, bronchioles and alveoli, there are infiltrates of lymphocytes, plasma cells and infectable macrophages. These cells and also the cells of the alveolar epithelium react with the secretion of various cytokines (IFN-γ, IL-4, IL-8, IL-9), chemokines and TNF-α, which can be detected in large amounts in lung secretions. Granulocytes migrate into the infected tissue, phagocytose virus–antibody complexes and, in turn, respond with increased production of IL-4, IL-6, IL-8 and TNF-α. All these processes together can be manifested as interstitial pneumonia.. It was found that the domain of the G protein situated between amino acids 184 and 203 is responsible for the immunopathological reactions.

Severe illnesses are quite common when babies are infected during the first 6 months of life; i.e. in a period in which maternal antibodies against respiratory syncytial virus are present in the blood of the child. This suggests a possible infection-enhancing mechanism by immunoglobulins. No bronchiolitis arises within the first 6 weeks after birth.

Immune Response and Diagnosis

In the course of infection with respiratory syncytial virus, IgM, IgA and IgG antibodies are synthesized and can be detected in ELISAs. During the disease in young children, the diagnosis is usually made by detection of viral RNA by RT-PCR or via identification of virus-infected cells in the salivary secretions by immunofluorescence tests. The virus can be cultivated in HeLa or Hep2 cell lines. Antibodies against the G and F proteins are neutralizing, and confer antiviral protection, especially after repeated infections. The viral subtypes A and B can be distinguished with the aid of neutralizing monoclonal antibodies against the G proteins. Animal studies suggest that MHC class I restricted cytotoxic T lymphocytes are decisively involved in the elimination of the virus from the organism. In patients with a defective cellular immune response, the virus can even be detected in the liver, myocardium and kidneys.

Therapy and Prophylaxis

A vaccine is not yet available. Formalin-inactivated viruses have proven to be unsuitable since the F protein is destroyed by chemical treatment and only antibodies against G protein can be generated. However, although they are virus-neutralizing, they are not able to prevent the spread of the virus by cell fusion. In addition, extremely severe cases of the disease have been observed in subsequent infections. This could be related to the phenomenon of antibody (immune) enhancement, which is known from dengue virus infections (► Sect. 14.5). The clinical treatment of patients who were infected with respiratory syncytial virus includes increased oxygen supply. Ribavirin inhibits viral translation and replication in vitro, however, its use as an aerosol is controversial and is only indicated for children with severe disease forms or immunocompromised adults; in the latter, ribavirin is also applied intravenously. In children with an extremely high risk of respiratory syncytial virus disease – e.g. prematurely born children – a monthly passive immunization with humanized monoclonal antibodies against the F protein (palivizumab) is performed in the winter months.

15.3.5.5 Human Metapneumovirus

Epidemiology and Transmission

In early summer 2001, a paramyxovirus of the subfamily *Pneumovirinae* was isolated for the first time by Bernadette van den Hoogen and colleagues from children with respiratory infections at the Institute of Albert Osterhaus in the Netherlands. The virus has been assigned to the genus *Metapneumovirus* on the basis of sequence analysis data. However, this virus has not adapted to humans in

recent years. Screening of human serum banks revealed that human metapneumovirus has been circulating for at least 50 years in humans, and has a worldwide distribution.

So far, two genotypes, A and B, have been characterized. Most sequence differences are located in the regions of the genome that encode the G and SH proteins. Initial studies showed that probably all children aged 5–10 years have IgG antibodies against the proteins of human metapneumovirus, thus they must have been infected with this pathogen. The transmission occurs through droplet infection or by contact with contaminated objects. The first infection with human metapneumovirus affects mainly children aged under 1 year; between 5 % and 10 % of children hospitalized with serious acute respiratory infections are infected with this virus. Owing to a decreasing seroprevalence rate, re-infections are frequently found in adults, who usually exhibit a mild course.

Clinical Features

The incubation period lasts roughly 4–6 days. The symptoms are identical to those caused by infection with respiratory syncytial virus and are usually manifested as mild cold-like symptoms; only very rarely are there life-threatening severe respiratory infections with fever, rhinitis, bronchitis and bronchiolitis. Middle-ear infections can developed in up to one third of cases.

Pathogenesis

Human metapneumovirus enters the upper respiratory tract via droplet infection. It proliferates in the cells of the mucosal epithelium and, migrating from cell to cell, spreads into the lower respiratory tract within 1–2 days, particularly in infants and young children. Like infections with respiratory syncytial virus, human metapneumovirus also gives rise to release – albeit weaker – of proinflammatory cytokines such as IL-6 and TNF-α in the epithelium of the airways.

Immune Response and Diagnosis

During infection with human metapneumovirus, IgM, IgA and IgG antibodies are synthesized, and can be detected in ELISAs. The F protein is immunodominant and induces the synthesis of neutralizing antibodies. The acute infection is diagnosed not serologically, but by detection of viral genomes in the pharyngeal samples using RT-PCR. Human metapneumovirus can be cultivated in vitro in kidney cell cultures from rhesus monkeys (LLC-MK2 cells) or green monkey (Vero cells); however, the development of the cytopathic effect as the typical sign of infection takes more than 2 weeks.

Therapy and Prophylaxis

A vaccine does not exist. The administration of ribavirin showed a reduction of replication and inflammatory activity in vitro and in the Balb/c mouse model. Similar to infections with respiratory syncytial virus, monoclonal neutralizing antibodies have proven to be suitable, by passive immunization, to prevent the infection in threatened children.

15.3.6 Animal Pathogenic Paramyxoviruses

Bovine parainfluenza virus 3 and canine parainfluenza virus 2 represent facultative pathogens which can cause shipping fever in cattle and kennel cough in dogs.

The host range of bovine parainfluenza virus 3 – a representative of the genus *Respirovirus* – is broadly diversified and includes ruminants (cattle, sheep) as well as horses, dogs and primates. However, in ruminants, especially in cattle, infections cause only clinically significant diseases. Shipping fever is a respiratory disease of cattle, and is observed particularly in relation to stress situations such as prolonged live animal transports. Bacterial pathogens such as *Pasteurella* can also be isolated from diseased animals, which additionally contribute to characterizing the clinical picture. Experimental infections with bovine parainfluenza 3 lead to mild symptoms in some animals, which are pathologically and anatomically associated with an interstitial pneumonia. A vaccine is available for the protection of cattle; however, its efficacy is limited because of the cause of the disease by various infectious agents.

Shipping fever of cattle is qualitatively similar to kennel cough of dogs, which is caused by canine parainfluenza virus 2 (a rubulavirus) along with *Bordetella bronchiseptica*. Even these infections develop frequently without signs of disease and, therefore, remain unrecognized without additional cofactors. Under unfavourable conditions for keeping animals, pneumonia can occur in diseased dogs, and can sometimes last for years. Both inactivated and live vaccines are available, but also have limited effectiveness, like the vaccine against infections with bovine parainfluenza virus 3.

15.3.6.1 Newcastle Disease Virus

Epidemiology and Transmission

A very important pathogen of animal infectious diseases is the virus of atypical avian influenza, which is better known as Newcastle disease virus. It has been assigned to the genus *Avulavirus*. In nearly all bird species, Newcastle disease virus causes a systemic disease. It is highly contagious, and thus spreads very quickly in a susceptible bird population.

Clinical Features

The incubation period is short and lasts about 2–3 days. The symptoms associated with the infection are very varied and range from diarrhoea, respiratory distress, reduced egg production and egg anomalies to torticollis (wry neck) and paralysis. Importantly, Newcastle disease virus can also infect humans and causes a mild and transient conjunctivitis.

Pathogenesis

Infections of chicken with Newcastle disease virus lead to disorders of the respiratory tract, gastrointestinal tract and central nervous system, depending on the virus isolate and the immune status of the chickens. The virulence of the isolates in

chickens is described by the terms "velogenic" (viscerotrop, highly virulent), "mesogenic" (moderately virulent) and "lentogenic" (avirulent). The degree of virulence correlates with the ability of the viral F protein to be cleaved by cellular proteases: the F protein of the velogenic strains is cleaved efficiently, whereas that of the lentogenic strains is cleaved very poorly.

Immune Response and Diagnosis

The clinical diagnosis of Newcastle disease is difficult owing to the widely diversified symptoms. A reliable serological diagnosis is achieved by examining the unvaccinated livestock by a haemagglutination-inhibition test or by isolating the virus from an egg of diseased animals.

Control and Prophylaxis

In most countries Newcastle disease is notifiable. Infected livestock is culled. Various effective live and inactivated are available. Vaccination of turkeys and chickens for all purposes is mandatory.

15.3.6.2 Rinderpest Virus

Epidemiology and Transmission

Rinderpest virus causes a disease in ruminants which was formerly found in Asia, Africa and Europe; the virus has never become established in the Americas or Australia. Today, it is considered largely eradicated owing to worldwide vaccination programmes. The virus is excreted from infected animals by virtually all secretions and excretions and is spread via direct contact. Chronic infections are rare, and do not play any significant epidemiological role. Together with foot-and-mouth disease virus (▶ Sect. 14.1.6), rinderpest virus is probably the most notable and, historically, the most important animal disease pathogen. This can be seen, inter alia, from the fact that rinderpest (also referred to as cattle plague) was the reason for the establishment of the world's first veterinary training centre by Claude Bourgelat in Lyon in 1761. Rinderpest was vigorously combated worldwide. A comparable disease is caused in small ruminants such as sheep and goats by peste-des-petits-ruminants virus, a pathogen that is related to rinderpest virus. However, its infections usually result in weakened symptoms or are clinically inapparent.

Clinical Features

The virus causes a highly fatal disease in cattle, which exhibit a morbidity and mortality of nearly 100 %. However, virus strains have also been described that cause a considerably milder clinical picture. The infection is often asymptomatic in wild ruminants and pigs; in African cattle breeds it generally develops as a milder disease. Classic rinderpest is characterized by high temperature and inflammation of almost all mucous membranes, which, as a result, can lead to extensive erosions. Furthermore, bloody diarrhoeas are characteristic.

Pathogenesis

Like all other members of morbilliviruses, rinderpest virus also has a strong tropism for endothelial cells and immunologically active cells such as monocytes, macrophages and dendritic cells. Like human pathogenic measles virus and canine distemper virus, it also uses SLAM (CDw150) as a cellular receptor for attachment. In the course of infection, haemorrhagies are caused in almost all mucosal regions owing to lytic infection of the cells. A destruction of cells in all lymphatic organs can also be found owing to lytic replication, particularly in the mesenteric lymph nodes and the spleen. This leads to a massive immune suppression and reactivation of latent infections by other pathogens.

For some time, molecular research into rinderpest virus has become possible by reverse genetics, i.e. by transcribing the RNA genome into complementary DNA, and its cloning into appropriate expression vectors, and so investigation of the virus and the pathogenesis of infection are feasible through targeted mutagenesis.

Immune Response and Diagnosis

An infection leads, provided that the animal survives, to long-lasting immunity. The diagnosis is performed by isolation of the virus in cell culture or in embryonated chicken eggs. The test material is the buffy coat fraction of a whole blood sample. Alternatively, RT-PCR has also been successfully used for detecting viral genomes.

Control and Prophylaxis

The General Rinderpest Eradication Programme of the Food and Agriculture Organization, which was aimed at the global eradication of rinderpest, finished in 2010. After elimination of variola virus (▶ Sect. 19.6), the second successful worldwide eradication of a virus was officially acknowledged and celebrated in June 2011. Several attenuated live vaccines are available and have been successfully employed in the past. This was achieved primarily by a global vaccination campaign. Although a recombinant vaccinia virus expressing the F protein of rinderpest virus has been used as a vaccine in recent years, the use of a classical live attenuated vaccine from cell cultures was the key to the success. This vaccine (Plowright vaccine) was relatively thermostable and did not require a complete cold chain. The last case of rinderpest was reported to the World Organisation for Animal Health in Kenya in September 2001. Today, vaccination is prohibited; in the case of a new outbreak of rinderpest, all animals of the infected livestock have to be culled, and this also applies for animals that are suspected of being infected.

15.3.6.3 Canine Distemper Virus

Epidemiology and Transmission

Canine distemper is an infectious disease of dogs and has been known for a long time. Whereas epidemic plagues were observed in the past, only sporadic outbreaks have been recorded in domesticated dogs since the availability of effective

vaccines. However, it has recently been shown that canine distemper is becoming a major problem for all wild carnivores, and that not only the family *Canidae* is affected, but also all meat-eating terrestrial mammals. After wild rabies, canine distemper causes the most deaths associated with infectious diseases. Therefore, the host spectrum of this virus is widely diversified. Besides dogs and other canines (wild dogs and foxes), it has also been isolated from clinically affected martens (*Mustelidae*), raccoons and seals. Furthermore, it has also been recently described in large cats which died of a distemper-like disease. Moreover, in the southwestern USA, canine distemper virus has even been isolated from collared peccaries (family *Tayassuidae*) which exhibited central nervous system disorders.

Clinical Features

The clinical picture of distemper is variable. Essentially, three main forms are distinguished: the respiratory, the gastrointestinal and the central nervous forms. After a period with non-specific symptoms such as fever and loss of appetite (anorexia), the typical respiratory, gastrointestinal or central nervous symptoms emerge; symptom combinations are also frequent. Non-purulent bronchopneumonia, catarrhal to haemorrhagic gastroenteritis and encephalitis are detectable, both pathologically and anatomically. The infection is frequently fatal. As a late sequela, animals that survive may develop a tremor-like trembling or twitching of individual muscles in the extremities or the head as a result of viral replication in the central nervous system. Special forms such as old dog encephalitis – possibly equivalent to SSPE as a late consequence of measles infection in humans (Sect. 15.3.5) – and hard pad disease (hyperkeratosis of sole pads and the nose) are rare, and are solely observed in old dogs.

An infection at the time of dental maturation, or during second dentition in the first few months of life, can cause defects in the formation of tooth enamel. These typical dental development anomalies are known as distemper-associated enamel hypoplasia.

Pathogenesis

The pathogenesis of distemper is well understood. The virus is transmitted by droplet infection, and replicates initially in the cells of the nasopharynx and the conjunctiva. Similar to human pathogenic measles virus and rinderpest virus, canine distemper virus also uses SLAM (CDw150) as a cellular receptor. This explains the lymphotropism of the virus. After a lymphocyte-associated viraemia, the virus enters the lymphoid organs, where it replicates lytically. From there, the virus spreads throughout the organism during a second, high-titre viraemia, reaching the lungs and intestines as well as the central nervous system. Possibly, the blood–brain barrier is destroyed by viral replication in endothelial cells – a process that facilitates the transfer of infected lymphocytes and macrophages into the central nervous system. Once the virus has arrived in the central nervous system, it infects glial cells and neurons. The extent of damage to the

central nervous system is dependent on the virus strain: there are some isolates that predominantly induce a polioencephalitis, and others that preponderantly cause a demyelination encephalitis.

Phocine Distemper

The sudden occurrence of seal deaths in the Wadden Sea caused a stir in 1988. At that time, approximately 2,300 seals (*Phoca vitulina*) were victims of the epidemic. Extensive virological investigations led to the identification of a virus as the aetiological agent; it had similarities to the causative agent of canine distemper. It is now generally recognized that the epidemic was also favoured by the seal overpopulation and the immunosuppression of the animals as a result of great environmental pollution at that time. The epidemic situation improved rapidly owing to the increasing infestation of the population. After isolation of phocine distemper virus 1 from seals from the North Sea, the characterization of isolates from animals from similar epidemics in Lake Baikal and the Mediterranean, as well as after examinations of stranded toothed whales in the Mediterranean and the North Sea, a group of closely related, but distinguishable morbilliviruses was identified. On the nucleotide sequence level, phocine distemper virus 1 exhibits roughly 65–85 % homology to canine distemper virus. Interestingly, sequence comparisons revealed that phocine distemper virus 2, which caused the seal epidemic at Lake Baikal, is actually a canine distemper virus. A number of other morbilliviruses have been isolated from various species of toothed whales, porpoises and dolphins, for instance dolphin morbillivirus and porpoise morbillivirus. However, they exhibit a closer relationship to rinderpest virus and measles virus. In 2002, a new epidemic was registered in seal populations in the North Sea and Baltic Sea which was caused by phocine distemper virus 1, and was the reason for the death of about 30,000 seals. Nevertheless, the cause of that epidemic is still unclear. It is speculated that grey seals (*Halichoerus grypus*) may have transmitted the virus to the seals and could be responsible for its spread from Arctic waters.

Immune Response and Diagnosis

Probably, surviving an infection results in lifelong immunity. The diagnosis of distemper is not always easy because the clinical picture is variable and the laboratory diagnosis is difficult. The detection of the virus from lymphatic tissue is possible; however, it is not always successful. Far commoner is the detection of viral proteins and virus particles by immunofluorescence in touch preparations of the conjunctiva, or the detection of the viral nucleic acid in lymphocytes by PCR. As the virus also proliferates in epithelial cells of the bladder, virus particles can also be detected by electron-microscopic analyses of epithelial cells excreted in the urine.

Control and Prophylaxis

Effective vaccines against canine distemper based on attenuated viruses are available. Inactivated vaccines have proven to be ineffective. Since 1960, domestic dogs have been consistently vaccinated; therefore, canine distemper has lost its epidemic character. However, the virus is still present even today. A lack of herd immunity, which, for example, can result from insufficient vaccination, can lead to epidemics of canine distemper. Such an epidemic outbreak occurred among domestic dogs in Scandinavia in 1995, which has shown quite plainly this correlation.

15.3.6.4 Bovine Respiratory Syncytial Virus

Epidemiology and Transmission

Bovine respiratory syncytial virus, a member of the subfamily *Pneumoviridae*, is widespread in cattle populations. Under certain conditions, it can cause severe respiratory diseases in calves during epidemic outbreaks. These frequently emerge in winter when a high infection pressure and suboptimal housing conditions prevail owing to the stabling of calves originating from different herds. The morbidity is very high; however, the mortality is low.

Clinical Features

Infections with bovine respiratory syncytial virus are usually mild or asymptomatic. In cases of severe diseases, the pathological and histological picture of bronchitis and interstitial pneumonia can be observed, which can develop into severe bronchopneumonia during the afore-described epidemics.

Pathogenesis

The virus replicates in epithelial cells of the nasopharyngeal region as well as in bronchi and bronchioles. The replication is lytic and is associated with the loss of cilia and necrosis of infected cells. In contrast to infections of humans with human respiratory syncytial virus, immunological complications have not been conclusively described.

Immune Response and Diagnosis

The diagnosis can be performed by isolation of the virus from nose smears of infected animals. However, in the evaluation of a positive isolation, the delimitation of a disease from the high number of subclinical infections is problematic. After infection, the animals are immune, but the immunoprotection is of short duration.

Control and Prophylaxis

Vaccines based on inactivated or attenuated viruses are available. However, the protection they confer, like that of a natural infection, is also short-lasting.

15.3.7 Human and Animal Pathogenic Paramyxoviruses

15.3.7.1 Henipaviruses

Epidemiology and Transmission

In 1994, acute systemic diseases emerged in riding horses from two adjacent stables in Australia; the causative agent was later identified as Hendra virus. Fourteen of 21 affected horses died during the outbreak. Moreover, a riding trainer died as well. Another affected person survived the infection. Two further outbreaks have been described in Australia, and were associated with the death of a farmer in Mackey and the death of a horse near Cairns. Hendra virus has not been isolated outside Australia to date. During the outbreaks, the affected horses did not spread the infection further. People were infected only when they had direct contact with body fluids of infected animals. Hendra virus was isolated from tissues of the affected horses and the trainer. After the RNA genome had been transcribed into the complementary DNA and sequenced, the virus was classified into the newly created genus *Henipavirus* in the family *Paramyxoviridae*. Various species of fruit bats have been identified as natural hosts of Hendra virus by serological and virological detection methods. The bats do not become sick, but they are presumably persistently infected with the virus. Viral transmission within bat populations occurs intrauterine or via virus-containing urine. The apparent rare transmission of Hendra virus to horses is possibly by the accidental ingestion of virus-containing placental or fetal material from fruit bats. A possible scenario, for instance, is pastures with roosting trees for bats under which the horses graze.

On the basis of the experience gained by the analysis of Hendra virus infections, it was possible to isolate another paramyxovirus that is transmitted by fruit bats, Menangle virus, in the Australian state of New South Wales in 1997. It was isolated from stillborn piglets. Human infections have not been described so far. The distribution of Menangle virus does not seem to be limited to Australia, as demonstrated by the detection of the virus in fruit bats in Malaysia.

A third paramyxovirus that is transmitted by fruit bats was isolated in the region of Nipah (Malaysia) in 1999. Regarding the number of deaths among infected people, Nipah virus is the most important of the three new paramyxoviruses. Overall, 265 cases were recorded in the outbreak in Malaysia in 1998–1999, in which more than 100 people, especially pig farmers and butchers, fell victims to the first Nipah virus epidemic, having died of encephalitis. The tropism of Nipah virus to infect cells of the respiratory tract promotes a transmission by droplet infection between swine, or between pigs and humans. In addition, there is evidence that the virus can be transmitted not only between infected animals and from infected animals to humans, but also from human to human. Furthermore, Nipah virus infections have also been described in one cat, two dogs and one horse. Further outbreaks were observed in India and Bangladesh between 2001 and 2008. In those epidemics, cows seemed to be

involved as carriers of the infection. Serological studies demonstrated that also in this case fruit-eating bats (*Pteropus* sp., *Hipposideros larvatus*) are the natural reservoir for Nipah virus, and more than 75 % of the bats have antibodies against it. Nipah virus RNA has been found in the urine of fruit bats, but also in fruits that have been nibbled by bats.

Clinical Features

Experimental Hendra virus infections in horses revealed the following main symptoms: interstitial pneumonia and systemic fibrinoid degeneration of blood vessels. Non-purulent encephalitis can also frequently be observed. On the other hand, experimental Menangle virus infections in swine lead to fertility disorders. Infected piglets exhibited widely different malformations and frequently an encephalomyelitis. Nipah virus infections in swine resulted particularly in bronchopneumonia.

Infected people develop symptoms of encephalitis in addition to a severe atypical pneumonia; the mortality rate is 37 % for the Malaysia strain and 67–100 % for the Indian strain of Nipah virus.

Pathogenesis

Hendra virus and Nipah virus are able to infect various cell types in animals. The pathogenesis of Nipah virus is well studied in swine. Following intranasal infection, the virus replicates in the epithelial cells of the nasopharyngeal cavity, entering the cells of the olfactory nerves and reaching the central nervous system along the axons, where it proliferates and causes lesions. During a viraemia, Nipah virus also infects the endothelial cells of blood vessels in the nasopharyngeal cavity, from where it infects lymphocytes, which carry the virus into the brain. The contribution of these two routes of infection of the central nervous system seems to be different in the various hosts: in swine, there is evidence that the haematogenous dissemination of Nipah virus is of subordinate significance for infection of the central nervous system. Cats are employed as another suitable animal model for studying the pathogenesis of Hendra virus and Nipah virus infections.

Immune Response and Diagnosis

Detection of the virus is performed by RT-PCR, i.e. by transcription of the viral RNA genomes into complementary DNA by a reverse transcriptase and a subsequent DNA amplification by PCR. There are no verified data concerning immunity after a natural infection.

Therapy and Prophylaxis

There are no vaccines against these viruses, and no antiviral chemotherapy is available.

15.4 Filoviruses

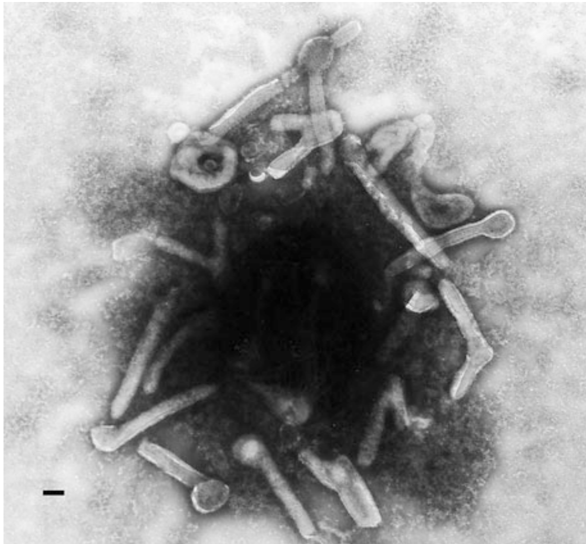


Table 15.8 Characteristic prototypes of filoviruses

Genus	Human/animal virus
Marburgvirus	Marburg marburgvirus
Ebolavirus	Zaire ebolavirus Sudan ebolavirus Tai Forest ebolavirus (Côte d'Ivoire) Bundibugyo ebolavirus (Uganda) Reston ebolavirus

The family *Filoviridae* belongs to the order *Mononegavirales*, along with the families *Rhabdoviridae*, *Bornaviridae* and *Paramyxoviridae*. Filoviruses do not exist under natural conditions in Europe. They include Marburg virus and Ebola virus, which usually cause a fatal haemorrhagic fever in humans, apes and Old World monkeys.

15.4.1 Classification and Characteristic Prototypes

The family *Filoviridae* includes the genera *Marburgvirus* and *Ebolavirus* (Table 15.8). With respect to the structure of their genome, they are more similar to paramyxoviruses than to rhabdoviruses. The serological relationship between members of the genera *Marburgvirus* and *Ebolavirus* is very small.

Reston ebolavirus was isolated from macaques from the Philippines; it has since also been detected in swine. Although Reston ebolavirus can infect humans, it does not cause diseases, but simply stimulates antibody production, in contrast to the Zaire ebolavirus, the Sudan ebolavirus and the other species of the genus. The natural hosts of Marburg virus and Ebola virus are probably bats.

Filoviruses Require Very Stringent Safety Precautions
The handling of filoviruses and research into them is associated with high safety requirements. Laboratories have to possess the highest safety standard (BSL-4 or S4). Only the development of genetic engineering techniques allowed the elucidation of the sequence of these viruses, as well as the analysis of the proteins encoded by them, and the construction of recombinant viruses with targeted mutations.

15.4.2 Structure

15.4.2.1 Virus Particle

The virions of filoviruses have a non-uniform, pleomorphous shape. They are similar to rhabdoviruses, but are considerably longer and filamentous (Latin *filus*, “thread”; Fig. 15.10). The filaments can be branched, U-shaped or spirally coiled. The particles have a constant diameter of 80 nm; however, their length is highly variable (up to 14,000 nm). On average, Marburg virus is about 665 nm long, whereas Ebola virus is 805 nm long. The filaments consist of a helical nucleocapsid, which is composed of the RNA genome and the viral proteins NP (nucleoprotein), P (also VP35), VP30 and L (RNA-dependent RNA polymerase). The nucleocapsid is surrounded by an envelope. The matrix proteins VP24 (minor) and VP40 (major) are associated with both the inner side of the envelope and the protein components of the nucleocapsid. The trimeric glycoproteins (GP) are embedded in the envelope and project approximately 7 nm from the surface of the virus (Table 15.9).

15.4.2.2 Genome Organization and Structure

The single-stranded, non-segmented RNA genome of filoviruses has a length of about 19,000 nucleotides. It comprises 18,957 nucleotides in Ebola virus and 19,099 nucleotides in Marburg virus. It has a negative-sense polarity. The RNA is non-infectious, and cannot be directly used as mRNA for protein synthesis. At the 3′ and 5′ ends of the genome there are non-coding sequences whose last nucleotide sequences are complementary, and probably exert functions similar to those of the leader and trailer regions of paramyxoviruses in initiation of transcription and synthesis of the positive-sense RNA during replication. The reading frames are located between the terminal non-coding regions in the following order: 3′-NP-VP35/P-VP40/M-GP-VP30-VP24-L-5′ (Fig. 15.11). An additional reading frame is situated in the GP gene region, which encodes a putative protein of

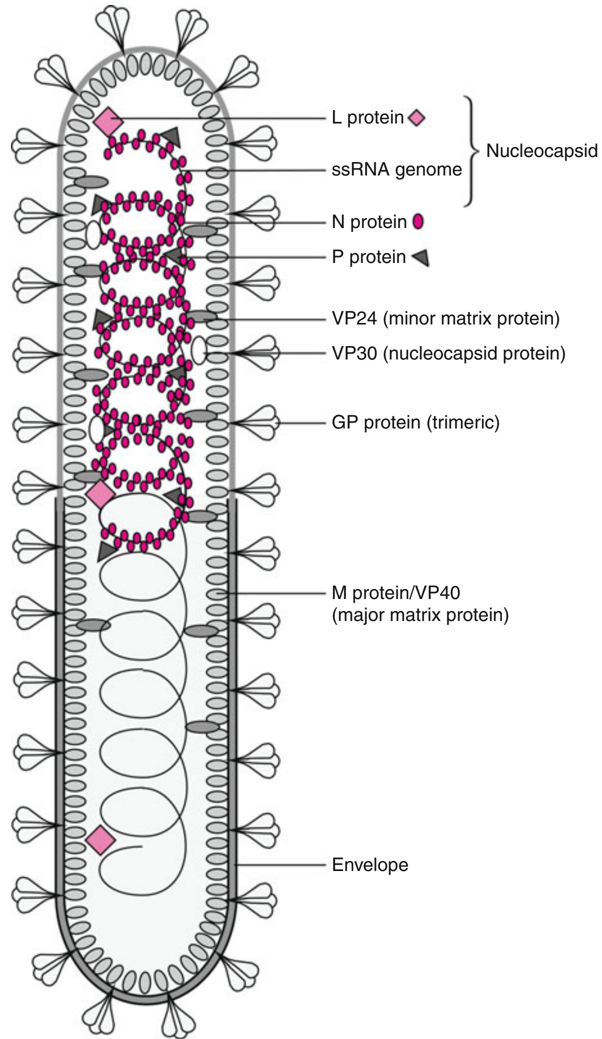


Fig. 15.10 Structure of an Ebola virus particle. The genome comprises a single-stranded RNA, which interacts with the N, P and L proteins, forming a helical nucleocapsid. The nucleocapsid is surrounded by an envelope, in which G proteins are inserted. The inner side of the envelope is associated with the matrix proteins VP24 (minor) and VP40 (major), which simultaneously bind to the other nucleocapsid components

approximately 15 kDa; however, the corresponding gene product has not been identified. In Ebola virus, the nucleotide sequences encoding the GP protein reside in two separate reading frames. The synthesis of the protein depends on the insertion of one nucleotide into the mRNA by RNA editing. If the mRNA is not edited, only the sGP protein will be synthesized (Fig. 15.11). At the initiation of transcription and termination sites there are sequences which are conserved in all genes and contain the pentanucleotide 3′-UAAUU-5′. The coding regions either are separated by short intergenic nucleotide sequences or overlap in a short segment. The overlaps are confined to the conserved transcription signals at the aforementioned pentanucleotide. Such overlapping genes are found in two

Table 15.9 Properties and functions of filovirus proteins

Protein	Molecular mass (kDa) Marburg virus	Ebola virus	Modification	Function
NP	96	104	Phosphorylated	Associated with the RNA genome; components of the nucleocapsid
VP35 (P)	32	35	Phosphorylated	Component of the nucleocapsid; interaction with matrix proteins, component of the transcriptase complex; inhibits interferon production
L	267	267		RNA-dependent RNA polymerase; nucleocapsid component
VP30	28	30	Phosphorylated	Component of the nucleocapsid; RNA binding; essential for transcription
VP40 (M)	38	40		Major matrix protein; is associated with the inner side of the viral envelope and with nucleocapsid components
VP24	24	24		Minor matrix protein; is associated with the inner side of the viral envelope and with nucleocapsid components
GP	170	120–150	N-glycosylated and O-glycosylated	Surface and envelope protein; homotrimer; attachment; induction of neutralizing antibodies, is cleaved by the Ebola virus protease furin into GP_1 (140 kDa) and GP_2 (26 kDa)
sGP	–	50	N-glycosylated and C-mannosylated	Secreted dimeric protein; decoy antigen for the inactivation of neutralizing antibodies?
Δ-peptide	–	6 kD	O-glycosylated	Peptide of 41 amino acids in length that is cleaved by the protease furin from the carboxy terminus of sGP

gene junction regions in the genome of Ebola virus, between the genes for VP35 and VP40 as well as between the cistrons encoding VP30 and VP24. In Marburg virus, only the genes that code for the proteins VP30 and VP24 overlap (Fig. 15.11).

15.4.3 Viral Proteins

A comparative overview of the known characteristics of the proteins of Marburg virus and Ebola virus is given in Table 15.9. The functions of these viral proteins are not well studied in each case. In many cases, it is assumed that they correspond to the polypeptides that are encoded in the analogous regions of the genome of rhabdoviruses and paramyxoviruses.

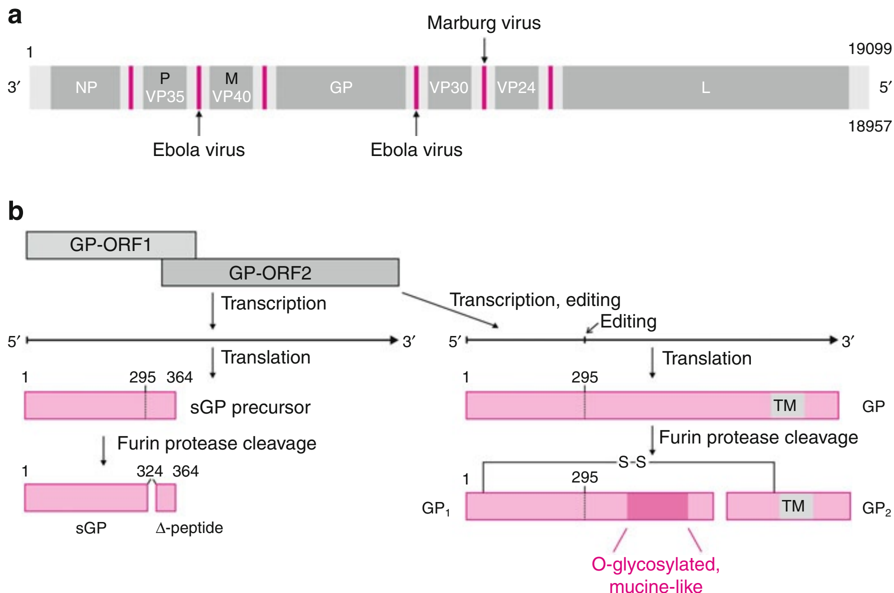


Fig. 15.11 (**a**) Genome organization of filoviruses. The genome, represented as a *bar*, consists of a single-stranded, negative-sense RNA molecule. The location and length of the different viral genes, which are transcribed in the course of the replication cycle, are indicated schematically. Regulatory sequences for stop and start of transcription are located at the ends and between the genes (shown in *light grey/red/light grey*). In Marburg virus, the 3′ non-coding sequence of the VP30-specific mRNA overlaps with the 5′ terminus of the VP24 transcripts (indicated by the *black arrow above the bar*). Similar overlaps are also found in Ebola virus at the junctions of the P/VP35 and M/VP40 genes as well as between the GP and VP30 genes (indicated by *black arrows* below the *bar*). (**b**) Genome organization of the Ebola virus GP gene and its expression products. The Ebola virus GP gene is composed of two overlapping open reading frames (ORF1 and ORF2). The viral RNA-dependent RNA polymerase synthesizes a single mRNA species from both open reading frames. If it is not edited (*left*), then the translation proceeds up to the stop codon at the end of ORF1, resulting in the synthesis of the sGP precursor protein, which is post-translationally cleaved into the mature sGP protein and the Δ-peptide. If the mRNA is edited (*arrow*), then ORF1 and ORF2 are translated together in the same reading frame up to the stop codon at the end of ORF2. This process leads to the synthesis of the GP protein, which is anchored in the envelope by the transmembrane region (*TM*). It is post-translationally cleaved into the GP_1 and GP_2 subunits, which are covalently linked by a disulphide bridge. The GP_1 protein contains an O-glycosylated, mucine-like domain at its carboxy-terminal moiety

15.4.3.1 Nucleocapsid Proteins

The nucleocapsid of filoviruses is constituted of a complex of RNA and proteins. The NP protein (96 and 104 kDa in Marburg virus and Ebola virus, respectively) is present in the highest concentration; it covers the entire length of the RNA genome, and is phosphorylated at serine and threonine residues. It is likely a functional analogue of the N/NP proteins of paramyxoviruses and rhabdoviruses and is most probably responsible for the helical structure of the RNA–protein complex in

the virion. VP35 is functionally equivalent to the P protein of the other members of the order *Mononegavirales*. It is involved in the transcription and replication processes as part of the RNA polymerase complex, and mediates the interaction of the nucleocapsid with the matrix proteins VP40 and VP30 on the inside of the viral envelope. In the cytoplasm of infected cells, VP35 binds to the light chain of dynein (DLC8); however, it is unclear how this interaction affects infection. Furthermore, it has been described that VP35 influences both the course and the pathogenesis of infection: it inhibits the synthesis of IFN-α in infected hosts. The L protein possesses RNA-dependent RNA polymerase activity. It has a theoretical molecular mass of 267 kDa in both Marburg virus and several strains of Ebola virus. In contrast to rhabdoviruses and paramyxoviruses, an additional component is found in the nucleocapsid of filoviruses, namely the phosphorylated VP30 protein. The RNA-binding VP30 is required for restarting mRNA synthesis during transcription of viral genes.

15.4.3.2 Envelope Proteins

The viral glycoprotein GP is the only protein exposed on the surface of virus particles; therefore, it is directly accessible to the immune system. It is present as a trimer, and is highly modified with carbohydrate residues. These account for half of the experimentally determined molecular mass of 170 and 125 kDa in Marburg virus and Ebola virus, respectively. The carbohydrate groups are linked to the amino acid residues by N-glycosidic and O-glycosidic bonds. *N*-Acetylneuraminic acid is found in Ebola virus as a terminal sugar, but not in the GP protein of Marburg virus, where it is replaced by galactose. As type I membrane proteins, the GP proteins are anchored to the cytoplasmic membrane of infected cells by a hydrophobic amino acid domain at the carboxy terminus. After it has been synthesized on the membrane of the endoplasmic reticulum, the amino-terminal signal peptide is removed from GP proteins; during their transport to the cell surface, the GP protein is cleaved by the protease furin into an external GP_1 subunit and a membrane-anchored GP_2 subunit in the Golgi vesicles. Both subunits are linked by a disulphide bridge, but a subset of GP_1 proteins are released from the virus particles and infected cells. The carboxy-terminal moiety of the GP_1 protein, i.e. the region that is located before the furin cleavage site, is rich in serine and threonine residues and has homology to mucine-like domains. A region containing hydrophobic amino acids is located at the amino terminus of the GP_2 protein, and provides the membrane-fusing activity of the protein. The protein domain that is situated before the transmembrane region is responsible for the trimerization of GP complexes. The reading frame for the GP protein of Ebola virus is also used for the synthesis of a second, shortened sGP protein, which interacts by cysteine bridges to form homodimers, and is secreted by infected cells (Fig. 15.11b). Its synthesis occurs when the mRNA of GP proteins is not modified by RNA editing. Therefore, its amino-terminal moiety is identical to that of the GP_1 protein and spans a region of 295 amino acid residues. On the other hand, the carboxy-terminal domains are different because translation occurs in another reading frame from the RNA editing site. Therefore, the sGP protein contains neither the O-glycosylated mucine-like

domain of GP_1 protein nor the transmembrane domain at the C-terminal region of the GP_2 protein; however, sGP possesses a WxxW consensus sequence for C-mannosylation at its carboxy-terminal domain. The protease furin cleaves sGP at its carboxy terminus, releasing the 40 amino acid long, O-glycosylated Δ-peptide. A possible function as a decoy antigen is discussed for sGP, i.e. it intercepts the neutralizing GP-specific antibodies, preventing their interaction with infectious virus particles.

VP40 is functionally equivalent to the matrix protein of paramyxoviruses and rhabdoviruses and links the inside of the envelope with the nucleocapsid. VP40 forms octameric complexes and is the particle-forming component of viral structural proteins. The third component is the VP24 protein, which is also associated with the envelope as a minor matrix protein.

15.4.4 Replication

The replication cycle of filoviruses is similar to that of the other members of *Mononegavirales*. Filoviruses are capable of infecting many different cell types and seem to interact unspecifically with various cell surface proteins: the complex of GP_1/GP_2 proteins interacts with TREM receptors (triggering receptors expressed on myeloid cells) on macrophages and neutrophils, several C-type lectins such as DC-SIGN on dendritic cells and macrophages as well as L-SIGN on hepatocytes. In addition, filovirus binding to Toll-like receptors 1 and 2 as well as to folate receptor α and heparan sulphate proteoglycans has also been described. The latter function as receptors for the infection of endothelial and epithelial cells, whereas endothelial cells of blood vessels have a major pathogenic significance as target cells. Marburg virus also interacts with the asialoglycoprotein receptor on hepatocytes, a C-type lectin that binds to terminal galactose residues of N-glycosidically linked sugar groups; therefore, the absence of terminal sialic acid residues as a modification of GP proteins of Marburg virus is likely important for the pathogenesis of this viral infection. Since the interaction between the virus and the cell can be inhibited by carbohydrates such as mannan, it is assumed that this occurs by contacts between the sugar modifications of GP proteins and the respective cellular receptors. In addition, there are probably further cell surface molecules that can act as receptors for the infection of other cells. Penetration of the particle into the cell occurs by receptor-mediated endocytosis; then, the fusogenic activity of GP protein induces the fusion between the envelope and the endosomal membrane. As a result, the nucleocapsid enters the cytoplasm, where all further steps are executed: the transcription of the genome into capped, polyadenylated mRNA species is catalysed by the protein complex NP–P/VP35–L. VP30 is essential for the resumption of transcription after skipping the intergenic nucleotides. The mRNAs are subsequently translated into the various proteins. It is assumed that a protein concentration gradient is also formed between the NP and the L polypeptides in filoviruses. A full-length positive-sense RNA, which serves as an antigenome template for replication, is only formed when sufficient amounts of NP proteins

are present in the cell. The assembly of the nucleocapsid components, which can be detected in cytoplasmic inclusion bodies, occurs concurrently with replication in the cytoplasm of the cell. The matrix protein VP40 is transported to the inside of the cytoplasmic membrane by the intracellular coat protein complex II (COPII) transport pathway. The protein molecules attach to the regions of the cytoplasmic membrane in which the trimeric GP_1/GP_2 complexes are anchored, which were glycosylated in the secretory pathway and cleaved by the protease furin after their synthesis. The glycosylation pattern is cell-type-specific; therefore, the modifications of GP proteins are different. Infectious viruses are released by budding from the lipid rafts on the cell surface.

15.4.5 Human and Animal Pathogenic Filoviruses

15.4.5.1 Marburg Virus and Ebola Virus

Epidemiology and Transmission

In Marburg in 1967, Slenczka Werner, Rudolf Siegert and Dietrich Peters isolated Marburg virus for the first time from people who had handled vervets (*Cercopithecus aethiops*) imported from Uganda. Twenty-five members of the laboratory staff became ill with haemorrhagic fever, and 7 of them died. In six cases, they transmitted the infection to family members and hospital staff. Simultaneously, the infection was also observed in Frankfurt and Belgrade. Even in those cases, the patients had been in contact with imported African green monkeys. In the following years, Marburg virus was sporadically detected in patients in East Africa in the region around Lake Victoria; however, a large outbreak occurred in Durba (Democratic Republic of the Congo) between 1998 and 2000. During this prolonged epidemic outbreak, a Marburg virus infection was diagnosed in 154 people, of whom 83 % died. A similar severe epidemic occurred in northern Angola in 2005, and claimed 227 victims.

Zaire ebolavirus and Sudan ebolavirus were originally detected during epidemics in the Democratic Republic of the Congo and Sudan in 1976, and were identified as filoviruses. A total of 500 people were infected with haemorrhagic fever in those cases. After sporadic individual cases, repeated new epidemics with hundreds of deaths have emerged in Sudan, the Democratic Republic of the Congo, Uganda and Gabon since in 1979. The mortality rate is between 50 % and 90 %. Infections with the highly virulent Zaire ebolavirus and Sudan ebolavirus are most often fatal. Infections with Tai Forest ebolavirus and the Bundibugyo ebolavirus are much rarer.

In Reston (USA) in 1989, Reston ebolavirus was isolated from macaques (*Macaca fascicularis*) which had been imported from the Philippines. It causes a deadly haemorrhagic fever in the monkeys, and it can apparently be transmitted to humans. Antibodies specifically against Reston ebolavirus were found in zookeepers who had contact with the infected monkeys; however, no diseases have been observed. In 2008, Reston ebolavirus was detected in diseased pigs in the Philippines, which in some cases died of the effects of the infection. The clinical

significance of Reston ebolavirus infection in pigs is unclear because the animals were also infected with the arterivirus porcine reproductive and respiratory syndrome virus (▶ Sect. 14.7). The fact that swine can be infected with Reston ebolavirus and serve as a host for the pathogen was confirmed in further studies in 2009. The outbreaks are followed with great concern, because swine are in close contact with humans and zoonotic transmissions are likely to be frequent. Indeed, antibodies against Reston ebolavirus have been found in the blood of people who come into contact with swine, and some people showed febrile diseases. Thus, there is a fundamental risk of a host change and also an associated increase of virulence.

Fruit-eating bats are deemed to be a reservoir for Ebola virus and also probably for Marburg virus. RNA genomes of Zaire ebolavirus were detected in liver and spleen samples of the bat species *Hypsignathus montrosus*, *Epomops franqueti* and *Myonycteris torquata*, and large quantities of specific antibodies against viral proteins were present in the blood of the bats. The bats were apparently infected with Zaire ebolavirus without developing disease symptoms. Great apes (gorillas, chimpanzees) and Old World monkeys which are either naturally or experimentally infected with the virus usually die of the disease; this applies particularly to Marburg virus and Zaire ebolavirus. The course of infection in apes resembles that in humans. Most of the Ebola virus epidemics in humans can be ascribed to initial contact with the blood of infected apes. Then, infected people transmit the virus by direct contact, by blood and other body fluids and probably also by droplet infection to hospital staff and family members. Under what conditions the virus is transmitted by its natural hosts, bats, to apes is not clear; however, similar to henipaviruses, virus-containing placental tissue is discussed as a possible source (Sect. 15.3).

Clinical Features

The symptoms of the disease are similar in infections with Marburg virus and Ebola virus. They appear after an incubation period of 4–7 days and are characterized by high temperature, grave headache and muscle pain as well as shivering. These preliminary symptoms are rapidly followed by sore throat, nausea, vomiting, diarrhoea and abdominal distress. Owing to infection and death of endothelial cells of blood vessels, bleeding in the conjunctiva and the throat mucosa appear very early, followed by severe haemorrhages in the gastrointestinal tract, the lungs and the oral mucosa after a few days. During this acute phase and to some extent long afterwards, viruses can be detected in all body fluids, such as pharyngeal lavage, urine, seminal fluid and the aqueous humour of the eye. The concentration of the virus in the blood increases continuously in fatal courses. Death occurs about 5–9 days after the onset of symptoms; it is caused by severe internal bleeding and related shock conditions. The symptomatic phase has a similar duration also in patients who survive the infection; however, a decrease of the concentration of the virus in the blood can be found, presumably caused by the induction of specific antibodies. The virus–antibody complexes are also blamed for the arthralgias that mainly occur during convalescence.

Pathogenesis

Filoviruses do not have pronounced cell specificity; they infect many different cell types of various tissues and destroy them. In the first phase of infection, the virus infects monocytes, macrophages and endothelial cells, which are the primary targets. After the first viraemia, the virus reproduces in endothelial cells of blood vessels, thus proliferating virtually in the entire organism. Cytoplasmic inclusion bodies comprising viral nucleocapsids can be detected in infected cells. Histopathologically, focal necroses are particularly found in the liver, whereas follicular necroses are especially observed in the spleen and lymph nodes. In the late stage of the disease, haemorrhages are observed in almost all organs; these haemorrhages are attributed to the infection-induced destruction of endothelial cells and the associated loss of integrity of blood vessels. Bleeding in the renal tubules is also an indication of severe injury in these organs. Fibrin and fibrin cleavage products can be found there. Abnormalities in blood coagulation parameters indicate a general perturbation of this process.

Infection of monocytes and macrophages induces the release of proinflammatory cytokines and chemokines. These include, in particular, TNF-α, IL-1β and macrophage inflammatory protein 1α, which additionally cause the migration of monocytes, macrophages and neutrophils to the infection site, thus exacerbating the inflammation, dilating the blood vessels and aggravating vascular damage. Blood vessel integrity is also affected by a direct viral activity, which is ascribed to the mucine-like domain of the GP_1 protein. If it is deleted, the protein loses its cytotoxic effect both in vitro and in explanted blood vessels. In these experiments, the GP_1 protein of Reston ebolavirus showed no cell-destroying effect in explants of human blood vessels, whereas the corresponding surface protein of the highly human pathogenic Zaire ebolavirus proved to be toxic for both human and rodent vessels. The evidence for the GP_1 protein as the component that is mainly responsible for the cytopathogenicity of the virus was confirmed by an artificially generated mutant. In this mutant, one nucleotide in the viral genome was modified in such a way that the RNA editing process, which is necessary for the synthesis of the GP_1/GP_2 proteins, takes place compulsorily, and the sGP protein is not synthesized. This mutant has proven to be substantially more cytotoxic than the wild-type virus. The sGP protein does not contain the mucine-like domain. This fact can be interpreted as evidence that under simultaneous synthesis of sGP, the cytotoxicity of the GP_1 protein is weakened, and thus the virus is attenuated.

Furthermore, the GP_1/GP_2 protein complex possesses an additional function that influences the pathogenesis of infection. When it is anchored in the cytoplasmic membrane of infected cells, its presence leads to the masking of various cell surface proteins such as MHC class I proteins, and even their own GP epitopes. The binding of components of the extracellular matrix is possibly responsible for this effect. As a result of this surface masking, infected cells appear depleted of MHC class I antigens and even GP-specific antibodies cannot recognize infected cells.

Besides the direct cytotoxic effect of filoviruses, which is responsible for the damage of the endothelium and blood vessels, filoviruses also affect the

non-specific immune response early during infection. The phosphoprotein VP35 prevents the synthesis of the class I interferons INF-α and INF-β by inhibiting phosphorylation of IRF-3. As a result, IRF-3 is not transported into the nucleus, and cannot function as a transcription factor to induce the expression of interferon genes. However, the minor matrix protein VP24 blocks the interferon-mediated signalling transduction cascade by preventing phosphorylation of protein p38 – a pivotal factor of the interferon-dependent mitogen-activated protein kinase signalling pathway. As a result of this, the expression of interferon-dependent genes remains inactivated, and the antiviral status is not established. The essential effect of the interferon-mediated defence response can be shown in animal systems: immunocompetent adult mice survived infections with Ebola virus; however, they died when they were concomitantly inoculated with antibodies against interferon. The same is true for mice in which the genes of the receptors for IFN-α and INF-β proteins or STAT1 were deleted. Since these defence mechanisms of the innate immune system are also responsible for the induction of the specific immune responses, besides their direct antiviral effect, they are either not established or are established only with delay.

Mucin and Mucin-Like Protein Domains

Mucins are highly glycosylated proteins and are a main cellular component of mucus-producing epithelial cells. In addition, there are proteins that exhibit mucin-like domains. All are now classified as members of a new protein family, the sialomucins. These represent proteins which are anchored in the cytoplasmic membrane either by classic transmembrane regions or by covalently linked aliphatic acid modifications in their amino acid sequence (e.g. glycosylphosphatidylinositol residues). Mucins and mucin-like proteins contain many threonine, serine and proline residues which are modified with carbohydrates. The sugar groups are linked to the amino acids by O-glycosidic bonds. Similar to cell adhesion molecules, sialomucins are involved in the mutual interaction of specific cell types and their interaction with the extracellular matrix. During such processes, signal transduction pathways are triggered which promote the release of various cytokines. Furthermore, it has been found that also the early stages of haematopoiesis are regulated by members of the mucin-like protein family. Mucin-like proteins have also been identified as surface components in helminths and protozoa, e.g. in *Trypanosoma cruzi*, the pathogen of sleeping sickness. It is supposed that they are responsible for penetration of the pathogens into tissue and cells and also for inducing the synthesis of cytokines and mediators of inflammation.

Immune Response and Diagnosis

Little is known about the antibody response. Ten to 14 days after infection, immunoglobulins mainly against the nucleoproteins, phosphoproteins and matrix

proteins (NP, VP35 and VP40) as well as the glycoproteins GP_1/GP_2 can be detected in survivors by ELISA and immunofluorescence tests. Glycoprotein-specific antibodies are able to inactivate the virus. During the last Ebola epidemics, it was investigated to what extent the immune reactions of survivors of Ebola virus infections differ from those of the deceased patients. Both patient groups exhibited nearly the same detectable virus concentrations during the first few days after the onset of symptoms. However, the peripheral blood virus concentration increased further in the group of patients who died in the course of the disease, whereas it remained at a lower level and then declined in the survivors.

There was an induction of IgM and IgG in the convalescents, which was followed by the clearance of viral antigens from the blood. In this phase, cytotoxic T lymphocytes were also activated, leading to the associated expression of FasL, perforin and IFN-γ, which were still detectable during the convalescence. Contrarily, no IgG antibodies were detected in patients who died of the infection. In the early phase of infection, in the group of patients who died there was activation of T cells and, in comparison with the group of patients who survived, increased production of IFN-γ, but they were, however, not able to control the viral infection. The expression of IFN-γ, FasL and perforin was temporarily further induced with progressive disease. This phase was followed by multiple apoptotic processes as shown by the increase of the levels of apoptosis markers in the serum. Presumably, this is a consequence of the increasing destruction of monocytes and endothelial cells of blood vessels by the disseminating viral infection.

Formerly, the diagnosis was once routinely made by isolation of the virus from serum or body secretions during the viraemic phase. Marburg virus can be propagated in Vero cell cultures (kidney cells from green monkeys), and the various species of Ebola virus can be propagated in MA104 cells (embryonal kidney cell line of a rhesus monkey). Currently, viral RNA from suitable biological materials is preferentially detected by PCR. Because of the great risk that emanates from these viruses for personnel, extensive safety measures have to be complied with in laboratories and hospitals. If the suspected diagnosis is performed by epidemiological correlation (e.g. staying in infected areas) and on the basis of medical history and symptoms, the patients have to be admitted immediately to such centres by special transport. The virological diagnosis must be performed only in approved laboratories.

Therapy and Prophylaxis

There is no vaccine that offers protection against Marburg virus and Ebola virus infections. Vaccines are being developed on the basis of recombinantly produced virus-like particles. A specific antiviral therapy is not possible; the “broad-spectrum antiviral agent” ribavirin has been applied without success against RNA virus infections. The application of immunoglobulin preparations from plasma of people who have survived the infection may confer a passive immune protection in acute outbreaks of Ebola virus epidemics. This notion is supported by animal experiments in which mice were inoculated with Ebola virus. Mice that survived the infection produced virus-specific immunoglobulins. When these immunoglobulins were administered to mice before infection, all mice survived.

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16 Viruses with Single-Stranded, Segmented, Negative-Sense RNA Genomes

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There are three virus families containing a negative-sense RNA genome, which does not exist as a continuous molecule, but is present in several segments. These are the families *Arenaviridae*, *Bunyaviridae* and *Orthomyxoviridae*. Similarly to members of the order *Mononegavirales* (▶ Chap. 15), they also require the presence of a special enzyme (RNA-dependent RNA polymerase) to perform the synthesis and replication of messenger RNA (mRNA); it reaches the cell along with other viral components during infection. A segmented genome enables the virus to generate reassortants. In this process, the RNA molecules of different virus strains are mixed or reshuffled in doubly infected cells during replication and morphogenesis. In this way, progeny viruses can obtain new combinations of

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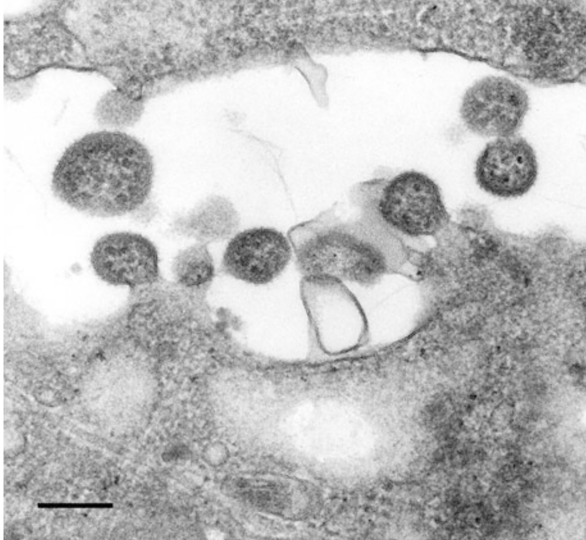
RNA segments and thus gain novel properties. This mechanism, which is referred to as antigenic shift, is particularly common and well studied in influenza A viruses, the causative agents of viral influenza or genuine flu (Sect. 16.3).

16.1 Arenaviruses



Arenaviruses, which are commonest in South America and Africa, cause preponderantly persistent infections in their natural hosts, rodents, which excrete them with the urine and saliva. In the case of contact with blood or contaminated excretion products, some arenaviruses can infect humans, causing fever and haemorrhagic diseases. Examples are Junín virus and Lassa virus as pathogens of Argentine haemorrhagic fever and Lassa fever, respectively. The prototype of the family is lymphocytic choriomeningitis virus (LCMV), which is found in the house mouse (*Mus musculus*) and causes, in rare cases, acute aseptic meningitis in humans. LCMV infection of mice is an important, well-established system to study the immune response. The name "arenavirus" is derived from the Latin word *arena* ("sand"), alluding to the granular structure that the virus particles exhibit in electron micrographs. The virions obtain this appearance by ribosomes, which they incorporate during morphogenesis.

16.1.1 Classification and Characteristic Prototypes

The family *Arenaviridae* comprises only one genus (*Arenavirus*). More than 20 arenavirus types are known and they are subdivided on the basis of their

geographical distribution into arenaviruses of the Old World (lymphocytic choriomeningitis Lassa serocomplex), which are widespread in Europe, Asia and Africa, and arenaviruses of the New World (Tacaribe serocomplex), which are found on the American continent (Table 16.1). LCMV is the only human pathogenic arenavirus with a worldwide distribution. In Africa, Lassa virus causes severe, often fatal haemorrhagic fever in humans. The New World arenaviruses are further subdivided on the basis of their serological properties into three clades (A–C), which solely exist on the American continent. Human pathogenic viruses of clade A are present in both North America (Whitewater Arroyo virus) and South America (Flexal virus). In addition, Junín virus, Machupo virus and Guanarito virus (clade B) are endemic in South America, and cause Argentine, Bolivian and Venezuelan human haemorrhagic fever, respectively. Another human pathogenic arenavirus, Sabiá virus has been identified in Brazil, but its natural host is unknown. In contrast to the other arenaviruses, Tacaribe virus, which is endemic to Trinidad, does not infect rodents as natural hosts, but infects fruit-eating bats. Besides them, a series of arenaviruses have been identified in different rodents for which no human pathogenic properties have been described so far (Table 16.1). Even New World arenaviruses of clade C (Latino virus, Pampa virus, Oliveros virus) have so far been isolated only from rodents in Argentina and Bolivia.

16.1.2 Structure

16.1.2.1 Virus Particle

Arenavirus particles are pleomorphic. They have a mostly spherical shape with variable diameters of 50–300 nm (Fig. 16.1), and consist of two nucleocapsid segments, which are surrounded by an envelope. Glycoproteins GP1 and GP2 are embedded in the envelope of LCMV. They are generated by proteolysis from a larger precursor protein, and stick out from the particle surface as club-like protrusions of 8–10 nm. The GP2 protein is anchored in the envelope, whereas the GP1 protein is non-covalently associated with the particle surface. The viral RNA genome segments L (for "large") and S (for "small") are situated inside the envelope, forming a complex with NP proteins (nucleoproteins, 63 kDa). Several copies of L and S segments can occasionally be encountered in the particles. Further virion components are L proteins, (RNA-dependent RNA polymerase, 250 kDa), relatively large amounts of Z proteins (11 kDa) and a protein kinase. The latter is able to phosphorylate NP proteins in vitro. It is not known whether this enzyme is virus-coded. Ribosomes of the host cell are incorporated during particle morphogenesis, and arise by budding from the cytoplasmic membrane. It is thought that this explains the polyadenylation and polyuridinylation enzymes which are detectable in the particles. The presence of ribosomes does not affect the infectivity of the virus.

Table 16.1 Characteristic prototypes of arenaviruses

Serogroup	Human virus[a]	Animal virus[b]	Natural host[c]
Old World viruses (Lassa–lymphocytic choriomeningitis serogroup)	LCMV	LCMV	*Mus musculus*
	Lassa virus	Lassa virus	*Mastomys natalensis*
	Lujo virus	Lujo virus	*unknown*
		Mopeia virus	*Mastomys natalensis*
		Mobala virus	*Praomys jacksonii*
		Ippy virus	*Arvicanthus* spp.
		Luna virus	*Mastomys natalensis* *Mus minutoides*
New World viruses (serogroup Tacaribe): clade A (North America)	Whitewater Arroyo virus	Whitewater Arroyo virus	*Neotoma albigula* *Neotoma mexicana* *Neotoma micropus* *Neotoma cinera*
		Taniami virus	*Sigmodon hispidus*
		Bear Canyon virus	*Peromyscus* spp.
New World viruses (serogroup Tacaribe): clade A (Central America and South America)	Flexal virus	Flexal virus	*Oryzomys* spp.
		Pichinde virus	*Oryzomys albigularis*
		Pirital virus	*Sigmodon alstoni*
		Paraná virus	*Oryzomys buccinatus*
		Allpahuayo virus	*Oecomys bicolour*
New World viruses (serogroup Tacaribe): clade B (South America)	Machupo virus	Machupo virus	*Calomys callosus*
	Guanarito virus	Guanarito virus	*Sigmodon hispidus*, Zygodontomy brevican
	Sabiá virus	Sabiá virus	Unknown
	Chapare virus	Chapare virus	Unknown
	Junín virus	Junín virus	*Calomys musculinus*
		Amapari virus	*Oryzomys capito*, *Neacomys guineae*
	Tacaribe virus	Cupixi virus	*Oryzomys capito*
		Tacaribe virus	*Artibeus* spp.[d]
New World viruses (serogroup Tacaribe): clade C (South America)	–	Latino virus Oliveros virus Pampa virus	*Calomys callosus* *Bolomys obscurus* *Bolomys* spp.

LMCV lymphocytic choriomeningitis virus

[a]Virus types that have proven to have the potential to infect humans. They were mostly isolated from patients

[b]Viruses that have been described as pathogenic for animals

[c]The species that are the natural hosts of the respective virus types, which persist in their hosts, and are transmitted by them

[d]Fruit-eating bat species

16.1.2.2 Genome Organization and Structure

The genome of arenaviruses consists of two single-stranded RNA segments, both of which exhibit an ambisense orientation, comprising altogether more than 10,000–12,000 nucleotides (Fig. 16.2). In LCMV, the S and L segments have

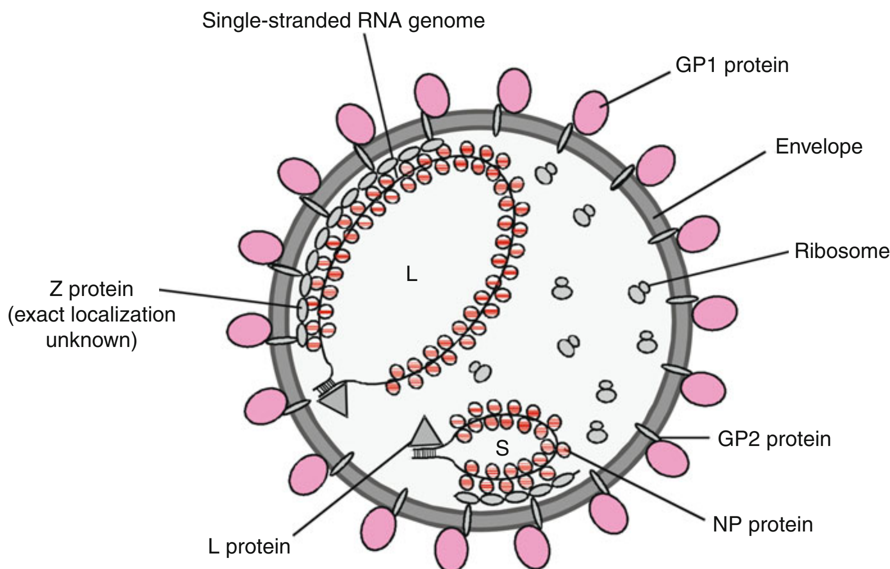


Fig. 16.1 Structure an arenavirus particle. The genome consists of two single-stranded negative-sense RNA segments, which associate with NP and L proteins into nucleocapsid complexes. The Z proteins presumably interact with the NP proteins. The terminal complementary sequences confer on the genome a quasi-circular shape. The nucleocapsids are surrounded by an envelope which is associated with viral glycoproteins G1 and G2. Subunits of cellular ribosomes are found as additional components inside the particle

a length of 3,376 and 7,219 nucleotides, respectively. Both segments are complexed nucleosome-like with NP proteins arranged throughout their entire length, forming helical nucleocapsids. Furthermore, some L protein molecules are also associated with them. There are 19 conserved nucleotides at the 3′ and 5′ ends of the segments, and they show inverted complementarity. Therefore, the complementary ends can hybridize into double-stranded regions, conferring on the genome a quasi-circular, panhandle-like configuration. In addition to these intramolecular base pairings, the terminal sequences can also interact with other RNA segments, so they are present as homodimers or heterodimers. The intermolecular hybrid formation is the reason why L and S segments are not always present in equal amounts within the particles. The conserved terminal sequences contain the promoters for transcription and the regulatory elements for replication.

The S segment encodes the GPC glycoprotein precursor of the GP1 and GP2 polypeptides as well as the NP protein (Fig. 16.2). Both genes do not overlap, and are separated by an intergenic region that forms a stable hairpin RNA structure. The NP protein is coded in the 3′ half of the S segment and is translated from an mRNA that is complementary to that region of the genome. Thus, this gene is present in

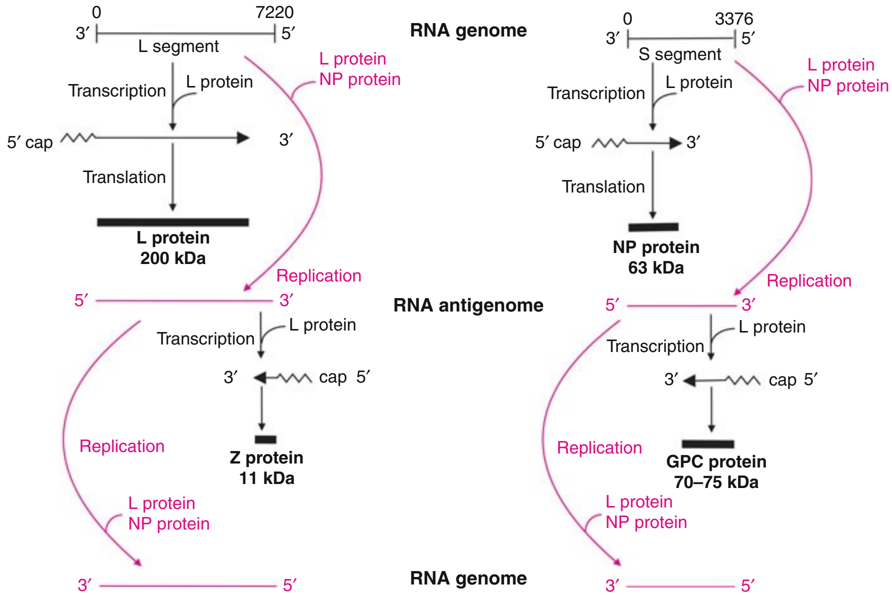


Fig. 16.2 Transcription, translation and genome replication in lymphocytic choriomeningitis virus. The L and S genome segments are transcribed into capped messenger RNA (mRNA) species. This step is catalysed by the L protein, which is codelivered into the cell as a component of the virus particles. L and NP proteins are translated from the synthesized mRNAs. If adequate amounts of newly synthesized NP protein are present in the cell, then uncapped RNA molecules with positive-sense polarity are synthesized (antigenomes). These serve both as templates for the synthesis of negative-sense RNA molecules, which correspond to the genome segments, and for the generation of capped mRNAs for the proteins Z and GPC (glycoprotein precursor). Both segments have an ambisense coding strategy

negative sense. The GPC protein is coded in the 5′ moiety of the segment, however in positive sense. As a prerequisite for the synthesis of this polypeptide, the corresponding genomic RNA segment must be transcribed into an antigenomic molecule, which subsequently serves as a template for the transcription of the GPC-specific mRNA. Thus, both reading frames are transcribed from opposite directions in a convergent fashion. They are separated by the intergenic region, which functions as a transcriptional termination signal.

Even the L segment has two non-overlapping reading frames arranged in ambisense orientation. The L protein is coded in the 3′ part of the segment in negative orientation (Fig. 16.2). In the 5′ region there is a reading frame in the opposite direction that encodes a Zn^{2+}-binding polypeptide, which is referred to as Z protein. A cytidine-rich intergenic region of about 200 nucleotides is situated between both reading frames. It forms specific secondary structures, which function as termination signals for mRNA synthesis.

16.1.3 Viral Proteins

16.1.3.1 Envelope Proteins

The glycoproteins of LCMV are synthesized as a precursor protein GPC with a molecular mass of approximately 70–75 kDa. Uncleaved GPC proteins form oligomeric complexes. A pre-GPC precursor protein of 82 kDa is synthesized in Lassa virus. Cellular signal peptidases cleave it into an unusually long amino-terminal signal peptide of 58 amino acids and the GPC precursor protein (76 kDa), which is anchored in the membrane of the endoplasmic reticulum by a carboxy-terminal hydrophobic domain. The enzyme subtilisin-kexin isoenzyme 1/site one protease (SKI-1/S1P), a cellular trypsin-like protease, cleaves the glycosylated GPC, which is probably present as a tetrameric protein in the Golgi or the post-Golgi compartment, into an amino-terminal GP1 peptide (40–46 kDa in LCMV and 40 kDa in Lassa virus) and a carboxy-terminal membrane-anchored GP2 subunit (35 and 36 kDa in LCMV and Lassa virus, respectively). In Lassa virus, the signal peptide remains associated with the GPC protein. This complex formation is a prerequisite for the cleavage of the GPC protein by SKI-1/S1P. The interaction between the signal peptide and the GP1 moiety of the GPC protein suggests that the cleavage site of the GPC protein becomes accessible to the protease by this conformational stabilization. In the mature virus particle, the GP1 and GP2 proteins form trimeric complexes. The GP1 trimers are interlinked via disulphide bridges, and remain non-covalently associated with the trimeric GP2 after cleavage. These complexes form the spike-like, protein protrusions (9.5 nm in length) on the surface of the virus. The GP1 proteins are responsible for the interaction with cellular receptor proteins, and neutralizing antibodies are produced against them during the course of infection. The GP2 proteins have fusogenic activity, and are linked with the nucleocapsid NP proteins by their carboxy-terminal cytoplasmic domains. Table 16.2 provides an overview of the molecular characteristics and functions of LCMV proteins.

16.1.3.2 Nucleoprotein

The NP proteins have a molecular mass of about 63 kDa and are associated with the RNA segments. In analogy to other negative-sense RNA viruses, it is believed that the NP protein of arenaviruses is also able to regulate the switch from the transcription mode to the replication mode (▶ Sects. 15.1–15.4, 16.2 and 16.3). Reproducible amounts of degradation products of the NP proteins are present in infected cells and in purified virus preparations. Furthermore, a phosphorylated variant of the NP protein is found in the late stages of the replication cycle. It is not known whether these products are involved in the regulation of transcription and replication.

16.1.3.3 RNA-Dependent RNA Polymerase

The L protein (200–250 kDa) is detected in small amounts in the virus particles and is associated with the nucleocapsids. The amino acid sequences of the L proteins of

Table 16.2 Molecular properties and functions of arenavirus proteins (LCMV)

Protein	Molecular mass (kDa)	Number of amino acid residues	Modification	Function
GPC	70–75	498	Glycosylated	Precursor protein of GP1 und GP2; interaction to form oligomers
GP1	40–46	262	Glycosylated	Amino-terminal part of GPC; external glycoprotein; induction of neutralizing antibodies; attachment; forms trimers, which are covalently linked by disulphide bonds
GP2	35	236	Glycosylated	Carboxy-terminal part of GPC; integral envelope protein; fusogenic activity; trimeric complexes, non-covalently associated with GP1
NP	63	558	Partially phosphorylated	Nucleocapsid component; complexed with RNA genome segments
L	250	2,211		RNA-dependent RNA polymerase; nucleocapsid component
Z	11	90	Myristoylated	RING finger motif; zinc finger protein; associated with the viral envelope; regulates particle formation and viral morphogenesis

the different strains of Lassa virus differ by up to 18 %. The RNA-dependent RNA polymerase activity has been assigned to a domain between amino acids 1,043 and 1,546 of the 2,218–2,221 residues that constitute the L protein. It is not clear whether it is modified or processed. The L protein must interact with NP proteins for successful transcription and replication of the genome segments; other viral and cellular factors are apparently not necessary.

16.1.3.4 Z Protein

The relatively hydrophobic Z protein (11 kDa) possesses the structural motif of RING finger proteins. It is a cysteine-rich zinc finger motif, which binds Zn^{2+} ions. Z proteins are found in large quantities in virus particles on the inner side of the envelope, and they are functionally similar to the matrix proteins of other negative-sense RNA viruses (rhabdoviruses, paramyxoviruses and orthomyxoviruses; ▶ Sects. 15.1, ▶ 15.3 and 16.3). In infected cells, Z proteins accumulate on the inside of the cytoplasmic membrane. The modification of the Z protein with myristic acid is responsible for this interaction. This aliphatic acid is covalently linked to the glycine residue at position 2 of the amino acid chain of the Z protein by the cellular transferase myristoyl coenzyme A:protein *N*-myristoyltransferase, after cotranslational removal of the amino-terminal methionine by a methionine aminopeptidase. In this context, the Z protein resembles to the matrix protein of retroviruses (▶ Sect. 18.1). Genetically engineered expression of the Z genes of Lassa virus and LCMV in eukaryotic cells has demonstrated the formation of membrane vesicles containing Z proteins, which are released from the cell surface and have

a size similar to that of the virus particles. Proline-rich amino acid sequences (PPPY in LCMV, PTAP and PPPY in Lassa virus) in the carboxy-terminal region of Z proteins are responsible for the formation of budding structures and vesicles. They have been designated as L domains (for "late") – a reference to their functional activity in morphogenesis during the late phase of the infection cycle. They regulate the accumulation of cellular proteins that are components of the vacuolar protein sorting pathway. These include the factors Tsf101, Vsp4A and VspB4, which are involved in the formation of intracellular membrane vesicles (vacuoles). In infected cells, membrane-associated Z proteins interact with NP proteins and are responsible for the accumulation of the genome segments into the newly arising virus particles. However, apart from these tasks in viral morphogenesis, the Z protein has additional functions. It inhibits the RNA polymerase activity of L proteins, thus influencing viral transcription and replication. It is detectable in the nucleus of infected cells; it binds to the cellular promyelocytic leukaemia protein by the RING finger domain and is responsible for the redistribution of promyelocytic leukaemia protein from the nucleus to the cytoplasm of infected cells. Promyelocytic leukaemia protein is a component of a multiprotein complex, the nuclear bodies, and also has a RING finger domain. Furthermore, the Z protein also interacts with the translation initiation factor 4E. This suggests that it may exert an inhibitory effect on the translation of capped mRNAs. Nonetheless, it is unclear whether these interactions of the Z protein with cellular polypeptides affect the infection course, and the establishment of viral persistence. Moreover, interaction of Z proteins with P proteins of the large ribosomal subunit has also been observed, but it remains unclear whether this interaction is able to give rise to the packaging of ribosomes into virus particles.

L Domains of Viral Structural Proteins Regulate Vesicle Formation

Cellular proteins have different functions. To be able to exert their activities correctly, proteins must be transported into certain cellular compartments after or during translation. This is usually mediated by certain amino acid sequences, so-called protein targeting or sorting signals, to which other proteins and factors can bind. Well-known examples are signal peptides, hydrophobic amino acid sequences at the amino terminus of proteins, which have to fulfil their role as membrane-anchored proteins. Signal-recognition particles bind cotranslationally to these signal sequences, and interact, in turn, with receptors for signal-recognition particles in the membrane of the endoplasmic reticulum. Thereby, the complex of mRNA, nascent amino acid chain and ribosome is transported to the endoplasmic reticulum membrane. The proteins are anchored in the membrane during translation, and are transported the cell surface as membrane proteins via Golgi vesicles. By means of other targeting signals, certain proteins are transported into the cell nucleus, mitochondria, lysosomes and vacuoles. For the targeted transport into vacuoles, proteins usually require proline-rich amino acid targeting signals such as PTAP, PSAP and PPxY as well as YxxL (where x represents any amino acid).

If such consensus sequences are present in viral proteins, then they give rise to an interaction with the components of the vacuolar protein sorting pathway. Such consensus sequences have been identified, among others, within the Gag proteins of human immunodeficiency virus and Rous sarcoma virus, the VP40 protein of Ebola virus, the matrix protein (M) of the vesicular stomatitis virus and in the Z proteins of LCMV and Lassa virus. They cause the incorporation of such proteins into vacuoles and vesicular membrane structures. Therefore, they have an essential role in the formation of vesicles, which are the precursors of the infectious virus particles of enveloped viruses.

16.1.4 Replication

It is not known in detail how arenavirus replication occurs. In many cases, the processes are deduced from those of bunyaviruses or influenza viruses. The various virus types use different cell surface proteins for attachment. In Old World arenaviruses and representatives of clade C of the New World arenaviruses, α-dystroglycan has been identified as a cellular receptor. The viruses bind to α-dystroglycan molecules through their GP1 proteins, in which amino acid residues 259 and 260 (based on the LCMV sequence) are important for the high-affinity interaction with the receptor. These positions are located adjacent to the cleavage site in the GPC precursor protein. If the GP1 proteins have phenylalanine or tyrosine and leucine or isoleucine at positions 259 and 260, respectively, then these virus types use α-dystroglycan as a receptor. α-Dystroglycan is a peripheral membrane protein that interacts with components of the extracellular matrix and is non-covalently associated with the membrane-anchored β-dystroglycan. Dystroglycan complexes are present in differing amounts on the cells of most organs. The other members of clade B of New World arenaviruses, such as Junín virus, Machupo virus and Sabiá virus, use transferrin receptor 1 for attachment. No cell receptor has been identified for the New World clade A arenaviruses.

After attachment, the particles presumably penetrate into the cells via receptor-mediated endocytosis and activation of the fusogene activity, as can be inferred by the cleavage of the GPC protein into subunits GP1 and GP2 in analogy to the HA protein of influenza viruses (Sect. 16.3). After the release of nucleocapsids, all processes occur in the cytoplasm. In the course of this process, nucleocapsids bind to the nuclear membrane. Possibly, nuclear factors are involved in replication, because viral propagation is very inefficient in anucleated cells.

In the initial steps of the replication cycle, the NP and L genes are transcribed; they are localized in negative sense on the S and L segments, respectively (Fig. 16.2). The 5′ ends of the mRNAs are capped, and the adjacent sequence of up to seven nucleotides is not encoded by the viral RNA segments but is of cellular origin. This suggests that arenaviruses, like influenza viruses, use a cap-stealing mechanism for transcription initiation, using the 5′-cap structures of cellular

mRNAs as primers (Sect. 16.3). The mRNA synthesis terminates at the hairpin-like secondary structures of the intergenic regions. The respective transcripts are translated into NP and L proteins on free ribosomes.

The switch from transcription to replication mode that gives rise to the synthesis of continuous RNA molecules is probably dependent on the amount of newly synthesized NP proteins (Fig. 16.2), a process that also requires a primer. However, its nature has not yet been completely elucidated. A guanosine residue is found at the 5′ termini of Tacaribe viruses that is neither present in base pairing nor coded in the viral RNA. Furthermore, short regions at the ends of the genome are heterogeneous. A model for the initiation of genome replication postulates that the L protein uses oligonucleotides as primers for the polymerization reaction; these are attached to the 3′ ends of the RNA segments. Subsequently, the antigenomic RNA molecules produced interact with NP proteins.

The mRNAs encoding the GPC and Z proteins are transcribed from the RNA antigenomes of the S and L segments, respectively. They end at the termination signals within the intergenic regions. GPC protein synthesis occurs on the endoplasmic reticulum membrane. The GPC protein is translocated into the lumen, and remains anchored in the membrane by a carboxy-terminal hydrophobic domain. The GPC proteins interact to form oligomers, are glycosylated and are cleaved into the GP1 and GP2 subunits by the SKI-1/S1P. Similarly as described for Lassa virus, processing of the GPC protein precursor is dependent on the formation of a stable complex of GPC and signal peptide, which has previously been cleaved by the signalase. The GP1 and GP2 complexes are transported to the cell surface via Golgi vesicles and form glycoprotein-rich regions in the cytoplasmic membrane. Simultaneously, the antigenomes are used as a template for the synthesis of continuous genomic RNA molecules, which associate with NP proteins into nucleocapsids.

Myristoylated Z proteins accumulate on the plasma membrane. They bind to the NP proteins of the nucleocapsid segments, which also interact with the cytoplasmic regions of GP2 subunits via NP proteins. In this manner, the initial budding structures emerge. Nucleocapsids are then surrounded by membranes enriched with Z and GP1/GP2 proteins, and are finally released from the cell surface, whereby ribosomes are also included into the virions. No mechanism seems to exist ensuring that each particle contains solely one S and one L segment. It has been shown in LCMV that reassortants can emerge by infecting cells with different virus strains simultaneously. In New World arenaviruses, there are indications that genetic recombination events can contribute to the formation of new virus variants when co-infections occur (see the box entitled "Whitewater Arroyo Virus").

16.1.4.1 Persistent LCMV Infections

LCMV infections are not cytolytic and have some peculiarities. After an initial proliferation stage, the virus establishes persistent infections in Vero cells (kidney cell cultures of green monkeys) or also in neurons. In this phase, it produces only a few offspring, the synthesis of GPC proteins decreases, and increasing concentrations of L proteins are detectable in the cytoplasm. The accumulation of

RNA-dependent RNA polymerase presumably suggests the formation of inactive enzyme complexes. Thus, the transcription and replication of the genomes are reduced. The cells continuously produce small amounts of infectious particles, so there are enough genome segments to ensure the transmission of the viral genetic information to daughter cells during cell division. The persistently infected cells cannot be re-infected with viruses that have been released into the culture; thus, an interference condition prevails. By an unknown mechanism, the persistent virus prevents cells being infected with further viruses.

16.1.5 Human and Animal Pathogenic Arenaviruses

16.1.5.1 Lymphocytic Choriomeningitis Virus

Epidemiology and Transmission

In 1933–1934, Charles Armstrong and Ralph Dougall Lillie isolated LCMV for the first time from a patient infected with St. Louis encephalitis virus, a flavivirus (▶ Sect. 14.5). They transmitted LCMV to mice. Erich Traub performed his pioneering research on LCMV infection of mice in Princeton between 1935 and 1938. Lymphocytic choriomeningitis was the first infectious disease in which a chronic virus carrier state was observed. It has almost exclusively immune pathological causes in the acute phase. By his research on LCMV, Michael B.A. Oldstone developed the theory of immune complex diseases. Rolf Zinkernagel demonstrated the MHC restriction of cytotoxic $CD8^+$ lymphocytes for the first time. For these investigations he was awarded the Nobel Prize in Physiology or Medicine in 1996.

Apart from the house mouse, LCMV infects various wild mouse species throughout the world. Research has revealed that – with regional variations – approximately 3–20 % of wild mice have a persistent LCMV infection in the USA. Pregnant mice transmit the virus vertically to the fetus, and in utero infected animals do not develop an immune response that would be able to eliminate the pathogen. Hence, chronic asymptomatic infections are established, and are associated with a high viral load in blood. The pathogens are excreted lifelong in urine, faeces, saliva, milk and semen. Virus-containing aerosols and dust are the main sources of zoonotic transmission to humans. This especially occurs during the cold season, when mice seek protection in houses. In addition, many laboratory infections are known from animal keepers who had contact with infected mice. The virus can be transmitted experimentally to several rodents, such as hamsters and guinea pigs, and even to monkeys. Wild mice can accidentally infect hamsters and guinea pigs, which are able to transmit the virus to humans (“hamster disease”). In such cases, some pet shops frequently function as “distributors”. Transmissions from infected people to other people are not known, with the exception of vertical transmission from LCMV-infected pregnant women to the fetus and certain transmissions through organ transplant (see the box entitled “Lymphocytic Choriomeningitis Virus – a Risk in Organ Transplants”).

Human LCMV infections are rare and generally asymptomatic. The seroprevalence in the USA ranges between 0.3 % in younger people and 5.4 % in people older than 30 years. The transmission frequency is much higher in rural areas or neglected residential buildings, in which mice reproduce efficiently owing to the accumulation of waste, than in urban residential areas.

LCMV – A Risk in Organ Transplants

In December 2003 and April 2005, symptoms of a severe LCMV infection were observed in two independent groups of patients who had received organs from two different donors. Seven of the eight infected patients died. The reasons for this were the organs of two donors who were apparently infected with LCMV, without having developed symptoms. Investigations revealed that the donor, along with other family members, had been infected by a golden hamster, which has recently been purchased in 2005. In the case of the other donor, the source of LCMV infection could not be determined. Viral RNA could not be detected in the blood of both donors any more. Therefore, it must be assumed that the viral genome segments remained latent in the cells of different tissues. These reports have shed new light on LCMV infection as a potential problem for graft recipients at least in the USA. However, because of the extreme rarity, general testing of donors seems to be technically and logistically too difficult and too expensive, like for the similar case of rabies virus transmission through transplanted organs (▶ Sect. 15.1), especially because in both instances they would not have resulted in the desired aim. A similar residual risk exists with all viral infections that are associated with a transient viraemia.

A few additional clusters have emerged in which LCMV was transmitted through donated organs. All of the five graft recipients died. In 2008, three deaths were documented in transplant recipients who had received organs from a donor and developed febrile diseases, of which they died 4–6 weeks after transplant. LCMV-like RNA sequences of a previously unknown arenavirus were demonstrated in still available organ samples of the donor. In December 2010, an organ donor died of general brain oedema following an episode of diabetic ketoacidosis. Both kidneys, liver and lung were transplanted to four recipients; in all four, severe post-transplant illness developed and two recipients died in February 2011. Through diagnostic methods, LCMV infection was diagnosed in all recipients. The LCMV infection of the immunosuppressed organ recipients probably resulted in a fatal outcome because LCMV is known to be effectively controllable by the cellular immune system. In such cases, a rapid diagnosis is very important: indeed, the only surviving patient received antiviral therapy with ribavirin in combination with a reduction of the drug-mediated immunosuppression.

Clinical Features

Human LCMV infections are frequently asymptomatic, or manifest themselves as a mild flu-like illness. The incubation period lasts on average 1–2 weeks. The actual lymphocytic choriomeningitis manifests itself, to some extent, as very severe headache, fever, weakness and nausea. The main symptom is neck stiffness, as a typical sign of meningitis. The disease lasts 5–7 days, but the symptoms can appear again later in two or three waves. Encephalitis, paralysis and other neurological symptoms are rarely observed. If pregnant woman become infected with LCMV, the virus can be transmitted to the embryo. Viral infections can lead to spontaneous abortion in early pregnancy, damaging the unborn child, especially during the first and second trimesters of pregnancy. Hydrocephalus, microencephaly, macroencephaly, chorioretinitis and non-immune hydrops fetalis have been described as possible consequences of LCMV infection in the fetus.

Pathogenesis

The virus is transmitted to the mucosa of the mouth and throat by contaminated aerosols, and is disseminated in the organism via the lymphatic system or the bloodstream. By binding to α-dystroglycan, it infects not only cells of the reticuloendothelial system, but also Schwann cells of the peripheral nervous system. In the latter, a non-lytic persistent infection is found. In vitro models have revealed that infected Schwann cells lose their ability to form compact myelin layers. This is possibly associated with the neurological phenomena which are found in infections with LCMV and other arenaviruses. In addition, leucopenia, i.e. a decline in the number of white blood cells, is a distinctive feature of all LCMV infections. In the very rare cases of human deaths, massive infiltration of mononuclear cells was detected in the brain, especially in the choroid plexus, but also in the meninges, ependyma and vascular endothelium. Similar infiltrations into the liver, lung and lymphatic system have also been found in the few systemic infections.

If the infection occurs in embryonal or neonatal mice, large amounts of virus can be detected in all organs, including the brain. Small amounts of antibodies against the NP, GP1 and GP2 proteins arise later. The infection is commonly asymptomatic. After birth, mice are persistently infected virus carriers and excrete large amounts of LCMV. The establishment of persistence is probably based on a peripheral tolerance of T cells, which is induced by the time of infection during the embryonic stage and the high concentration of the virus during infection. Glomerulonephritis, a kidney inflammation, may appear some months later, and is caused by immune complexes. Immune complexes are also found in arterial walls and in the choroid plexus of the brain. They consist of viral proteins, the complement components C1q and antibodies, and can attach to cellular Fc receptors by the Fc region of the immunoglobulins.

By contrast, if adult mice are infected with LCMV, like in humans an acute lymphocytic choriomeningitis arises, which is characterized by very dense

infiltrations of T and B lymphocytes, natural killer cells and plasma cells as well as monocytes into the infected tissues. Similar effects can be found in kidneys, liver, salivary glands, pancreas, lungs and the lymphatic system. Necroses and haemorrhages appear in the lymph nodes. Furthermore, serous pleurisy and peritonitis as well as elevated respiratory rate and increased vascular permeability can be detected. These symptoms are similar to those of haemorrhagic fever (Sect. 16.1.5). Increased levels of TNF-α, interferon-α and interferon-β as well as of other cytokines can be detected in early stages. These induce particularly the expression of MHC class I complexes on the surface of infected and uninfected cells, making them, in connection with viral peptide epitopes, preferential targets of cytotoxic T lymphocytes. Cytokines also induce the proliferation of natural killer cells, which, however, are not decisive in eliminating the virus. Individual virus strains differ greatly in their ability to induce sufficient amounts of interferon, thus differing in their virulence. An effective induction of interferon synthesis leads to reduced amounts of the virus during the early infection stages. $CD8^+$ T lymphocytes play the most important role in eliminating viruses and protecting from reinfections. $CD4^+$ T lymphocytes are also cytotoxic, albeit to a lesser extent; however, they contribute most notably to activation of $CD8^+$ T lymphocytes and to antibody production by secreting cytokines such as interleukin-2 and interferon-γ. Virus-specific B and T cells proliferate, the LCMV production is controlled and the pathogen is eliminated from the organism.

Immune Response and Diagnosis

IgM and IgG antibodies against NP, GP1 and GP2 proteins are generated in the course of acute LCMV infections, and $CD8^+$ and $CD4_+$ T lymphocytes and activated natural killer cells are produced as well. Epitopes that are recognized by cytotoxic T cells have been identified in all proteins in the mouse system. The serological diagnosis is based on the detection of antibodies by ELISA (IgM for acute infection, IgG for high prevalence). LCMV can be detected by reverse transcription PCR (RT-PCR) from suitable material.

Lymphocytic Choriomeningitis of Mice is Caused by Cytotoxic T Cells

Lymphocytic choriomeningitis in immunocompetent mature mice is triggered by immune pathogenic mechanisms, more precisely by $CD8^+$ T lymphocytes. This was demonstrated by the following experiments: X-ray irradiation or administration of immunosuppressive agents prevents the development of the disease in adult mice, which, like newborn mice, establish an asymptomatic, persistent infection. The same applies to mice from which the thymus has been removed after delivery because they are not able to produce cytotoxic T cells. If T cells of sick mice are injected into these mice, they will develop a typical lymphocytic choriomeningitis.

Therapy and Prophylaxis

There is no vaccine against LCMV infection. Treatment with ribavirin can help to mitigate the symptoms in especially severe cases.

16.1.5.2 The Pathogens of Haemorrhagic Fever

Epidemiology and Transmission

Lassa virus, Junín virus, Machupo virus and Guanarito virus cause persistent infections in certain rodents (Table 16.1), which subsequently excrete the pathogens in the urine, faeces and saliva throughout life. Like hantaviruses (Sect. 16.2), the diseases caused by these viruses are confined to the geographical regions in which the respective rodent species exist under natural conditions: Lassa fever in West Africa and haemorrhagic fever caused by Junín virus, Machupo virus, Sabiá virus and Guanarito virus in Argentina, Bolivia, Brazil and Venezuela, respectively. Junín virus had no significance for human health up to the first half of the twentieth century when the carriers (*Calomys musculinus*, *C. laucha*) did not come into contact with human settlements. In contrast to other rodents, the intensification of agriculture in the Pampa regions of Argentina has led to a massive spread of mouse species that are virus carrier, thus gaining access to the human population. Lassa viruses can be subdivided into four strains according to their different geographical distribution (Nigeria, Guinea, Liberia, Sierra Leone). Approximately 20 % of infections are associated with diseases for which a mortality rate of 10–30 % is assumed. The number of new emerging Lassa virus infections in West Africa is estimated to be 100,000–500,000 annually, and is associated with several thousand victims. Travellers to such countries are considerably endangered in the case of exposure. Already several, generally fatal Lassa virus infections have recently been imported to Europe by travellers from Africa.

The viruses are transmitted by virus-containing dust (aerosols) or by direct contact with rodents. Epidemiological studies on the transmission of Lassa fever in West Africa have revealed that the viruses infect primarily those people who hunt mice and other small rodents as a food source. The pathogens existing in the blood of animals reach the bloodstream through small injuries and induce the infection. The viruses causing South American haemorrhagic fever are mainly transmitted by virus-containing aerosols that arise during grain harvest. Infected rodents are killed in the harvesting equipment. Their blood is intermingled with the resulting dust, which is inhaled by harvest workers, and reaches the mucous membranes. Hence, infections are especially found in people who work with or near these machines during the harvest season. In rare cases, the viruses can also be transmitted to other people by direct contact with infected patients. In the case of Lassa fever and Machupo fever, there are nosocomial infections, which probably arise in hospitals through the use of inadequately sterilized instruments. Such human-to-human transmissions have not been observed in infections with Junín virus, Sabiá virus and Guanarito virus.

Whitewater Arroyo Virus

Whitewater Arroyo virus causes severe human respiratory diseases that are associated with haemorrhagic symptoms and liver failure. It is the only known virus that belongs to the serogroup of Tacaribe viruses and causes haemorrhagic fever in infected individuals in North America. Whitewater Arroyo virus was isolated from three people who died in 1999 and 2000 in California. Mice of the genus *Neotma* were identified as the natural hosts. By analysis of the nucleotide sequences of the S segment of the genome, indications emerged that suggest that this highly human pathogenic virus may have evolved by genetic recombination in rodents which were simultaneously infected with different arenavirus types. This illustrates the high pathogenic potential of this virus group.

Clinical Features

Lassa fever has an incubation period of up to 3 weeks. It is primarily manifested by sudden onset of high fever, headache, back, neck and joint pain, pharyngitis, vomiting and diarrhoea. Many patients experience bleeding of mucous membranes, excretion of protein in the urine, swelling of the face and neck and signs of hepatitis and/or encephalitis. As late complications, the infection causes pericarditis, hearing loss and inflammation of the retina and testes. Lassa fever has an exceptional position among the viral haemorrhagic fevers, as during the shock syndrome the generalized endothelial dysfunction and the thrombocytopenia are clearly in the foreground. The cause of death is usually cardiovascular failure due to internal bleeding. In Argentine and Bolivian haemorrhagic fever, no symptoms can be found in the respiratory tract, but the haemorrhagic symptoms are much more prominent. If untreated, the disease is associated with a high mortality rate: approximately 20–30 % of infected people die, even in spite of intensive medical assistance. In pregnant women, the complication rate is even higher, as stillbirths can almost always be expected.

Pathogenesis

After entering the body via inhalation or skin wounds, the virus spreads by the bloodstream. It mainly infects cells of the reticuloendothelial system in many organs, such as liver, lung and placenta. Direct damage of tissues is relatively low. The pathogenesis of the disease is largely unclear. Focal necroses especially in liver, spleen and adrenal glands can be found in Lassa fever. Severe cases of the disease develop the leaky capillary syndrome, which leads to shock with cardiovascular failure in 30–40 % of cases. Increased bleeding of skin and mucous membranes of the gastrointestinal tract can be observed in Argentine and Bolivian haemorrhagic fever, which also exhibit a significant increase in vascular permeability. Thus, the typical symptoms of haemorrhage are more evident in these infections than in Lassa fever, which is in general clinically uncharacteristic.

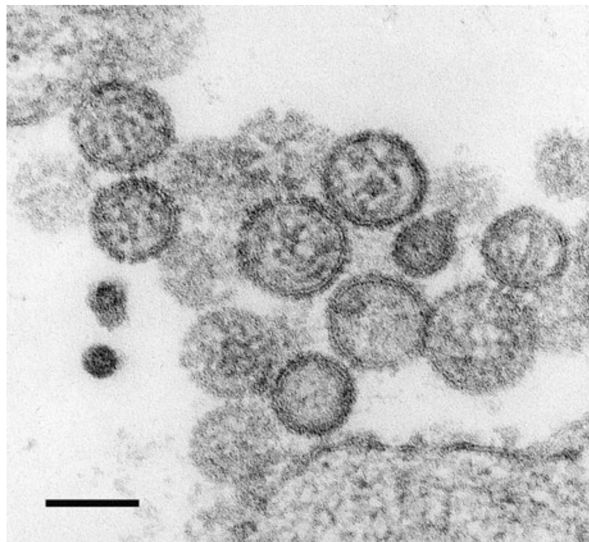
Immune Response and Diagnosis

In Lassa fever, a low IgM response and an already beginning IgG reaction can be observed at the onset of infection. Neutralizing antibodies can frequently be detected only in low titres later, during convalescence. Therefore, the virus is eliminated not by antibodies, but probably by cytotoxic T cells of the cellular immune response, as similarly shown in LCMV infections of mice. The virus persists for a long time after the disease, and is detectable in serum or urine. By contrast, in South American haemorrhagic fever, antibodies against the NP and G proteins become detectable in serum only 2–4 weeks after infection by immunofluorescence tests or ELISA. Since the NP protein contains highly conserved sequences, NP-specific antibodies are frequently cross-reactive. The presence of IgM antibodies or a fourfold increase of IgG titre in serum is considered as a sign of an acute infection. The immunoglobulins directed against surface proteins are neutralizing, and react considerably more specifically. G-protein-specific antibodies, which are detected in the neutralization test, can be used to determine the virus type and immune status. However, as they become detectable only late in the course of infection, their detection can generally not be taken into account to diagnose acute infections. RT-PCR analyses in authorized laboratories are essential for the clinical diagnosis of acute infections. Owing to the possible nosocomial transmission of Lassa virus, the diagnosis must be made as fast as possible.

Therapy and Prophylaxis

A vaccine against Lassa virus has been developed which is based on recombinant vaccinia viruses that express GP proteins. The use of this vaccine prevents severe diseases and deaths in monkeys. Even in such cases, antibodies are not produced, so the protective effect is probably based on the cellular immune response. In animal experiments, an attenuated Junín virus has proven to be successful as well. This vaccine (Candid 1) was tested in Argentina in a double-blind study including 6,500 men employed in agriculture and showed a good protective effect. Since then, several hundred thousand people have been vaccinated with this live attenuated vaccine in regions where Argentine haemorrhagic fever is endemic. This measure has led to a significant reduction of the disease. The treatment of patients with both Lassa fever and South American haemorrhagic fever with high doses of intravenously administered ribavirin leads to significant weakened disease courses. Orally administered ribavirin is also used prophylactically. Furthermore, the immunotherapeutic administration of convalescent sera (sera from survivors that contain neutralizing antibodies against the virus) gave rise to a significant reduction of the mortality rate. However, this approach is quite controversial considering the AIDS problem in Africa. Otherwise, the treatment is aimed at preventing cardiovascular shock, if possible under intensive medical care. Depending on the carrier, the control of rodent populations through traps, poison, cats, etc., as well as appropriate hygiene within human dwellings is necessary and reasonable in urban and rural areas.

16.2 Bunyaviruses



The family *Bunyaviridae* comprises many virus types that infect various organisms. The name is derived from the town Bunyamwera in Uganda, where Bunyamwera virus was first isolated. All bunyaviruses have a tripartite segmented, single-stranded, negative-sense RNA genome. Bunyaviruses exist under natural conditions in different mammals, and are predominantly transmitted by arthropod bites. Humans are only rarely infected. In such cases, bunyaviruses usually cause febrile diseases that are associated with rash and joint pain. In some cases, such as infections with Crimean–Congo haemorrhagic fever virus (CCHFV), the symptoms of a haemorrhagic fever develop. Infections with La Crosse virus and with other members of the California virus serogroup are, especially in children, a major cause of encephalitis on the American continent. A direct transmission of the virus among humans occurs only in exceptional cases, e.g. by nosocomial infections in hospitals; only CCHFV is relatively easily transmitted by infected people. Since all virus types rely on a particular insect, tick or rodent species as a carrier, their geographical incidence depends on the distribution areas of these organisms. Many human pathogenic bunyaviruses are endemic in tropical and subtropical regions. The genus *Hantavirus* is an exception: Some members of this genus, such as Puumala virus and Dobrava–Belgrade virus, are also present in Europe. These viruses are excreted and transmitted by the faeces of chronically infected rodents. In humans, they cause, depending on the virus type, infections of the kidneys, which can be manifested as a mild disease (epidemic nephropathy, renal insufficiency with proteinuria), but also as a febrile haemorrhagic disease associated with an impairment of kidney function and complete renal failure (haemorrhagic fever with renal syndrome, HFRS).

Hantaviruses, e.g. Sin Nombre virus, that have been isolated from rodents on the American continent may cause a severe lung disease with high mortality (hantavirus cardiopulmonary syndrome, HCPS) (Table 16.3).

16.2.1 Classification and Characteristic Prototypes

More than 350 different types of bunyaviruses have been identified. They are subdivided into five genera: *Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, *Hantavirus* and *Tospovirus*. The latter encompass the plant-specific bunyaviruses, and will not be discussed in this book. The classification is based on the host specificity and molecular properties. These particularly include the conserved terminal sequences of the genome segments, which are largely identical within the genus, but also considering the arrangement of genes on the segments. On the basis of the serological properties of their N proteins, the viruses are classified into serogroups within the individual genera. The distinction between the different virus types is based primarily on the characteristic reactivity of their glycoproteins in neutralization or haemagglutination-inhibition tests.

A Few Bunyaviruses Are Transmitted by Insects in Europe

Sandflies of the genera *Phlebotomus*, *Sergentomyia* and *Lutzomyia* are carriers of sandfly fever in the European and Asian Mediterranean area, Arab and African countries, the Middle East and Central Asia. This disease is caused by different types of sandfly fever viruses. These include the sandfly fever virus types Naples, Sicily and Toscana, which cause Naples or Tuscany fever. In humans, these infections usually provoke a mild, febrile disease that can be associated with skin rash and/or an aseptic meningitis, and lasts about 2–4 days. Since the antigenic epitopes of the Gn and Gc surface proteins differ among the different virus types, recurrent infections and diseases are not rare in endemic regions.

16.2.2 Structure

16.2.2.1 Virus Particle

Bunyavirus particles are pleomorphic, i.e. multiform. They are predominantly spherical with a diameter of 100–120 nm (Fig. 16.3). Even so, also oval or filamentous particles with a length of more than 200 nm can be observed in electron micrographs. The particles are constituted of three nucleocapsid segments that are surrounded by an envelope. The envelope is associated with a complex of two glycoproteins (Gn and Gc, in older literature referred to as G1 and G2, see also the box entitled "The Gn and Gc Proteins of Bunyaviruses Were Formerly Called G1 and G2"), which project about 10 nm from the surface of the virus, and arise by proteolytic cleavage from a common precursor protein (GPC). Hydrophobic amino acid sequences at the carboxy-terminal domains of Gn and Gc proteins are responsible for anchoring in

Table 16.3 Characteristic prototypes of bunyaviruses

Genus	Serogroup	Human virus/distribution	Animal virus/distribution	Carrier/natural hosts
Orthobunyavirus	California encephalitis viruses	California encephalitis virus/North America	California encephalitis virus/North America	Mosquitoes (*Culicoides*, *Culex*, *Aedes* spp.)
		La Crosse virus/North America	La Crosse virus/North America	
		Snowshoe hare virus/North America	Snowshoe hare virus/North America	
		Tahyna virus/Europe	Tahyna virus/Europe	
	Simbu viruses	Oropouche virus/South America	Akabane virus/Japan	Mosquitoes (*Culicoides*, *Culex*, *Aedes* spp.)
			Aino virus/Japan	
			Shamonda virus/Japan	
			Shino virus/Japan	
	Group C	Apeu virus/South America	Apeu virus/South America	Mosquitoes (*Culicoides*, *Culex*, *Aedes* spp.)
	Bwamba viruses	Bwamba virus/Africa	Bwamba virus/Africa	Mosquitoes (*Culicoides*, *Culex*, *Aedes* spp.)
		Pongola virus/Africa	Pongola virus/Africa	
	Bunyamwera viruses	Bunyamwera virus/Africa	Bunyamwera virus/Africa	Mosquitoes (*Culicoides*, *Culex*, *Aedes* spp.)
		Nyando virus/Africa	Nyando virus/Africa	
		Cache Valley virus/America	Cache Valley virus/America	
		Fort Sherman virus/Central America	Fort Sherman virus/Central America	
		Xingu virus/South America	Xingu virus/South America	
Phlebovirus		Sandfly fever virus Toscana/southern Europe		Biting flies (*Phlebomotus* spp.)
		Sandfly fever virus Sicily/southern Europe		
		Sandfly fever virus Naples/southern Europe		
		Rift Valley fever virus/Africa	Rift Valley fever virus/Africa	Mosquitoes (*Culicoides*, *Culex*, *Aedes* spp.), ticks (*Hyalomma* spp.)
			Avian Uukuniemi virus/Europe	Ticks

(*continued*)

Table 16.3 (continued)

Genus	Serogroup	Human virus/distribution	Animal virus/distribution	Carrier/natural hosts
Nairovirus		Crimean–Congo haemorrhagic fever virus/Middle East, Turkey, Afghanistan	Crimean–Congo haemorrhagic fever virus/Middle East, Turkey, Afghanistan	Mosquitoes (*Culicoides* spp.), ticks
			Dugbe virus (Nairobi sheep disease virus)/Africa	Ticks
Hantavirus		Hantaan virus/Southeast Asia	Hantaan virus/Southeast Asia	*Apodemus agrarius*
		Seoul virus/Southeast Asia	Seoul virus/Southeast Asia	*Rattus norvegicus*, *Rattus rattus*
		Dobrava–Belgrade virus/southeastern Europe	Dobrava–Belgrade virus/southeastern Europe	*Apodemus flavicollis*
		Tula virus/central and eastern Europe	Tula virus/central and eastern Europe	*Microtus arvalis*, *Microtus rossiaemeridioalis*
		Puumala virus/central Europe	Puumala virus/central Europe	*Clethrionomys glareolus*
		Sin Nombre virus/America	Sin Nombre virus/America	*Peromyscus leucopus*, *Peromyscus maniculatus*
		Andes virus/America	Andes virus/America	*Oligoryzomys longicaudatus*

This table includes only serogroups and virus types which are known to cause disease in humans, or are considered relatively well-characterized prototypes

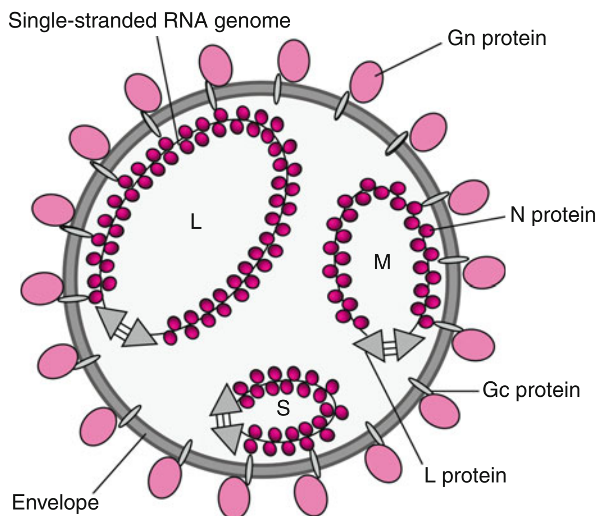


Fig. 16.3 Structure of a bunyavirus particle (hantavirus). The genome consists of three single-stranded, negative-sense RNA segments, which are complexed with N and L proteins into helical nucleocapsids. Terminal complementary sequences confer on them a quasi-circular shape. The nucleocapsids are surrounded by an envelope, in which the complexes of the viral Gn and Gc proteins are intercalated

the envelope. The molecular masses are different in the various virus types; masses for Gn range between 30 and 60 kDa (55 kDa in hantaviruses, 34–38 kDa in La Crosse virus), whereas those of Gc range between 70 and 125 kDa (70 kDa in hantaviruses, 125 kDa in La Crosse virus). In some members of the genus *Nairovirus*, another protein seems to be associated with the envelope, and is also formed by proteolytic cleavage of the glycoprotein precursor GPC. The helical nucleocapsids are composed of single-stranded RNA complexed with N protein (20–50 kDa; 48 kDa in hantaviruses, 27 kDa in La Crosse virus). About 2,100 N protein units are found per particle. In addition, the virions contain approximately 25 copies of the L protein that are associated with the nucleocapsid. They usually have molecular masses of 240 and 270 kDa (247 kDa in hantaviruses, 263 kDa in La Crosse virus); however, the L protein of CCHFV is significantly larger, exhibiting an estimated mass of 448 kDa. L proteins function as RNA-dependent RNA polymerases.

The Gn and Gc Proteins of Bunyaviruses Were Formerly Called G1 and G2

In the various types of bunyaviruses, the cleavage product of G proteins exhibiting the greater molecular mass was traditionally called G1 until a few years ago. However, since the GPC precursor protein is cleaved at quite different sites in the various virus types, the G1 protein corresponded in some virus types to the amino terminus, as is the case, for example, in hantaviruses, but in others it matches with the carboxy-terminal moiety. This led to repeated misunderstandings and confusion, as functionally similar G protein domains did not always have the same name in the literature. In principle, it has recently been agreed to designate as "Gn" glycoproteins derived from the amino-terminal domain of the GPC protein. Accordingly, the proteins derived from the carboxy-terminal moiety are denominated "Gc" in recent literature.

Table 16.4 Conserved terminal sequences of bunyavirus genomes

Genus	3′ end	5′ end
Bunyavirus	UCAUCACAUGA...	...UCGUGUGAUUGA
Hantavirus	AUCAUCAUCUG...	...AUGAUGAU
Nairovirus	AGAGUUUCU...	...AGAAACUCU
Phlebovirus	UCUCGUUAG...	...CUAACGAGA

16.2.2.2 Genome Organization and Structure

The single-stranded genome of bunyaviruses is present in three segments. There are complementary sequences with a length of eight to 11 nucleotides at the 3′ and 5′ termini, and they form double-stranded regions (Table 16.4). Therefore, the RNA molecules are present in a quasi-circular, panhandle-like shape. The conserved ends of the genome contain *cis*-active consensus sequences for the initiation of transcription and replication. The segments encompass a total of nearly 12,000 nucleotides; only the representatives of the genus *Nairovirus*, such as CCHFV, have, with roughly 18,000 RNA nucleotides, a much larger coding capacity (Fig. 16.4).

In the vast majority of bunyaviruses, the L segment ("L" for "large") has an average of 6,330–9,000 nucleotides and encodes the L protein. Only the L segment of nairoviruses is significantly larger (more than 12,000 nucleotides). The M segment ("M" for "middle") is 2,300–5,000 nucleotides long and contains the genetic information for the G proteins as well as – in members of the genera *Bunyavirus* and *Phlebovirus* – for the NSm protein (for "non-structural protein, middle segment"). The S segment ("S" for "small") has a length of approximately 960–3,000 nucleotides. It encodes the N protein in the genera *Nairovirus* and *Hantavirus*. The genus *Orthobunyavirus* has a second gene (*NSs*), which is translated from the same mRNA species by using an alternative start codon. The S segment of phleboviruses has ambisense polarity: the antigenome strand, which arises as an intermediate product during replication, serves as a template for the synthesis of an mRNA species that is used for the translation of the NSs protein (for "non-structural protein, small segment"). The reading frames do not overlap. The intergenic regions appear to form stem-loop-like, partially double stranded secondary structures that serve as termination signals for transcription. Table 16.5 gives an overview of the different characteristics of bunyavirus genomes.

16.2.3 Viral Proteins

16.2.3.1 Structural Proteins

Envelope Proteins

The G proteins of hantaviruses are generated by proteolytic cleavage of the GPC precursor protein (about 125 kDa) by a cellular protease which is associated with the Golgi vesicles or the endoplasmic reticulum. This processing leads to the emergence of an amino-terminal polypeptide Gn (55 kDa) and a carboxy-terminal protein Gc (70 kDa), which assemble into a heteromultimeric complex.

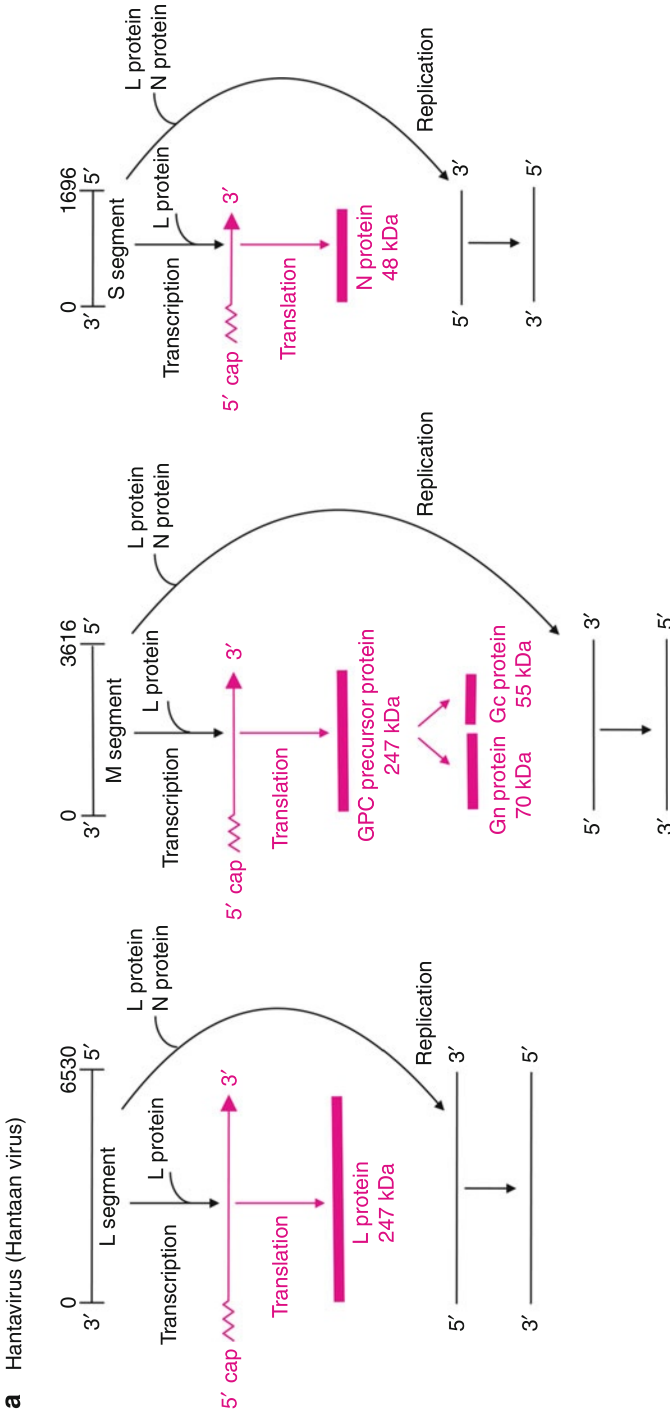


Fig. 16.4 (continued)

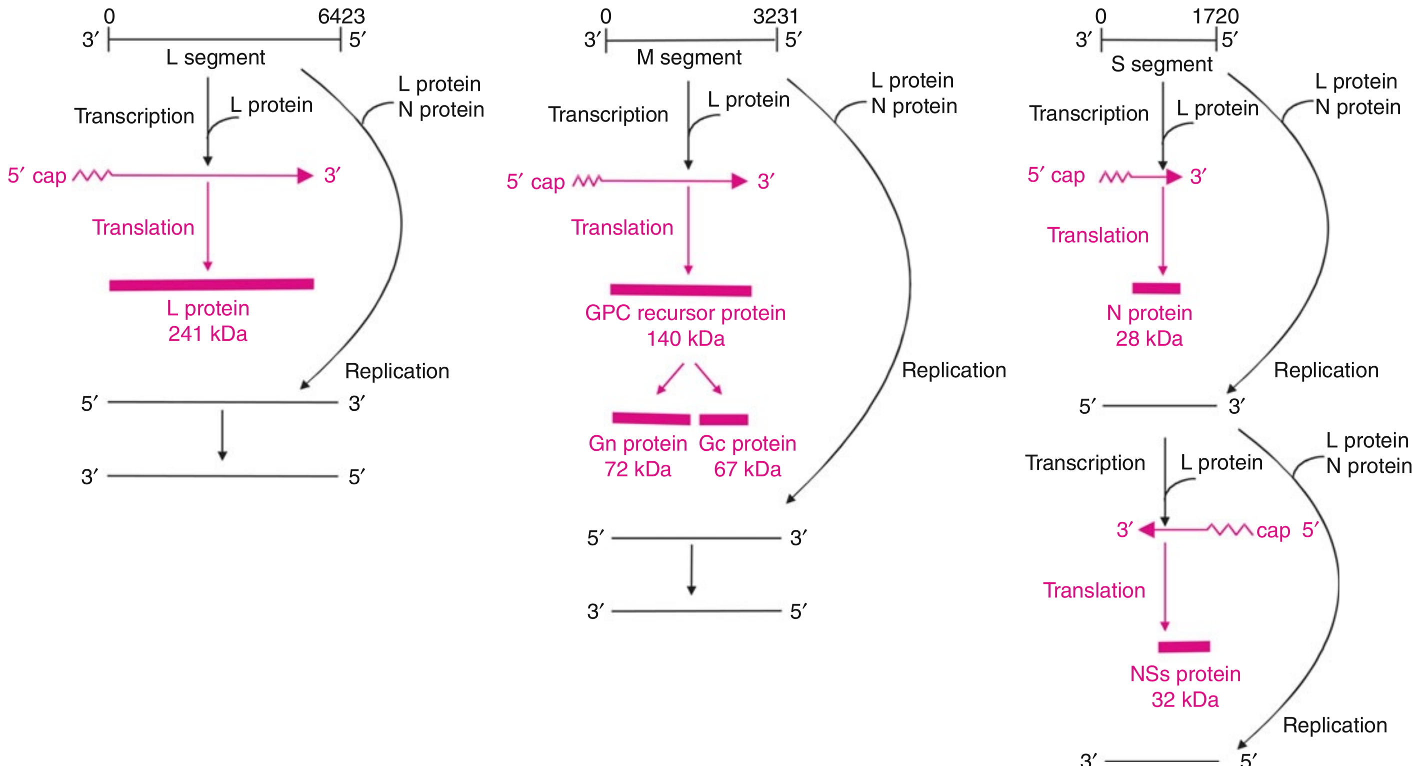


Fig. 16.4 (continued)

Comparative amino acid sequence analysis between different hantavirus types revealed a homology of 43 and 55 % among the Gc and Gn proteins, respectively. In nairoviruses, a relatively large fragment is cleaved from the amino-terminal region of the GPC precursor protein. It probably corresponds to the third glycoprotein (173 kDa) that can occasionally be found in virus preparations. The Gn and Gc proteins (35 and 73 kDa) of nairoviruses are generated by cleavage of the central and carboxy-terminal region of the precursor protein. In addition to the Gn and Gc proteins, a smaller protein, NSm, is also generated by proteolysis of the precursor polypeptide in bunyaviruses and phleboviruses. In orthobunyaviruses, it is located between the amino-terminal Gn protein (32 kDa) and Gc protein (110 kDa) and has a molecular mass of 18 kDa. Conversely, the NSm protein of Rift Valley fever virus, a phlebovirus, is situated at the amino terminus of the GPC protein. Its function has not been conclusively elucidated: deletion of the NSm coding sequences does not affect the replication of Maguari virus in cell culture. In bunyaviruses, the NSm protein seems to be inserted along with the Gn and Gc proteins in the membrane of Golgi vesicles and appears to be important for the morphogenesis of virus particles.

The virus attaches to the cellular receptors by the cysteine-rich, acetylated Gn and Gc proteins. In orthobunyaviruses, the Gc protein is responsible for binding to the receptors on the surface of both insect and mammalian cells. Integrins $\alpha_5\beta_3$ are involved, along with other cellular components (30-kDa protein, 70-kDa protein), in the attachment of the nephropathic hantaviruses to the cell surface; by contrast, non-pathogenic hantaviruses such as Prospect Hill virus and Tula virus bind to $\alpha_5\beta_1$ integrin. It is discussed whether binding of hantaviruses to $\alpha_v\beta_3$ integrins also affects their roles in intercellular interactions, such as platelet aggregation. This might contribute to abolishing the barrier function of blood vessels and capillaries. Binding of hantaviruses to $\alpha_v\beta_3$ integrins impedes the β_3-directed migration of endothelial cells. In addition, the HCPS-associated hantaviruses have a sequence motif within the cytoplasmic domain of their Gc proteins which activates cellular tyrosine-dependent kinases. These regulate not only the immunological defence, but also endothelial cell function. Whether the activation of these kinases affects the pathogenesis of HCPS virus infections is also unclear. Nevertheless, it seems to be responsible for the different potential of hantaviruses to induce the interferon response, as it enables non-pathogenic strains to induce the synthesis of

Fig. 16.4 Transcription, translation and genome replication in the family *Bunyaviridae*. **a** Hantavirus (Hantaan virus). **b** Phlebovirus (Uukuniemi virus). The L, M and S segments of the genome are transcribed into capped mRNA species; the capped RNA oligonucleotides used as primers are derived from cellular transcripts, and are cleaved from them. This step is catalysed by the L protein that is codelivered into the cell as a component of the virus particle. The L and GPC (Gn and Gc as well as Gn, NSm and Gc) proteins are translated from the synthesized mRNAs. If adequate amounts of newly synthesized N proteins are present in the cell, uncapped RNA molecules with positive-sense polarity are synthesized (antigenomes). These are used as templates for synthesizing negative-sense RNA molecules, which correspond to the genome segments. In Uukuniemi virus, an additional mRNA is transcribed from the S segment, which directs the translation of the NSs protein. Hence, the S segment also has an ambisense coding capacity

Table 16.5 Properties of genome segments of bunyaviruses and their encoded proteins

Segment		*Orthobunyavirus*	*Hantavirus*	*Nairovirus*[a]	*Phlebovirus*		Function
L	Nucleotides	6,875	6,530	12,164	6,423[b]	6,404[c]	
	Proteins kDa	L/259	L/247	L/448	L/241	L/247	RNA-dependent RNA polymerase
M	Nucleotides	4,458	3,616	4,888	3,231	3,884	
	Proteins[d] kDa	Gn/32	Gn/70	Gn/35	Gn/72	NSm/14	Gn/Gc proteins: glycosylated surface proteins, which are generated by proteolytic cleavage of the GPC precursor protein; attachment, fusion, induction of neutralizing antibodies
		NSm/18	Gc/55	Gc/73	Gc/67	Gn/55	
		Gc/110				Gc/62	NSm protein: integral membrane protein of Golgi vesicles; for morphogenesis
S	Nucleotides	961	1,696	1,677	1,720b	1,690	
	Proteins kDa	N/26	N/48	N/48–50	N/28	N/28	N protein: RNA binding; RNA chaperone (double-stranded RNA helicase); cap-snatching; ribosome binding; main component of the nucleocapsid
		NSs/11[e]			NSs/32[f]	NSs/29[f]	NSs protein: anti-interferon effect

The theoretical molecular masses derived from the amino acid sequence are given

[a]Crimean–Congo haemorrhagic fever virus

[b]Values in the entire column apply to Uukuniemi virus

[c]Values in the entire column pertain to Rift Valley fever virus

[d]The proteins are in the order specified on the precursor protein from which they are cleaved by proteases

[e]The orthobunyavirus NSs protein is translated using an alternative start codon

[f]The phlebovirus NSs protein is synthesized from a subgenomic RNA in opposite orientation

interferon-γ. In this process, the cytoplasmic domain interacts with TNF receptor associated factor family associated nuclear factor κB (NFκB) activator binding kinase 1 (TBK1), activates it and gives rise to the phosphorylation of interferon-regulating factor (IRF) 1 and IκB, the inhibitor of NFκB, two prerequisites for the expression of the interferon-γ gene. There are also indications that the cytoplasmic domain of pathogenic strains inhibits the double-stranded RNA helicase retinoic acid inducible gene I (RIG-I), which, in turn, is essential for the induction of the interferon-dependent signalling pathways that are mediated by viral double-stranded RNA.

The proteolysis of GPC releases a fusion activity associated with the Gc protein, which is also dependent on acid pH values and induces structural rearrangements in the Gc protein. The Gn and Gc proteins appear to mediate, similarly to the HA_1/HA_2 protein complex of influenza viruses (Sect. 16.3), the fusion between the viral envelope and the endosomal membrane after penetration of the virus particle.

The Gn proteins possess sequences that determine their localization in the Golgi membrane. These amino acids prevent virus proteins from being transported to the cell surface via Golgi vesicles. The retention of proteins is important for the formation of infectious particles, as morphogenesis occurs in this cell compartment. The nucleocapsids attach to G proteins and new virions are formed by budding in the Golgi vesicles. This does not apply to some hantaviruses that cause HCPS in humans because their Gn and Gc proteins are anchored in the cytoplasmic membrane. The newly formed virus particles are released directly from the cell surface.

Components of the Nucleocapsid

The N protein is coded in the S segment of the genome. It has a molecular mass of nearly 50 kDa in hantaviruses, whereas its molecular mass is just 26 and 28 kDa in orthobunyaviruses and phleboviruses, respectively. Large amounts of N proteins are synthesized in infected cells. They form aggregates in the cytoplasm, and these can be detected as inclusion bodies or filaments. N protein trimers associate with genomic RNA molecules, thus being the principal component of nucleocapsids and virus particles. The amino and carboxy termini as well as a central region of the N protein contain highly conserved basic amino acid residues. In hantaviruses, the domains responsible for oligomerization are found in the amino-terminal and carboxy-terminal regions of the N protein, whereas the central domain (amino acids 175–217) is responsible for the interaction with RNA (Fig. 16.5). Furthermore, the N protein of hantaviruses interacts with high affinity with the double-stranded, panhandle-like RNA regions at the ends of the viral genome segments. It also binds, albeit with lower affinity, to double-stranded RNA structures at the ends of the positive-sense replication intermediates. This interaction elicits a latent RNA chaperone activity of the N protein that unwinds double-stranded RNA regions in the 3′–5′ direction. Subsequently, the trimeric N protein remains associated with the 5′ end of RNA segments and induces the binding of the small ribosomal subunit. Presumably, this process is also essential for binding of L proteins, and for the initiation of transcription and genome replication. In addition, N proteins are involved in the cap-snatching process and in the morphogenesis of virus particles. As main components of the

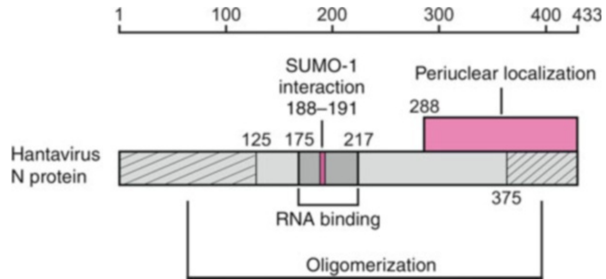


Fig. 16.5 The hantavirus N protein and its functional domains. *Numbers* refer to the amino acid positions, beginning at the amino terminus. *SUMO-1* small ubiquitin-like modifier 1

nucleocapsid segments, they bind to the cytoplasmic domains of the envelope-anchored Gn and Gc proteins. Furthermore, N proteins also interact with cellular proteins: through its carboxy-terminal domain, the N protein of hantaviruses binds to the cellular Daxx protein, a transrepressor protein that suppresses induction of apoptosis. Possibly, the interaction with the N protein hinders this regulatory function and is responsible for the induction of Fas-dependent apoptosis, which is elicited in hantavirus-infected cells. Moreover, the hantavirus N protein seems to interact with small ubiquitin-like modifier 1 (SUMO-1) influencing the sumoylation of cellular proteins; however, it is still unclear what cellular functions are affected. The N proteins of La Crosse virus, Rift Valley fever virus and CCHFV bind to MxA proteins, which are induced by interferon-α (▶ Chap. 8, Sect. 16.3). Thereby, N proteins are held in the perinuclear space, and are not available for replication of the RNA segments, thus limiting viral infection. This has not yet been shown for hantaviruses.

The L protein has RNA-dependent RNA polymerase activity. It is essential for both the transcription of the genome into mRNA species and genome replication. The hantavirus L protein has a theoretical molecular mass of 247 kDa, which approximately corresponds to that of the purified protein from virus preparations. Its polymerase activity is dependent on Mn^{2+} ions. The L proteins of different hantavirus types have a pronounced homology (70–80 %). Apart from nairoviruses, the L proteins of the other genera have a similar size: 263 kDa in La Crosse virus and 239 kDa in phleboviruses. The L protein of CCHFV, a representative of the genus *Nairovirus*, has a significantly higher molecular mass of 448 kDa. In comparison with orthobunyaviruses, phleboviruses and hantaviruses, it has approximately 1,800 additional amino acids at the amino-terminal region. In this characteristic region of the L protein of nairoviruses, a sequence motif with similarity to ovarian-tumour-like proteases (OTU-like proteases) resides close to the amino terminus. OTU proteases are cysteine proteases of the papain family. In this amino-terminal domain, the L proteins of nairoviruses show similarities to the corresponding proteins from different plant viruses, such as carlaviruses and foveaviruses. In these viruses, the OTU-like protease cleaves itself from the precursor protein by an autocatalytic cleavage process, and generates by further cleavage reactions, besides the RNA-dependent RNA polymerase, also a helicase that unwinds double-stranded RNA structures during genome replication. The finding that nairoviruses possess an OTU-like domain in the L protein strongly suggests that they may be polyproteins which

are autocatalytically cleaved into different subunits. In addition, it has been shown that OTU proteases have deubiquitinating activity. Whether the L proteins of nairoviruses also have such functions, and whether such activities are necessary during the infection cycle, has not yet been clarified.

16.2.3.2 Non-Structural Proteins

Some members of the family *Bunyaviridae* encode two non-structural proteins. The NSm protein, a cleavage product of the GPC precursor protein, has already been mentioned.

An additional NSs protein (11 kDa) is coded in the same open reading frame of the N protein in the S segment of orthobunyaviruses. It is translated in another reading frame by using an alternative start codon. Therefore, both proteins are not homologous. The NSs protein of phleboviruses (32 kDa in Uukuniemi virus) is also encoded in the S segment, but in reverse orientation to the N protein. Thus, the two open reading frames are translated in convergent directions, but are separated by an intergenic region in which the transcription termination signals reside. The NSs protein of Rift-Valley fever virus and La Crosse virus is not necessary for infection and replication in cell culture. However, there are also contradictory data concerning the function of NSs proteins among the different viruses. It promotes replication and transcription of the RNA segments in an in vitro system of Rift-Valley fever virus, but a contrary effect has been observed in bunyaviruses. In La Crosse virus, NSs deletion mutants showed, in comparison with the wild-type virus, a decreased shutdown of cellular functions, and reduced apoptosis induction. Similar effects have been found in corresponding deletion mutants of Rift Valley fever virus, but these effects were strongly dependent on the cells used for infection. Furthermore, the NSs protein of Rift Valley fever virus functions as an interferon antagonist; it binds to the p44 subunit of cellular transcription factor TFIIH and inhibits the function of RNA polymerase II. As a result, the transcription of cellular genes, including interferon genes, is impaired. The NSs protein of La Crosse virus suppresses the process of RNA interference, in which gene expression is inhibited by short double-stranded "interfering" RNA molecules (small interfering RNA) in a sequence-specific manner: a transcription inhibition of the viral M segment caused by RNA interference was abolished by the NSs protein. NSs proteins can influence the course and pathogenesis of infection by allowing the virus to inhibit interferon production and thereby to evade the immune responses mediated by interferon-α and interferon-β. Conversely, virus mutants without normal NSs functions are good inducers of class I interferon and have proven to be highly attenuated in animals.

16.2.4 Replication

Many details of bunyavirus replication have not been elucidated so far, including the question of the identity of the cellular receptors to which the viruses bind. Integrins $\alpha_v\beta_3$ have been identified as cellular interaction partners of most hantaviruses. On the part of the virus, this step is mediated by Gc proteins. An interaction

with gC1qR/p32 has also been shown for Hantaan virus. It is a glycosylated cell surface protein which interacts with the complement component C1q. After binding to the cell surface, virus particles penetrate into the cytoplasm via endocytosis: acidification of the interior of the vesicle – presumably promoted by an ion pump in the endosomal membrane – is a prerequisite for rearrangement of the Gc protein, and for induction of its associated fusogenic activity. Subsequently, the nucleocapsid is released into the cytoplasm, where all further steps of the replication cycle occur in association with perinuclear membrane compartments, like in flaviviruses and togaviruses (▶ Sects. 14.5 and ▶ 14.6).

Transcription of the genome segments is done by the RNA-dependent RNA polymerase activity of the L protein, which is associated with the nucleocapsid (Fig. 16.4). Initiation is – similarly as in orthomyxoviruses – primer-dependent. The transcripts have a methylated cap structure at their 5′ ends and ten to 18 additional nucleotides that are derived from cellular mRNAs. Like orthomyxoviruses, bunyaviruses have developed the mechanism of cap-stealing (also known as cap-snatching) (Sect. 16.3), which is promoted by the multifunctional N protein. It finds its way into the processing bodies (P bodies) in the cytoplasm, the cellular foci in which defective or non-active transcripts are degraded. During this process, the cellular decapping enzyme DCP2/DCP1 removes the 5′-cap ends by cleaving them along with an oligonucleotide sequence from the transcripts. The N protein binds to 5′-capped oligonucleotides, and it collects and transports them to the viral genome segments. They serve as primers for the synthesis of viral mRNAs, during which their 3′-OH ends are used as starting points for elongation. Concurrently, the RNA-chaperone activity of the N protein separates the double-stranded structure of genome segments into single strands. The N proteins are also responsible for binding of the small ribosomal subunit to the 5′-capped ends. In this process, they replace the entire cellular eIF4F complex composed of the cap-binding protein eIF4E, the RNA helicase eIF4A and the protein eIF4G.

The production of long transcripts is reliant on the simultaneous synthesis of proteins. Probably, ribosomes bind to the 5′ end of the mRNA during transcription, thus preventing the formation of a double-stranded RNA between the mRNA and the genome, which would impede transcription elongation. On the other hand, host cell proteins can also interact with nascent mRNA, suppressing the formation of RNA/RNA hybrids. The transcription does not proceed up to the ends of the genome segments, but stops about 50–100 nucleotides before. The transcription of the S segment of phleboviruses ends at a termination signal located approximately in the middle of the molecule (Fig. 16.4b). The 3′ ends of mRNA are apparently not polyadenylated.

Accordingly, the translation of mRNAs into proteins is initiated during transcription. The GPC precursor proteins are translocated through the membrane of the endoplasmic reticulum by an amino-terminal signal sequence and anchored there. They are then processed by a protease that is associated with the compartment, giving rise to the proteins Gn and Gc (in some cases also NSm). Subsequently, the proteins are modified by glycosylation and addition of aliphatic acid residues.

After that, a complete, continuous, complementary RNA must be synthesized. This negative-sense RNA strand is not capped and does not have additional

nucleotides at the 5′ terminus – whether other primers are required for initiation is not known. The activity of the L protein must be modified for the synthesis of antigenomes; possibly, this happens through interaction with the newly synthesized N proteins. Thereafter, the antigenomes form complexes with N proteins and serve as templates for the synthesis of new viral genome segments with negative-sense polarity. This process is suppressed by interferon in human cells infected with Rift Valley virus, La Crosse virus or CCHFV by functional inactivation of new N proteins via complex formation with the interferon-induced MxA protein, thus blocking replication. In the case of the S segment of phleboviruses, which is also used in antisense orientation like the genome segments of arenaviruses (Sect. 16.1), the positive RNA strand serves not only for the synthesis of new negative strands, but also for the transcription of NSs-specific mRNAs (Fig. 16.4b).

The synthesized genome segments interact with N and L proteins, forming nucleocapsids. With the exception of the respiratory hantaviruses, which assemble at the cytoplasmic membrane, electron microscopy data indicate that morphogenesis of bunyaviruses occurs on the membranes of the Golgi vesicles, where G proteins accumulate in high concentrations, and whose intracytoplasmic moieties presumably interact with nucleocapsids via their N protein domains. The Golgi membrane envelopes nucleocapsid segments, the particles produced invaginate into the lumen of Golgi vesicles, and are subsequently transported to the cell surface. The vesicles merge with the cytoplasmic membrane, and the viruses are released into the environment.

Similar to orthomyxoviruses, in bunyaviruses there is also no evidence for the existence of a mechanism that ensures that each virus particle receives the correct combination of nucleocapsid segments, which is essential for infectivity. Reassortants can be generated by co-infections with different virus variants in the same culture in vitro, a process that can also occur under natural conditions.

16.2.5 Human Pathogenic Bunyaviruses

16.2.5.1 Hantaviruses

Epidemiology and Transmission

In humans, hantaviruses cause HFRS (hemorrhagic fever with renal syndrome) and HCPS (hantavirus cardiopulmonary syndrome). The infections are almost always transmitted by infected rodents to humans; only for the South American Andes virus, which causes HCPS, have infectious transmissions from person to person been repeatedly detected. Most transmissions are due to contact with virus-contaminated dust originating from animal excrements. Occasionally, infections have also been found in laboratory staff and animal keepers who are employed in animal facilities or involved in experiments.

The name “hantavirus” is derived from the river Hantaan in Korea. The first registered diseases occurred in this region during the Korean War around 1950. Hantaan virus, which was identified in 1978, infects the Korean striped field mouse (*Apodemus agrarius corea*) and persists in this mouse species. It is excreted with the saliva, urine and faeces, and thereby is transmitted to humans.

In rodents, disease signs have only been found in experimentally infected hamsters or newborn severe combined immunodeficiency (SCID) mice – the latter possess neither a humoral nor a cellular immune system and die of encephalitis after infection with Hantaan virus. The transmission to other mouse species or laboratory animals as well as cultivation of the virus in cell cultures is difficult. Today, the Hantaan virus can be reproduced in Vero E6 cells (kidney cell cultures of green monkeys). The infection does not cause a pronounced cytopathic effect in the cells.

Hantaan virus is widespread in Asia and eastern Russia, but infections have also been occasionally reported in southern Europe. Several serotypes of hantaviruses are known which are related to Hantaan virus. They have in common that each of them has adapted to a specific mouse species in which they persist. Hence, the occurrence of the infection in humans is determined by the geographical distribution of the different hosts. Transmissions to other mouse species, even to closely related ones, have proven to be unsuccessful in almost all cases. Puumala virus is widespread in Scandinavia and Europe. It causes persistent infections in bank voles (*Clethrionomys glareolus*), and is excreted in the saliva, urine and faeces. Thus, the virus is present in contaminated soil and can be transmitted to the human mucosa via dust and aerosols. In central Europe, up to approximately 4 % of the population has antibodies against this virus. It causes the epidemic nephropathy (renal failure with proteinuria after fever), a disease similar to Korean haemorrhagic fever, which is, however, far less severe and without haemorrhagic symptoms. Infections are often asymptomatic.

In addition to Puumala virus, Tula virus and Dobrava–Belgrade virus are common in Europe, and the latter is present in two variants: Dobrava–Belgrade Aa and Af. Tula virus is transmitted by the common vole (*Microtus arvalis*), Dobrava–Belgrade virus Aa is transmitted by the striped field mouse (*Apodemus agrarius*) and Dobrava–Belgrade virus Af is transmitted by the yellow-necked field mouse (*Apodemus flavicollis*). They occasionally cause severe diseases, courses of which are similar to those of Hantaan virus infections in Southeast Asia. Seoul virus infects various rat species (Table 16.5). It was originally endemic in East Asia, but today, it has been carried by infected rats on ships to almost all port cities, thus disseminating it throughout the world. Human infections are frequently severe. It is estimated that Seoul virus and Hantaan virus cause about 100,000 cases of HFRS in humans in China and Southeast Asia annually.

Further members of the genus *Hantavirus* have been isolated in the USA and other countries of the American continent in recent years. In 1993, a severe epidemic appeared in a reservation of Navajo Indians in the southwestern USA which was associated with fulminant pulmonary and cardiac failure. Different names were used for this virus in the first descriptions (Four Corners virus, Sin Nombre virus and Muerto Canyon virus). Consensus has been reached for the name Sin Nombre virus. It is transmitted through excreta of infected deer mice (*Peromyscus maniculatus*) and causes a very severe disease of the respiratory tract with possible lung and heart failure, called hantavirus cardiopulmonary syndrome (HCPS). Interestingly, ancient Indian lores suggest similar occurrences in the past, in relation to climate-caused mouse infections. Since the discovery of Sin Nombre virus, further serotypes of hantaviruses have been identified on the

American continent whose infections also cause HCPS in humans. Since the first epidemic, approximately 1,000 cases have been documented, and more than 50 % of cases were fatal.

The Discovery of Hantaviruses

Hantaviruses attracted international attention for the first time during the Korean War in the early 1950s. More than 3,000 UN soldiers had to be hospitalized because of Korean fever, which was associated with kidney failure and shock; about 7 % died. The causative agent of Korean haemorrhagic fever remained unknown until 1978, when Hantaan virus was isolated from the Korean striped field mouse (*Apodemus agrarius corea*) by Ho Wang Lee and colleagues, who assigned it to the family of bunyaviruses. They observed that sera from patients with Korea fever reacted with proteins in the lungs of the Korean striped field mouse. The virus could be transmitted to that mouse species, in which it causes a persistent infection. However, Hantaan virus and its associated disease, HFRS, have probably been widespread in Korea for a long time. Today, it is estimated that the different types of hantaviruses cause 150,000 cases of HFRS annually worldwide.

Clinical Features

The main symptoms of hantavirus infections are fever accompanied with or without haemorrhages, renal disease and HCPS. Depending on the virus type, HFRS can be manifested in differing severities. Only about a third of hantavirus-infected individuals develop a haemorrhage; in the other cases, the disease evolves as a flu-like illness with high temperature and muscle pain. The incubation period lasts on average 1–2 weeks; longer periods of more than 6 weeks have rarely been reported. The first symptoms (fever, chills and muscle pain) begin suddenly and can be associated with intense headache and back pain. This phase lasts 3–7 days. In about 30 % of cases, these initial symptoms are followed by haemorrhagic symptoms, which manifest themselves as punctate bleedings (petechiae) in the conjunctiva and mucosal membranes. Bleedings in the gastrointestinal tract and the lungs can also follow. These patients develop a thrombocytopenia, and the virus as well as blood and proteins can be detected in their urine as signs of nephropathy. A blood pressure drop occurs during the first week of the disease and can lead to hypovolaemic shock states in up to 15 % of patients (shock following blood or fluid loss), and is accompanied by a fatal outcome in about one third of cases. Following the recovery to normal blood pressure, a continuous decreased urine production (oliguria) with azotaemia follows for approximately 3–7 days, and is caused by direct and indirect impairment of kidney function. In this phase, deaths due to heart failure, pulmonary oedema and cerebral haemorrhage can also occur, being caused, for example, by capillary haemorrhage and intravascular coagulation with disseminated intravascular coagulopathy. An increased urine production of 3–6 L per day indicates the disease has been overcome (polyuric phase).

However, the recovery period up to the formation of normal electrolyte values lasts up to 3 months. In addition to nephropathy, some virus types can also damage the liver. In the considerably milder epidemic nephropathy, no haemorrhagic symptoms are usually found, and shock signs virtually do not occur.

HCPS is initially characterized also by fever, muscle ache and headache for 3–5 days, but it has a severe clinical course. A pronounced, radiologically detectable pulmonary oedema rapidly develops, and this is caused by increased capillary permeability in the lung endothelium, and severely impairs oxygen exchange in the lungs. Patients display severe respiratory distress (tachypnoea) and symptoms of progressive hypoxaemia, and thus need to be artificially ventilated within 24 h. A consequence of this acute respiratory distress syndrome is a decreased number of thrombocytes, which is associated with coagulation problems, disseminated intravascular coagulopathy and circulatory shock with acute right-sided heart strain, hypotension and cardiovascular failure. This combination of symptoms affecting the lungs, heart and circulation entails a mortality rate of more than 50 %. Nonetheless, patients who survive recover rapidly.

Pathogenesis

The virus is transmitted by aerosols into the mucosa of the mouth, nose and throat, and during the further course of infection infects endothelial cells and macrophages. It binds to $\alpha_v\beta_3$ integrin heterodimers on the surface of target cells, thus being disseminated throughout the organism. In infected people, the virus can be detected particularly in the kidney, but owing to viraemic dissemination also in the liver, spleen, lungs and heart. The extent of histopathological alterations with scattered haemorrhages and destruction of renal tubules and medulla is dependent on the severity of the disease. Viral glycoproteins can be found in the cytoplasm of epithelial cells of renal tubules. Cell destruction with necrotic and apoptotic signs seems to be caused partially by the viral infection. The differentially expressed ability of pathogenic and non-pathogenic virus strains to induce the interferon-mediated, non-specific immune response seems to be determined by the amino acid sequences in the cytoplasmic domain of the glycoprotein components (Sect. 16.2.3). Conversely, the severity of the disease appears to be associated with the number of cytotoxic T lymphocytes, which are produced during infection and that recognize virus-infected cells. The number of $CD8^+$ T lymphocytes is significantly higher in patients infected with Sin Nombre virus with severe HCPS than in those with less serious diseases. In addition, the severity of infection also seems to be associated with certain HLA types. Following Puumala virus infections, patients with haplotypes HLA-B8 and DRB1*0301 develop more frequently severe forms of endemic nephropathy than carriers of the HLA-B27 type.

Immunologically mature rodents, which are susceptible to the various hantavirus types, establish an asymptomatic and persistent infection. The rodents develop a viraemic phase that can lasts for several months. In this stage, large amounts of virus can be detected in the blood and the various organs. The rodents excrete the virus especially in the urine, but also in the saliva. If newborn mice are infected, the virus replicates particularly in the capillary endothelial cells of the kidneys,

lungs and brain, and the viraemia lasts about 2 weeks. As a result, the mice die of inflammatory lesions in these tissues. The occurrence of disease signs suggests that the virus spreads unhindered in the immunologically non-competent mice, developing its cytopathogenicity directly by destruction of infected cells. Furthermore, there is evidence that cell destruction is secondarily determined by both TNF and the cellular immune response. If TNF receptors are blocked by monoclonal antibodies, haemorrhagic symptoms do not emerge.

Immune Response and Diagnosis

The detection of viral RNA by RT-PCR is mandatory for the diagnosis of acute infections. In patients, IgM antibodies against N proteins can be detected by ELISA about 7 days after infection, that is generally at the onset of symptoms. The IgM concentration decreases below the detection limit after 3–6 months. IgG immunoglobulins are produced relatively soon after IgM antibodies and probably remain present lifelong. Care must be taken concerning possible cross-reactivities. Antibodies against G proteins are neutralizing, and inhibit attachment as well as the fusogenic and haemagglutination activities. Iatrogenic IgM negativity can be a diagnostic problem in patients when plasma exchange is performed therapeutic reasons. Cross-reacting IgM antibodies against G proteins of different serotypes, such as Puumula virus, Dobrava–Belgrade virus and Hantaan virus, are mainly found in the early phase of infection. In the case of unclear serological findings, clarification by Western blotting is important. Virus-specific antibodies and immune complexes are temporarily excreted in the urine of patients. However, their occurrence does not correlate with the severity of infection. It remains to be elucidated whether they are important for the pathogenesis of infection.

Therapy and Prophylaxis

To date, there is no approved vaccine. Formaldehyde-inactivated virus preparations from brains of infected rodents have been tested for efficacy in Korea and China. A protective effect against HFRS was evinced, but neutralizing antibodies could only be detected in half of all vaccinees. Vaccines based on inactivated viruses, which were grown in cell culture, showed a lower rate of side effects and induced better production of neutralizing antibodies. However, the concentration of neutralizing antibodies decreased rapidly in the case of both vaccines, so a long-lasting protection is presently very doubtful.

Ribavirin has been used as therapy for HFSR in some studies. The therapy showed a reduction of morbidity and mortality. This effect could not be unambiguously confirmed in HCPS patients. In model systems in which rodents were infected with Sin Nombre virus, treatments with ribavirin accompanied with antiviral antibody-containing plasma samples from subjects with past infections or with antibodies directed against the integrin β_3 chain have proven to be successful. Even interferon-β showed very good inhibition of hantavirus replication. However, whether interferon-β can be applied for treatment of severe HFRS or HCPS has not been definitively proven. The best precaution is to avoid contact with infected soil or dust, e.g. by wearing masks. However, this is usually not easily

feasible in exposed individuals (agricultural, sewage and road construction workers). Laboratory rodents should be tested for persistent hantavirus infections in order to reduce the risk to animal keepers and staff to a minimum.

16.2.6 Animal and Human Pathogenic Bunyaviruses

Pathogens causing animal diseases are found in every genus of the family *Bunyaviridae*, with the exception of the genera *Tospovirus* and *Hantavirus*. The viruses can be transmitted, to some extent, by insect stings or tick bites to other animal species and even to humans. Because human infections occur only in exceptional cases owing the distribution of the viruses, they will be described only briefly below. These viruses are regulated by animal health regulations because of their zoonotic potential. Therefore, bunyavirus research demands high safety precautions concerning laboratory equipment, and these must comply with the regulations for biosafety level 4.

Of particular veterinary importance are Rift Valley fever virus and Nairobi sheep disease virus. In addition, CCHFV and Akabane virus have become increasingly important. The latter is widespread in Australia, New Zealand, Asia and Africa. Akabane virus infections cause fetal deformities in ruminants (cattle, sheep, goats) such as microencephaly and arthrogryposis. When animals are infected after birth, they show, like infected mothers, no symptoms; human diseases have never been observed to date. Akabane virus is transmitted by mosquitoes of the genera *Culicoides*, *Culex* and *Aedes*. A vaccine based on inactivated viruses is available and is applied in endemic regions.

CCHFV, a nairovirus, can be transmitted from infected animals to humans by ticks of the genus *Hyalomma* and causes a severe viral haemorrhagic fever. The geographical distribution of CCHFV extends from China to Central Asia and from the Middle East to eastern Europe and Africa. Since the virus is also transmitted directly by diseased people, it has medical importance relating to travel.

La Crosse virus is a member of the group of California encephalitis viruses. In the USA, it is the commonest cause of viral encephalitis in childhood, which can be associated with neuronal sequelae (paralysis). The virus is transmitted by mosquitoes (*Aedes triseriatus*). Infected mosquitoes can transmit the virus vertically to their offspring through the eggs. Reassortants are preferentially generated in mosquito larvae that are infected with different bunyaviruses during diapause (quiescence period). Rodents, such as squirrels and chipmunks, are the reservoir. A similar role is played by snowshoe hare virus in other regions of North America. Both viruses do not have veterinary importance.

Increasing Infections with CCHFV

CCHFV is widespread in vast regions of eastern Europe, Asia and Africa. It is transmitted by ticks of the genus *Hyalomma*, which pass on the virus to the various developmental stages of ticks. In this way, virus-infected endemic tick populations can rapidly be generated. Among mammals, CCHFV frequently infects bovines and ovines. These animals are a reservoir for the

virus, and develop a viraemia in the course of infection, which is sufficient to allow sucking ticks to ingest the pathogens with the blood. If people are bitten by infected ticks, or have direct contact with the infected blood of animals (e.g., butchers or workers in slaughterhouses), then they become seriously ill and develop the symptoms of a haemorrhagic fever. Between 20 % and 40 % of patients die. There are no vaccines to protect humans and animals. However, these are become increasingly important because the infected ticks seem to spread continuously, thus further disseminating the virus. This virus does not have veterinary importance and its infections have a subclinical course in cattle and sheep.

16.2.6.1 Rift Valley Fever Virus

Epidemiology and Transmission

Rift Valley fever virus is named after a region in East Africa from which it was originally isolated from sick cattle. However, it is widespread throughout the African continent and is an important bovine pathogen. The virus has not yet been found outside of Africa. Its particular significance resides in its zoonotic potential. Sheep, cattle and goats are highly susceptible. Young animals are particularly sensitive. Humans are considered to be moderately susceptible, and about 10 % of infections have a fatal outcome. Rift Valley fever virus is transmitted by various mosquito species of the genera *Aedes* and *Culex*. The virus remains as a reservoir within the population of *Aedes* spp. through transovarial transmission. Especially *Aedes* mosquitoes that deposit their eggs in moist soil and not in water play an important role for outbreaks. The eggs can survive in dry soil for years, and the larvae are able to hatch out after intensive rainfall. Suitable climatic conditions can lead to massive insect proliferation, and thus to efficient propagation and to the appearance of epidemics, during which diseases also occur in humans; for instance, there were about 200,000 cases in Egypt in 1978. Climatic changes in southern and central Europe may also contribute, together with the settlement of pertinent mosquito species, to the spread of Rift Valley fever virus in temperate zones – a scenario that has repeatedly been discussed in recent years. Apart from mosquitoes, wild and domesticated ruminants are another reservoir; they are infected in endemic cycles, and are viraemic for several days. Viruses are present in sufficiently high concentrations to be able to infect sucking mosquitoes. It is important that transmission of the virus is not necessarily associated with mosquitoes: tissue from dead animals or contaminated bedding in stalls can contain sufficient amounts of viruses to transmit the pathogens to animals and humans.

Clinical Features

After an incubation period of a few hours to a few days, depending on the susceptibility of the species, the animals develop a systemic infection with primarily gastrointestinal symptoms including vomiting and diarrhoea, which are associated with an infectious hepatitis. In the further course of the disease, an epidemic

emergence of abortions occurs in herds infected with Rift Valley fever virus. Up to 100 % of pregnant sheep or cattle show abortions. This symptom is also known as "abortion storm".

In humans, most infections are subclinical; however, albeit rarely, severe and fatal diseases are regularly observed. The mortality rate is 5–10 %. The symptoms are similar to those of a flu. After a short incubation period of 2–6 days there is fever, severe headache, eye ache and generalized muscle pain, as well as photophobia. Mild encephalitis, retinitis, and haemorrhagic fever with hepatitis are complications.

Pathogenesis

The viruses enter the bloodstream with the salivary gland secretions of mosquitoes and infect monocytes and macrophages, which transport them to the lymph nodes. The virus spreads by infecting other cells during the primary viraemia. In this way, it reaches its target organs such as the liver, the central nervous system and – in pregnant animals – the fetus, where it multiplies lytically. Under appropriate culture conditions, the polymerase complexes of Toscana virus and Rift Valley fever virus are capable of performing transcription and replication of the genome segments of both viruses. Inasmuch as the geographical distribution of these two types of phlebovirus overlaps, the emergence of reassortants is at least theoretically possible.

Immune Response and Diagnosis

Animals that survive an infection with Rift Valley fever virus gain long-lasting protective immunity. The diagnosis can be achieved by cultivating the virus in Vero cells, by detecting viral proteins in infected tissues (lymphoid tissue, liver, fetus) or by amplification of viral genomes using PCR. The serological proof of infection is also possible by analysing serum pairs in neutralization tests or ELISA. The same is true for diagnosis in humans, but the acute infection is detected by RT-PCR.

Control and Prophylaxis

Several attenuated live vaccines or inactivated vaccines are available for animals. Mutations in the NSs gene lead to a loss of the anti-interferon effect of NSs proteins. Such virus mutants induce large amounts of interferon, and are highly attenuated in infected animals. Control is implemented in the form of vaccination programmes prior the period during which mosquitoes fly, as well as by draining their breeding grounds and by using DDT to kill the insect larvae. There is neither an approved vaccine for human use, nor an antiviral therapy.

16.2.6.2 Nairobi Sheep Disease Virus

Epidemiology and Transmission

Dugbe virus or Nairobi sheep disease virus is widespread in East Africa. It induces a severe haemorrhagic enteritis in small ruminants, i.e. in sheep and goats. In addition to the endemic areas in East Africa, a few sporadic cases of Nairobi sheep disease have been reported in India and Sri Lanka. The virus is predominantly transmitted by the brown tick (*Rhipicephalus appendiculatus*). It is able to persist in

the tick for years, and is transmitted to larvae during various developmental stages. Occasionally observed human infections in laboratories were associated with mild and transient flu-like symptoms.

Clinical Features

Nairobi sheep disease virus causes severe systemic infections in sheep and goats, and its main symptom is haemorrhagic enteritis with diarrhoea. Pregnant ewes abort frequently. Up to 90 % of infected animals die.

Pathogenesis

There are only few data on the pathogenesis of this tick-borne infectious disease of sheep and goats.

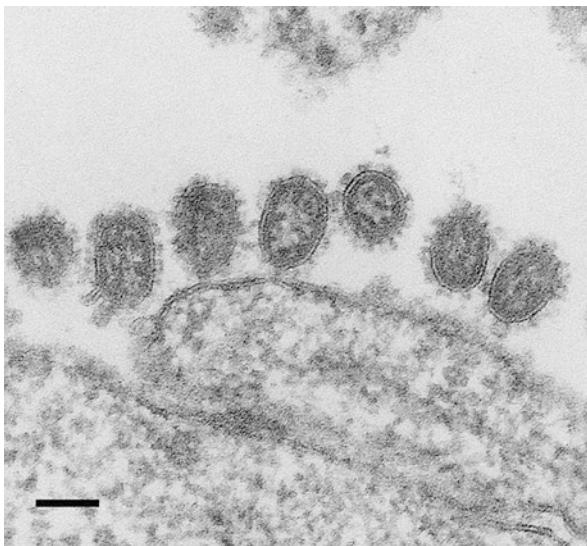
Immune Response and Diagnosis

The diagnosis is mainly made by isolating the pathogen from the spleen, lung and intestinal lymph nodes and propagation in cell culture. Many viral proteins can be detected by immunofluorescence.

Control and Prophylaxis

Both a live vaccine based on attenuated viruses that are not transmitted to ticks and an inactivated vaccine are available and effective.

16.3 Orthomyxoviruses



The best known prototypes of this virus family are the different types of influenza A viruses that cause the classic human flu. The first indication that the causative

agent of swine influenza is a virus was provided by the findings of Richard Shope that were published in 1931: he found that this epizootic disease is caused by filterable agents. Wilson Smith, Christopher Andrewes and Patrick Laidlaw isolated influenza A viruses from humans for the first time in1933. Influenza B viruses were described by Thomas Francis in 1940, and in 1955 Werner Schaefer in Tübingen showed that a fatal disease of poultry is caused by influenza viruses (classic avian influenza). All these viruses belong to the family *Orthomyxoviridae*, the members of which are characterized by a segmented, single-stranded, negative-sense RNA genome.

In particular, influenza A viruses are known to cause acute, highly febrile respiratory diseases that should not be confounded with the common cold. Influenza or flu occurs periodically as a pandemic, which usually originates in Southeast Asia and China, and spreads from there around the world. Influenza pandemics are associated with a large number of deaths, not only in older people, but also among adolescents. The Spanish flu claimed more than 20 million victims in 1918–1919, considerably exceeding the number of people killed in the concomitantly raging First World War. After a pandemic outbreak, influenza viruses cause subsequent small-scale epidemics by the process of antigenic drift, which primarily occurs during the winter period. It is estimated that in “normal” seasonal winter epidemics, influenza A virus infections cause several thousand deaths each year (e.g. between 3,000 and 49,000 cases in the USA).

16.3.1 Classification and Characteristic Prototypes

Because of the different molecular properties and the serological characteristics of their NP and M proteins, we distinguish between influenza virus types A, B and C. They are classified into three different genera (Table 16.6). Influenza A viruses and influenza B viruses have eight genome segments, but differ in several properties. Influenza C viruses possess not only a different number of genome segments (seven), but also other surface proteins. Influenza A viruses and influenza B viruses encode a haemagglutinin and a neuraminidase, whereas influenza C viruses unify these two traits into a single surface protein, the haemagglutinin–esterase fusion (HEF) protein. Influenza A viruses can infect humans and a variety of other mammals and birds, whereas influenza B viruses have been isolated only from humans and seals, and influenza C viruses have been isolated solely from humans and swine. Further orthomyxoviruses are Quaranfil virus, Johnston Atoll virus, Thogoto virus and Dhori virus, which are widespread in Africa, Asia and southern Europe, and are transmitted by ticks. They constitute the genera *Quaranjavirus* and *Thogotovirus*, have six genome segments and cause febrile diseases with abortions in animals (bird, sheep, cattle and goats) and neurological symptoms in humans. Infectious salmon anaemia virus has recently been classified into the newly created genus *Isavirus*. A common feature of all previously examined orthomyxoviruses is that their reproduction is inhibited by the interferon-induced Mx proteins (see the box entitled “Innate Defence Against Influenza Viruses and Other Negative-Sense RNA Viruses”).

Table 16.6 Characteristic representatives of orthomyxoviruses

Genus	Human virus	Animal virus
Influenza A virus	Influenza A viruses	Influenza A viruses (pigs, horses, seals, turkeys, ducks, gulls, etc.)
Influenza B virus	Influenza B viruses	Influenza B viruses (seals)
Influenza C virus	Influenza C viruses	Influenza C viruses (swine)
Thogotovirus		Thogoto virus, Dhori virus (ticks, cattle, sheep, goats, rodents)
Isavirus		Infectious salmon anaemia virus (*Salmonidae*)
Quaranjavirus		Johnston Atoll virus (ticks, birds) Quaranfil virus (ticks, birds)

16.3.2 Structure

16.3.2.1 Virus Particle

Virus particles of orthomyxoviruses are pleomorphic, i.e. they differ greatly in size and shape. They have mainly spherical forms with a diameter of about 120 nm, but filamentous virions also are found. They are composed of segmented nucleocapsids that are surrounded by an envelope, in which glycosylated viral proteins (spikes) are embedded (Fig. 16.6). In influenza A viruses and influenza B viruses these proteins are a haemagglutinin (HA protein), which is a trimeric complex protruding about 7–8 nm from the particle surface, and a neuramidase (NA protein), whose active form is a homotetramer. The HA protein is responsible for attachment of the virus to *N*-acetylneuraminic acid molecules on the surface of host cells. In addition, it is able to fuse membranes and to agglutinate erythrocytes. The NA protein cleaves terminal *N*-acetylneuraminic acids from complex carbohydrates, thus ensuring the release of newly synthesized virus particles. Instead of HA and NA proteins, influenza C viruses have only a single surface protein, namely the trimeric HEF protein. In addition, influenza A viruses have a small tetrameric M2 protein, which is anchored in the membrane in a low copy number of approximately 20–60 molecules, and exerts the function of a proton channel. The matrix protein M1 is associated with the inner side of the envelope and coats it.

The envelope encompasses the viral nucleocapsids. These are constituted of eight single-stranded RNA segments in influenza A viruses and influenza B viruses as well as in infectious salmon anaemia virus. Influenza C virus contains seven segments. The RNA segments are complexed with NP proteins throughout their entire length. In addition, each nucleocapsid segment is also associated with the proteins of the polymerase complex, PB1, PB2 and PA (Fig. 16.6).

16.3.2.2 Genome Organization and Structure

The segmented RNA genome of influenza A viruses, influenza B viruses and influenza C viruses encompasses about 13,600, 14,600 and 12,900 nucleotides (Table 16.7). The 3′ and 5′ ends of the segments have short complementary regions and form double strands, through which the RNA molecules are held in a quasi-circular, panhandle-like shape. These regions do not encode proteins, but contain

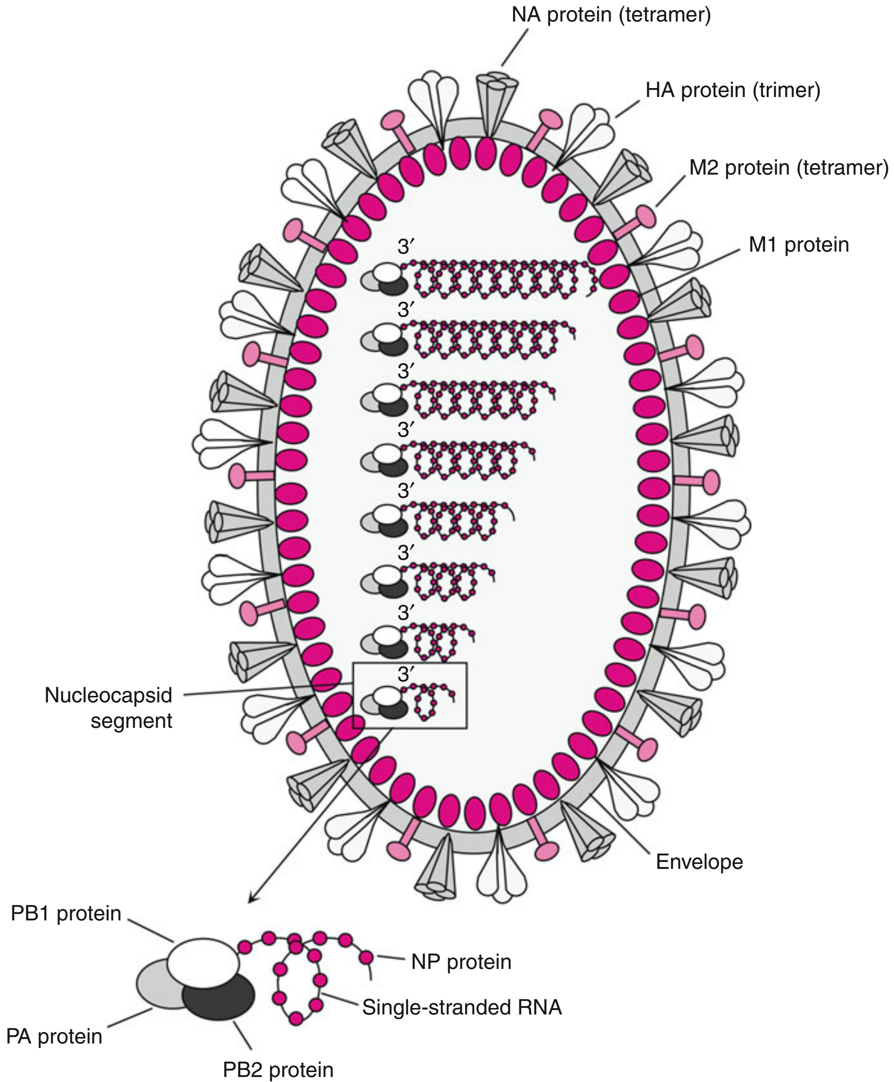


Fig. 16.6 Structure of an influenza A virus particle. The single-stranded RNA genome is constituted of eight segments, which are complexed with NP proteins. The PB1, PB2 and PA proteins of the polymerase complex are associated with the 3′ termini. The nucleocapsid segments are surrounded by an envelope, in which the surface proteins HA, NA and M2 are intercalated. The M1 protein is located on the inner side of the envelope, where it forms a protein layer

the regulatory sequences for transcription and replication. The basic, arginine-rich NP proteins are associated with the single-stranded RNA, whereby each NP molecule covers a section of about 20 nucleotides. Furthermore, each particle contains approximately 50 polymerase complexes binding to the RNA segments.

Table 16.7 RNA genome segments of influenza viruses and their encoded proteins

Segment	Influenza A virus[a] Length (nucleotides)	Influenza A virus[a] Protein	Influenza B virus Length (nucleotides)	Influenza B virus Protein	Influenza C virus Length (nucleotides)	Influenza C virus Protein
1	2,341	PB2	2,386	PB1	2,350	P1
2	2,341	PB1, PB1-F2, PB1 A/40	2,396	PB2	2,350	P2
3	2,233	PA	2,304	PA	2,150	P3
4	1,778	HA	1,882	HA	2,000	HEF
5	1,565	NP	1,841	NP	1,750	NP
6	1,413	NA	1,557	NA, NB	1,150	M1, CM2
7	1,027	M1, (A/)M2	1,191	M1, BM2	975	NS1, NS2
8	890	NS1, NS2	1,096	NS1, NS2	–	–

[a]Influenza A virus strain A/PR/8/34

They consist of the proteins PB1, PB2 and PA and are preferentially associated with the 3′ ends of the genome segments.

Each segment of the influenza virus genome codes for specific viral proteins, usually one per segment. Only the two small RNA segments of influenza A viruses and influenza C viruses as well as segments six, seven and eight of influenza B viruses encode two polypeptides, which are translated from alternatively spliced mRNA species (Table 16.7).

16.3.3 Viral Proteins

16.3.3.1 Structural Proteins

Envelope Proteins

Haemagglutinin is a trimeric protein complex. Comparisons of the amino acid sequences of various influenza A viruses and influenza B viruses suggest that they are very closely related, and their HA proteins have a similar structure. To date, 16 different variants of the HA protein (H1 to H16) have been identified in the group of influenza A viruses, and they determine the virus subtype (Table 16.8). The H16 variant was first described in the spring of 2005; it was discovered in Mediterranean gulls in Sweden. The 16 HA variants differ in their amino acid sequence, in their cleavage pattern by cellular proteases, in their receptor specificity and in their recognition by antibodies. Recently an additional HA variant (H17) has been described in an influenza A-like virus that was isolated from fruit-eating bats in Guatemala (*Sturnira lilinm*). The receptor binding properties and the cleavage pattern of H17 are different from H1-H16, however.

The precursor protein HA_0 has a length of approximately 560 amino acids, and possesses both a signal peptide at the amino terminus and a hydrophobic domain at its carboxy terminus, which anchors the protein in the cytoplasmic membrane of the

Table 16.8 Distribution of the different haemagglutinin subtypes in mammals and birds

HA subtype	Humans	Swine	Horses	Dogs	Water birds (wild ducks)	Seabirds (gulls, etc.)	Commercial poultry (chickens, geese, etc.)
H1	++	++			+	+	++
H2	+	+			+	+	+
H3	++	+	++	+/−	++		+
H4					++	+	+
H5	+/−[a]				+	+	++
H6					++	+	+
H7	+/−[a]		+		+	+	++
H8					+		+
H9	+/−[a]	+/−			+	++	++
H10					+	+	+
H11					+	+	+
H12					+	+	
H13						++	+
H14					+		
H15					+	+	
H16						+	

+/− rare, + regular, ++ frequent
[a]Sporadic transmission of avian influenza viruses to humans

infected cell and in the viral envelope. Three cysteine residues within the transmembrane region are palmitoylated. This serves for tight anchoring of the HA trimer in membrane microdomains, called lipid rafts, which are rich in glycosphingolipids and cholesterol. This tight anchorage seems to be important for the successful fusion between the envelope and the endosomal membrane at the beginning of the infection cycle. The nascent amino acid chain of the HA_0 precursor protein is translocated through the endoplasmic reticulum membrane during translation, and the signal peptide is removed by cleavage. During transport to the cell surface through the Golgi apparatus, HA_0 proteins assemble into trimers, and are post-translationally modified by addition of sugar groups. Palmitic acid residues are added to the carboxy-terminal transmembrane region. Intracellular and extracellular trypsin-like proteases cleave the precursor protein HA_0 into the amino-terminal moiety HA_1 (36 kDa, in modified form roughly 50 kDa) and HA_2 (26 kDa). In the course of this, an arginine residue is cleaved from the carboxy terminus of the arising HA_1 protein. This cleavage reaction is absolutely necessary for viral infectivity and is an important pathogenicity factor. The proteases involved in this process include:

1. The protease furin of Golgi vesicles.
2. The subtilisin-like proteases, which are present in the cells of many tissue types. They are involved, among other processes, in post-translational processing of hormones and growth factor receptors.

3. The mini-plasmin, which is formed from the precursor products plasminogen and plasmin especially in the epithelial cells of segmental bronchia,
4. The protease Clara, which is secreted by the so-called Clara cells (secretory epithelial cells of the lung capillary net) of the terminal, respiratory bronchioles.
5. Type 2 serine proteases, which are anchored in the cytoplasmic membrane, such as the proteases TMPRSS2, TMPRSS4 and HAT.

Which of the proteases is used for processing of the HA_0 protein is determined by the amino acid sequence at the cleavage site. If the site contains several basic arginines and lysines, the intracellular furin-like and subtilisin-like enzymes are preferably used. Thus, newly formed viruses already have the haemagglutinin in its processed version (HA_1 and HA_2) in their envelope and are consequently infectious immediately after release from the cell surface. These proteases preferentially recognize the amino acid sequence ariginine–X–serine/lysine–arginine in front of the cleavage site. Such polybasic cleavage sites are characteristic of the highly pathogenic avian influenza strains of subtypes H5 and H7, the causative agents of avian influenza (Table 16.8). In contrast, low-pathogenicity avian influenza viruses and human influenza virus types with haemagglutinins H1, H2 and H3 have mainly only one basic amino acid in front of the protease cleavage site. Such monobasic cleavage sites are not efficiently recognized by the intracellular enzymes. The processing and, consequently, the induction of infectivity are performed by tissue-specific, local proteases only after release of the virus from infected cells. Such an enzyme is the protease Clara in the bronchial epithelium, whose activity requires the sequence glutamine/glutamic acid–X–arginine in front of the cleavage site. It is thought that these differences are essential for the pathogenicity and virulence of the various influenza virus subtypes (Sect. 16.3.5). HA_1 and HA_2 remain linked by a disulphide bridge (Fig. 16.7a). Through the cleavage, a region of non-polar, hydrophobic amino acids becomes exposed at the amino terminus of the HA_2 protein, which thus develops its fusogenic activity to merge the viral envelope with the endosomal membrane at the beginning of the replication cycle (compare this with the activity of the F protein of paramyxoviruses; ▶ Sect.15.3.3).

Attachment of the virus to terminal *N*-acetylneuraminic acid (sialic acid) residues, which exist as a modification of membrane proteins or lipids on the cell surface, is mediated by a globular domain in the HA_1 protein. Several amino acids are involved in the binding process, and come in close proximity to each other by three-dimensional folding (Fig. 16.7b, c). The type of linkage between the terminal sialic acid residues and the penultimate sugar residue of the carbohydrate modification is important for interaction. In this case, the penultimate residue is a galactose molecule. The sialic acid can be linked with it by either an α-(2,3) or an α-(2,6) glycosidic bond. The amino acids at positions 226 and 228 in the HA protein appear to be mainly responsible for determining which variant is bound. However, they are not directly involved in the interaction, but influence the conformation of the binding site. If leucine or serine residues are present in these positions, the HA complexes will preferentially bind to *N*-acetylneuraminic acid residues that are linked with the galactose by an α-(2,6) glycosidic bond. Conversely, the replacement of leucine by glutamine as well as serine by glycine

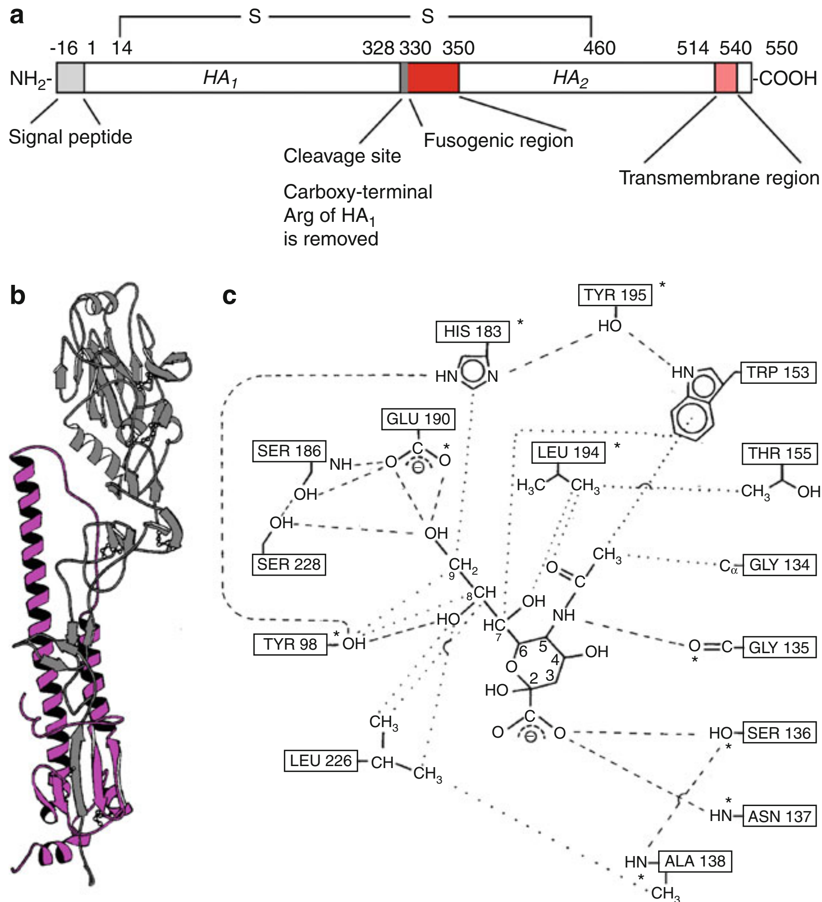


Fig. 16.7 Haemagglutinin of influenza A viruses. **a** The HA protein and localization of functionally important domains. The *numbers* refer to the positions in the amino acid sequence of the haemagglutinin of strain A/Aichi/2/68/H3N2 (starting at the first amino acid of the amino terminus after cleavage of the signal peptide). Indicated are also the cleavage site, the fusogenic region, the transmembrane region and the cysteine residues that form a disulphide bridge, through which the two cleavage products remain linked to each other. **b** Crystallographic structure of the HA_1–HA_2 complex after cleavage, depicted as a ribbon model. *Arrows* represent β-sheet structures, and *cylinders* represent α-helices. The HA_1 and HA_2 proteins are depicted in *grey* and *red*, respectively. The natural HA_2 protein additionally contains the transmembrane domain and the short cytoplasmatic moiety, which were removed for technical reasons to improve purification and crystallization of the external part of the protein complex. The attachment site, through which the HA_1 protein interacts with terminal neuraminic acid residues, resides in a region of HA_1 that faces away from the envelope, where the amino acids form a globular domain. **c** Interactions of the HA protein with *N*-acetylneuraminic acid. Shown are the different amino acids (according to their positions in HA_1), which interact via hydrogen bridge bonds (*dashed lines*) and van der Waals contacts (*dotted lines*) with the sugar residue in the *centre*. *Stars* indicate the amino acid residues that are highly conserved among different influenza A virus strains (**b** From Lamb and Krug; **c** from Wharton et al. 1989)

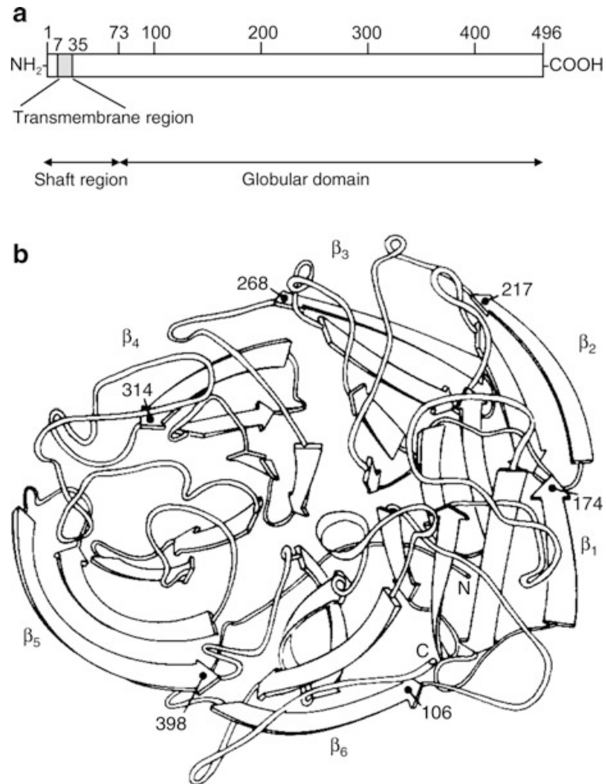


Fig. 16.8 Neuraminidase of influenza A viruses. **a** The NA protein and localization of functionally important domains. *Numbers* refer to the positions in the amino acid sequence of the neuraminidase of strain A/Tokyo/3/67/H3N2 (starting at the first amino acid of the amino terminus). Depicted are the transmembrane region and the domains that shape the "shaft" and the domain. **b** Crystallographic structure of the neuraminidase globular domain, depicted as a ribbon model. *Arrows* represent β-sheet structures. The six large β-sheet structures are named β_1 to β_6 according to their position in the protein. *N* and *C* indicate the amino terminus and the carboxy terminus of the protein, respectively. the transmembrane region was removed proteolytically (From Colman 1989)

leads to the recognition of the α-(2,3) glycosidic bond. This distinction plays an important role in determining the host tropism of the viruses and, consequently, their pathogenicity. It is crucial for the efficiency of zoonotic viral transmission between different species.

The HEF protein of influenza C viruses has a molecular mass of 88 kDa and differs significantly in terms of function and sequence from other influenza viruses. It integrates the receptor-binding and fusogenic properties with the receptor-destroying activity (neuraminidase). Similar to HA proteins, it exists as a trimer, and must be cleaved by a cellular protease to activate its fusogenic activity. Influenza C viruses use *N*-acetyl-9-*O*-acetylneuraminic acid as a receptor. The receptor-destroying property resides in a sialic acid-*O*-acetylesterase activity. Antibodies directed against HA or HEF proteins are able to neutralize the respective viruses. Slightly different functions are found in infectious salmon anaemia virus: It binds to *N*-acetyl-4-*O*-acetylneuraminic acid and has an receptor-destroying esterase activity, which cleaves 4-*O*-acetyl groups from the carbohydrate complexes.

The NA protein of influenza A viruses and influenza B viruses is active as a tetramer. NA protein monomers have a length of approximately 460 amino acids. The hydrophobic domain responsible for membrane anchoring is situated near the amino terminus. An amino-terminal signal peptide does not exist (Fig. 16.8a).

Therefore, the glycosylated NA proteins are type II membrane proteins whose amino terminus is localized not in the lumen of the endoplasmic reticulum, but in the cytoplasm of the cell. X-ray structure analyses revealed that NA proteins possess a "shaft or stalk" moiety that is adjacent to the transmembrane region. The rest of the NA protein, approximately 390 amino acid residues, is folded into a globular structure. This domain is composed of six β-sheet regions, each consisting of four β-sheets. From the top view, they resemble an airplane propeller with six rotor blades (Fig. 16.8b). The membrane-anchored NA protein is a mushroom-shaped homotetramer, where the mutually interacting globular domains form the "mushroom cap". After infection, the function of this enzyme is to remove neuraminic acid residues (which serve as virus receptor) from the cell surface,. This process plays an important role in the release of virus particles by preventing the interaction the HA proteins of newly produced virions with membrane components of infection-destroyed cells. In addition, this process probably prevents the particles from sticking to each other, as the viruses do not have their own terminal neuraminic acid residues on the surface because they are removed by the enzyme.

Like HA proteins, NA proteins of different virus isolates differ from each other. Overall, nine subtypes of NA proteins (N1 to N9) are known from influenza A virus. Just as for HA proteins, antibodies with neutralizing properties are also produced against NA proteins. Although they do not inhibit binding of the virus to the receptor, they prevent, to some extent, dissemination within the organism. Hence, the combination of the different HA and NA proteins in a virus isolate determines the antigenic properties of the respective influenza virus subtype.

Besides the NA gene, the genetic information of a further glycosylated envelope protein has been found on genome segment 6 of influenza B viruses. The NB protein (12 kDa) is encoded by a reading frame that overlaps with the neuraminidase NA gene and begins four nucleotides before it. The protein is characterized by a hydrophobic domain that acts as a transmembrane region, anchoring the protein in the envelope of infectious particles. The function of the NB protein has not yet been conclusively elucidated, but there are indications that it forms an ion channel, similar to the BM2 protein. Genetically engineered virus mutants which do not express the NB protein exhibit similar proliferation rates as wild-type influenza B viruses in vitro. By contrast, a delayed replication cycle can be observed in infected mice.

Matrix proteins (M1) are present in large amounts in the virus particles. They have a molecular mass of 28 kDa, are associated with the inner side of the envelope and form a layer, in which they interact with each other. M1 proteins do not have a transmembrane region, but they seem to be associated with the viral envelope by interacting with the cytoplasmic moieties of HA, NA and M2 proteins. Simultaneously, they are also linked with NP proteins of nucleocapsids. Thus, they also play an important role in packaging nucleocapsids into the emergent virus particles.

The M2 protein of influenza A viruses (A/M2 protein) along with the M1 protein is encoded by genome segment 7. For its synthesis, the M1-specific mRNA is spliced. The eight amino-terminal amino acids of both proteins are identical. The reading frame changes starting from the splice site, leading to a different amino acid sequence. The M2 protein has a molecular mass of roughly 15 kDa and is present in

large amounts in infected cells. Contrarily, it is present only in a few copies in the virus particles. It is modified by fatty acid and anchored in the membrane by a sequence of 20 hydrophobic amino acids (amino acids 24–44). The M2 protein is present in the viral envelope as a tetrameric complex. The transmembrane regions of the four proteins interact and form an ion channel, for whose activity the amino acid motif $H_{37}XXXW_{41}$ is essential. In the early stage of infection, and after penetration of the virus particle via endocytosis, the proton channel function generates the acidification of the virus inside, causing thereby a structural rearrangement in the M1 protein. This leads to a relaxation of the interaction of the M1 proteins with the nucleocapsids so that they are subsequently transported into the nucleus. M2 proteins are embedded in the membrane of Golgi vesicles in a late stage of the infection cycle; by regulating the pH, they prevent the premature structural rearrangement of HA_1/HA_2 proteins, and thereby the associated induction of the fusion activity. Amantadine, an antiviral agent, interacts with the amino acids of the proton channel and inhibits its function (▶ Chap. 9). Mutations in the M2 gene, particularly those that replace hydrophobic amino acids with polar residues at the entrance to the channel, confer resistance against amantadine (e.g. replacement of alanine with serine at position 30).

In influenza B viruses, the seventh genome segment encodes a protein that is functionally analogous to M2 (BM2, 18 kDa). It is translated along with the M1 protein of influenza viruses from a bicistronic mRNA. The translational start codon of BM2 overlaps by one nucleotide with the stop codon of M1. The BM2 protein acts as an ion channel similar to the A/M2 protein of influenza A viruses. A comparison of the amino acid sequence reveals little homology, but the $H_{19}XXXW_{23}$ motif is conserved. Nonetheless, amantadin does not influence the function of the BM2 protein in influenza B viruses. Therefore, influenza B viruses cannot be inhibited by amantadin or similar drugs. BM2 has been detected in the cytoplasmic membrane of infected cells and in the viral envelope. Genetically engineered virus mutants that have a reduced BM2 protein content in their particles exhibit decreased infectivity. In influenza C viruses, the sixth genome segment encodes besides the M1 gene product also an analogous ion channel protein, referred to as CM2.

Components of the Nucleocapsid

The NP protein is the principal component of nucleocapsids. The RNA genome segments are associated throughout their entire length with the NP polypeptide. It is rich in basic arginine residues, has a domain that mediates its transport into the nucleus and has a molecular mass of approximately 55 kDa. In its free, non-RNA-bound form, it is important for the correct execution of genome replication during the life cycle of the virus. The NP protein sequence of the different influenza viruses is highly conserved, and determines the corresponding virus type. It possess important T-cell epitopes that are presented in complex with MHC class I proteins by infected cells, and thus is important for the induction of the host cellular immune response, and the elimination of virus-infected cells from the organism.

The P protein complexes, constituted of the components PB1, PB2 and PA that are non-covalently linked into heterotrimeric complexes, are present in about 50 units per virus particle and are preferentially associated with the ends of the genome segments. They possess RNA-dependent RNA polymerase activity. Each of the proteins has a molecular mass between 80 kDa and 90 kDa and is endowed with nuclear transport signal sequences. The PB1 and PB2 proteins are rich in basic amino acids. Contrarily, PA is an acidic protein. The PB2 protein binds to the 5′-cap structure of cellular mRNA molecules (they are used as primers for viral mRNA synthesis), which are subsequently cleaved after ten to 13 nucleotides from the ends of the cellular transcripts by the viral endonuclease, which resides in the PA subunit (cap-snatching). The PB1 subunit possesses the polymerase activity and is responsible for chain elongation. Apart from the endonuclease activity, the functions of the PA protein are not completely elucidated. It is necessary for replication of the genome segments and especially for the step in which the positive-sense antigenome segments are used as templates for the synthesis of genomic RNAs. In the course of this, the PA protein is bound to the 12 amino-terminal amino acids of the PB1 protein. If the complex formation is impaired, by mutating nucleotides encoding this domain of the PB1, then the RNA polymerase activity is drastically reduced and the viruses replicate poorly or not at all. It has also been reported that phosphorylation of PB1 by the cellular protein kinase C affects the polymerase activity. Furthermore, it has recently been discovered that the PA protein acts as a serine protease. The serine at position 624 is part of the active centre. The enzymatic activity is optimal at 37 °C, and is impaired at low temperatures (33 °C). What function the PA protease exerts during infection remains to be elucidated. It is only known that it is not necessary for the catalytic processes of transcription and replication of the viral RNA segments.

The Elucidation of the Haemagglutinin Structure Provided Important Data for Understanding Protein Function

In 1981, Ian Wilson, John Skehel and Don Wiley unveiled the structure of the HA complex by X-ray structural analysis. It was the first viral envelope protein for which this was achieved. For this purpose, they cleaved the surface-exposed moiety of the complex by treatment with bromelain and obtained a soluble trimer. These and further investigations revealed that attachment to *N*-acetylneuraminic acid is mediated by a globular domain composed of eight antiparallel β-sheets of the HA_1 subunit. Its amino terminus is localized close to the envelope (Fig. 16.7b). The subsequent 63 amino acids have an extended conformation and form a kind of spacer or shaft between the envelope and the adjoining globular domain. Binding to the cellular receptor *N*-acetylneuraminic is mediated by a pocket-like-shaped structure that is inaccessible to antibody molecules. Owing to the folding of the amino acid chain in the globular domain of the protein, the neuraminic acid binding site is surrounded by protein regions that represent B-cell epitopes. Neutralizing antibodies, which are produced by the host during

infection, are directed against these regions. The various influenza A virus types and variants differ especially with respect to the sequence of these epitopes. Like picornaviruses (▶ Sect. 14.1), the actual receptor binding site of influenza viruses is not surface-exposed and is thus not subject to selection pressure by antibodies.

The part of the HA_1 protein which is adjacent to the globular domain is folded so that the carboxy terminus is situated near the envelope. The amino and carboxy-terminal regions of the HA_1 protein can interact with each other and form a stem-like structure along with the amino-terminal region of the HA_2 protein (Fig. 16.7b). During the attachment and penetration processes of virus particles in the cellular endosomal compartment, the structure of the HA_1–HA_2 complex is subject to large structural rearrangements by the associated acidification, so the hydrophobic region at the amino terminus of the HA_2 protein can enter the adjacent endosomal membrane, thus mediating membrane fusion.

16.3.3.2 Non-Structural Proteins

The two non-structural proteins NS1 and NS2/NEP are coded on segment 8 (or segment 7 in influenza C viruses), the smallest influenza virus genome.

NS1 Protein

The NS1 proteins of influenza A viruses and influenza B viruses have a molecular mass of 26 and 40 kDa, respectively. Older data suggest that they influence splicing of viral transcripts. Nevertheless, they are primarily virulence factors that affect cellular antiviral defence mechanisms in multifarious ways. They form dimers and are transported to the nucleus after their synthesis. Phosphorylation by the cellular protein kinase C seems to be necessary for their functional activities. The amino-terminal NS1 domains form a structure motif of six α-helices which is responsible for binding to double-stranded RNA. Particularly, the arginine residue at position 38 of the NS1 protein of influenza A viruses is essential for this interaction. The carboxy-terminal region constitutes the effector domain: the influenza A virus NS1 protein harbours there the nuclear transport signal and sequences which are responsible for the interactions with various cellular proteins. Alterations in individual amino acids of the effector domain are crucial for the activity of the NS1 protein: for example, it has been shown that the glutamic acid residue at position 92 is particularly important for the virus to evade the antiviral effect of interferon. Such a sequence was found in isolates of the subtype H5N1, which was initially transmitted from poultry to humans in Hong Kong in 1997, and caused severe diseases with high mortality.

NS1 proteins interfere in several ways with the basic immune response of the organism:

1. Influenza virus infections induce the synthesis of interferon-α and interferon-β in the affected lung epithelial cells, but especially in dendritic cells, and they

inhibit viral replication in a dose-dependent manner. To express interferon genes, different transcription factors must coordinately interact, among others NFκB, IRF-3 and IRF-7. Interferon-α and interferon-β mediate antiviral responses such as induction of MxA genes in neighbouring cells and contribute to preventing spread of the infection in the tissue (▶ Chap. 8). Binding of NS1 to NFκB impedes its activation, thus affecting the expression of interferon genes.

2. Apart from activating gene expression, interferon-mediated defence strategies are dependent on the presence of double-stranded RNA molecules. These are produced as intermediates during transcription of viral genes, and in the course of genome replication. By binding to double-stranded RNA, the NS1 protein prevents the activation of 2′-5′-oligoadenylate synthetase mediated by double-stranded RNA. This enzyme is induced by the interferon effect, and it activates the cellular RNase L. RNase L degrades single-stranded RNA molecules and impairs both viral and cellular gene expression. The virus inhibits this defence mechanism by the interaction of NS1 proteins with double-stranded RNA.
3. The NS1 protein prevents the activation of protein kinase R (PKR) as well. PKR is activated during the non-specific immune response by interaction with double-stranded RNA, which is synthesized during transcription and replication of the viral genome segments, or by interaction with specific cellular proteins. It phosphorylates the α subunit of translation initiation factor eIF2, thus inhibiting the synthesis of viral and cellular proteins. The inhibitory effect of NS1 proteins appears to be based on a direct interaction of NS1 proteins with PKR, and is not based on the previously described indirect way by interaction with double-stranded RNA.
4. The influenza A virus NS1 protein acts as an inhibitor of 3′ processing of newly synthesized cellular mRNAs. It binds to the 30-kDa subunit of cleavage and polyadenylation specificity factor (CPSF) and to poly(A)-binding protein II (PABII) and inhibits their function during post-transcriptional modification of mRNA precursor molecules, which do not become polyadenylated, and are consequently not transported from the nucleus. This process interferes with the general cell metabolism, and also prevents the activation of the interferon-dependent immune response. During attachment, binding of influenza A viruses to the cell surface triggers a signalling cascade that activates IRF-3 and IRF-7. Both factors move into the nucleus and form, along with other transcription factors (p300, CREB-binding protein), the virus-activated factor (VAF) complex. The VAF complex induces the expression of genes which are regulated by interferon-stimulated regulatory elements (▶ Chap. 8), and predominantly encode antiviral proteins. Since the NS1 protein prevents the export of mRNAs from the nucleus by inhibiting polyadenylation, the synthesis of these antiviral factors does not occur – part of the basic defence of the infected cell is suppressed. In contrast to influenza A viruses, the NS1 protein of influenza B viruses does not interact with CPSF and PABII; accordingly, it does not inhibit the post-transcriptional modification of mRNA precursors. However, the NS1 proteins of influenza B viruses bind to the protein encoded by interferon-stimulated gene 15 (ISG15). The synthesis of this antiviral factor is

induced by IRF-3-mediated activation. Whether this process also contributes to the shutdown of the non-specific immune response is still unclear.

NS2/NEP Protein

The transcripts that direct the synthesis of the NS2/NEP protein are generated by splicing from the NS1-specific mRNA (Fig. 16.9b). The NS2 protein is also called NEP (for "nuclear export protein"). Furthermore, it is found in low concentrations in virus particles. In the course of the infection cycle, the NS2/NEP protein is responsible for the export of newly synthesized viral nucleocapsid segments (viral ribonucleoprotein segments, vRNPs) from the nucleus to the cytoplasm. These are closely linked with the histones of the nuclear matrix by interacting with the viral NP proteins during the replication cycle. Some of the newly synthesized M1 matrix proteins are transported into the nucleus, where they break the tight association of vRNPs with histones. The viral NS2/NEP protein presumably interacts with M1-complexed vRNPs and causes the accumulation of the cellular exportin (chromosome region maintenance 1 protein (CRM1). This promotes the transport of proteins that possess a nuclear export signal from the nucleus to the cytoplasm, and interacts with the nucleoporins in the nuclear membrane. Such a leucine-rich nuclear export signal is located in the amino-terminal region of NS2/NEP proteins.

PB1-F2 and PB1 N40 Proteins

A further non-structural protein has been identified in influenza A viruses. It is encoded by an open reading frame that resides in the second genome segment within the PB1 gene, but is translated in a different reading frame; therefore it is referred to as PB1-F2 protein (for "PB1 frame 2"; 10 kDa). Like the PB1 N40 protein, it is translated not from alternatively spliced transcripts, but by using alternative start codons that are located near the mRNA 5′-cap structure. The PB1-F2 protein encompasses 87 amino acids and acts as a virulence factor: Recombinant influenza A viruses in which PB1-F2 translation has been blocked by altering the start codon by in vitro mutagenesis exhibit a significantly reduced pathogenicity in mice models. The substitution of the amino acid serine at position 66 with an asparagine residue also gives rise to the generation of an attenuated virus mutant.

PB1-F2 proteins are localized in both the cytoplasm and the nucleus. Furthermore, they also accumulate in the membranes of mitochondria. Responsible for this behaviour are amino acids in the carboxy terminus of PB1-F2, which act as a mitochondrial targeting sequence signal. The PB1-F2 protein interacts with two proteins of the mitochondrial membrane, namely the protein adenine translocator 3 (ANT3) in the inner mitochondrial membrane and the protein voltage-dependent anion channel 1 (VDAC1) in the outer mitochondrial membrane. This promotes the formation of a pore complex (permeability transition pore complex), which renders the mitochondrial membrane permeable, and induces the release of mitochondrial products such as cytochrome *c* into the cytoplasm. As a consequence, apoptosis is elicited in these cells. This proapoptotic function seems to depend on the phosphorylation of PB1-F2 and is cell-specific. It becomes important particularly in

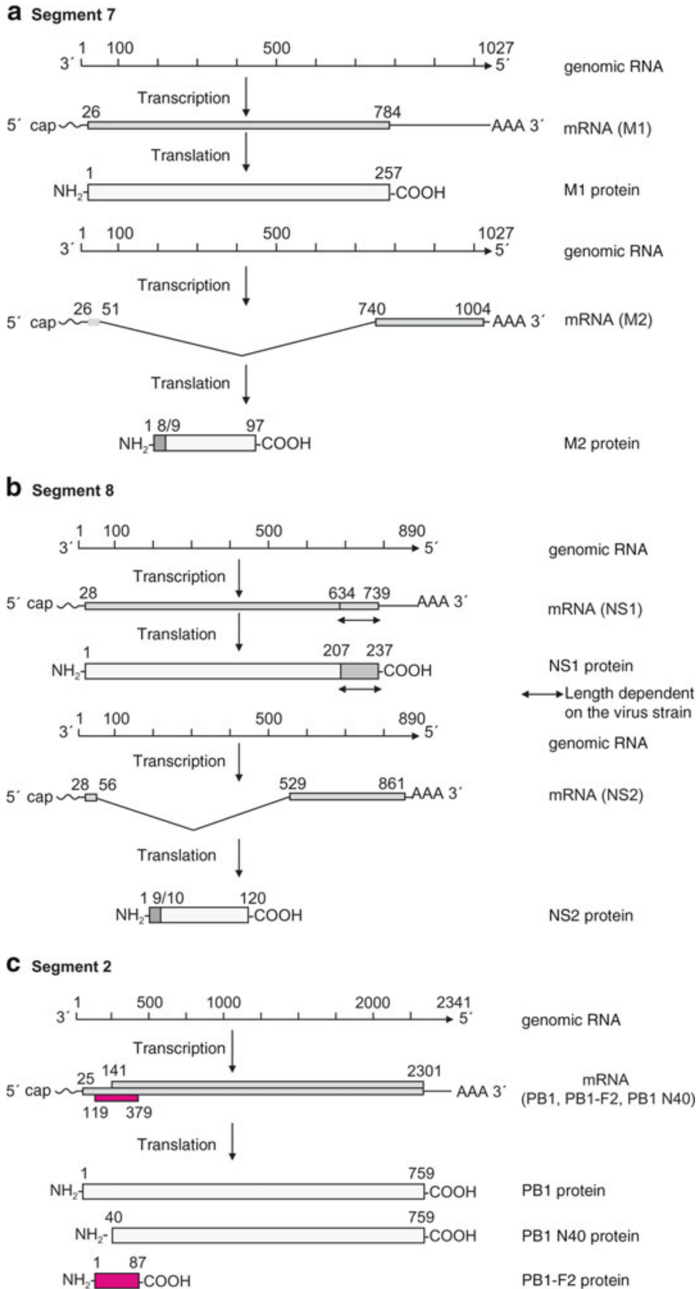


Fig. 16.9 Genome organization of the two small RNA segments and the second largest RNA segment of influenza A viruses.

a Genome segment 7. The segment is transcribed. The M1 protein is translated from the unspliced mRNA. A further mRNA form is generated by splicing. It is used for translating the M2 protein.

infected lymphocytes, and other immunologically active cells such as monocytes and alveolar macrophages. Possibly, these processes give rise to a specific suppression of the immune response in the epithelial cells of the infected respiratory tract. This not only hinders the immune system from coping with the influenza virus infection, but it also facilitates superinfections with bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Haemophilus influenzae*. Moreover, the PB1-F2 protein seems to have further activities: It forms a complex with the PB1 protein, which is also encoded on the second-largest RNA segment and acts as an RNA-dependent RNA polymerase. This interaction leads to an enhancement of the enzymatic activity of PB1. In cells infected with PB1-F2-negative influenza A viruses, the PB1 protein was predominantly localized in the cytoplasm and not in the nucleus.

It has recently been described that, in addition to PB1 und PB1-F2 proteins, the second RNA segment encodes a third protein. This non-structural protein has been denominated PB1 N40. It is translated from the same reading frame that encodes PB1, however by using the AUG start codon at position 40 (Fig. 16.9c). Accordingly, PB1 N40 is an amino-terminal truncated version of the PB1 protein, and does not exhibit RNA polymerase activity, but interacts with PB1, influencing its function in a similar fashion as PB1-F2.

Table 16.9 provides an overview of the characteristic properties of influenza virus proteins

16.3.4 Replication

By their HA or HEF envelope proteins orthomyxoviruses bind to *N*-acetylneuraminic acid (influenza A virus, influenza B virus), to *N*-acetyl-9-*O*-acetylneuraminic acid (influenza C virus) or to *N*-acetyl-4-*O*-acetylneuraminic acid (infectious salmon anaemia virus) on the cell surface. Attached virus particles are enclosed by the cytoplasmic membrane, and penetrate into the cell as intracellular vesicles via receptor-mediated endocytosis. Thus, the nucleocapsids are surrounded by two membranes. The HA protein changes its conformation by the cellular-regulated acidification of endosomal vesicles. As a result, the fusogenic region at

Fig. 16.9 (continued) Its eight amino-terminal amino acid residues are identical to those of the M1 protein. The reading frame changes at the splice site, and thus the protein sequence changes. **b** Genome segment 8. The segment is transcribed. The NS1 protein is translated from the unspliced mRNA. A further mRNA form is generated by splicing. It serves to translate the NS2 protein, whose amino-terminal amino acid residues are identical to those of the NS1 protein. The reading frame changes at the splice site, and hence the protein sequence changes.
c Genome segment 2. The segment is transcribed. PB1 as well as the PB1-F2 and PB1 N40 proteins are translated from the unspliced mRNA. The PB1 N40 protein is translated from an alternative AUG start codon by using the same reading frame and constitutes an amino-terminal truncated PB1 protein lacking the first 39 residues. The translational start of the PB1-F2 protein is initiated at an AUG codon of a reading frame that is shifted by one (+1) nucleotide

Table 16.9 Properties and functions of influenza virus proteins

Protein	Influenza A virus	Influenza B virus	Influenza C virus	Function
Structural proteins				
HA	77 kDa, glycosylated, acylated	77 kDa, glycosylated	–	Precursor protein for HA1 und HA2; induction of neutralizing antibodies
HA1	50 kDa, glycosylated	50 kDa, glycosylated	–	Amino-terminal part of HA; attachment to neuraminic acid, haemagglutination
HA2	26 kDa, glycosylated, acylated	26 kDa, glycosylated	–	Carboxy-terminal part of HA; membrane fusion
HEF	–	–	88 kDa, glycosylated	Hemagglutination, receptor binding, membrane fusion, induction of neutralizing antibodies; acetylesterase
HEF1	–	–	65 kDa, glycosylated	Amino-terminal part of HEF
HEF2	–	–	30 kDa, glycosylated	Carboxy-terminal part of HEF
NA	56 kDa, glycosylated	57 kDa, glycosylated	–	Neuraminidase, cleavage of terminal neuraminic acid residues; important for release of particles; induction of neutralizing antibodies
M1	28 kDa	28 kDa	30 kDa	Matrix protein; associated with the interior side of the viral envelope; functionally active during morphogenesis
A/M2	15 kDa			Integral envelope protein; ion channel protein, active early during release of nucleocapsids from endosomes
BM2		18 kDa		
CM2			18 kDa	
NP	55 kDa	55 kDa	60 kDa	Principal constituent of the nucleocapsid; baic; nuclear transport signal
PB1	90 kDa, phosphorylated	85 kDa	89 kDa	Component of the nucleocapsid and the polymerase complex; RNA-dependent RNA polymerase; basic
PB2	80 kDa	88 kDa	85 kDa	Component of the nucleocapsid and the polymerase complex; binding to 5′-cap structures; basic
PA	83 kDa	83 kDa	82 kDa	Component of the nucleocapsid and the polymerase complex; acid serine protease activity
NS2/NEP	11 kDa	11 kDa	14 kDa	Regulatory protein; present in small amounts in the virion; export of vRNPs from the nucleus into the cytoplasm

(*continued*)

Table 16.9 (continued)

Protein	Influenza A virus	Influenza B virus	Influenza C virus	Function
NB	–	12 kDa	–	Glycosylated membrane protein; is present in virions; ion channel protein?
Non-structural proteins				
NS1	26 kDa, phosphorylated	40 kDa	25 kDa	Nuclear protein; RNA slicing inhibitor; regulates export of spliced mRNAs; inhibits NFκB activation and expression of IFN-α und IFN-β
PB1-F2	10 kDa, phosphorylated	–	–	Virulence factor; accumulates in the mitochondrial membrane; induces apoptosis of immunologically active cells (alveolar macrophages); regulates the RNA polymerase activity of PB1
PB1 N40	85 kDa	?	?	Amino-terminally truncated version of PB1, regulates RNA polymerase activity of PB1

vRNP viral ribonucleoprotein segments, *mRNA* messenger RNAs, *NFκB* nuclear factor κB, *IFN-α* interferon-α, *IFN-β* interferon-β

the amino terminus of the HA_2 fragment comes close to the endosomal membrane. The hydrophobic nature of the fusion-promoting amino acids allows the incorporation of the amino terminus of HA_2 into the endosomal membrane and induces the fusion of the two lipid bilayers. The fusogenic activity of the HA protein largely resembles that of the F protein of paramyxoviruses (▶ Sect. 15.3.3). Similarly, it must also be cleaved to become activated (▶ Fig. 15.6). The main difference between the two proteins is that, in paramyxoviruses, membrane fusion is induced by binding of the virus to the cell surface, whereby the merging process occurs between the cytoplasmic membrane and the viral envelope. Such a direct membrane fusion is not possible in orthomyxoviruses. On the other hand, acidification of the endosome is an absolute requirement for activating the fusogenic activity of the HA protein. The merging of endosomes with the viral envelope leads to the release of nucleoprotein complexes from the vesicles into the cytoplasm (Fig. 16.10).

Concurrently, acidification by the proton channel of the M2 protein disintegrates the interaction between NP proteins and M1 proteins. In such a way, the segmented nucleocapsids reach the cytoplasm, and are then transported to the nucleus, where transcription and replication then occur. Therefore, influenza viruses are an exception among the RNA viruses because they replicate in the nucleus. Similar processes are only observed with bornaviruses (see ▶ Sect. 15.2). Initially, the ribonucleoprotein complex serves as a template for the production of viral mRNA molecules. The pertinent promoters reside in the 3′-terminal sequences of the different segments, are situated in front of the transcription initiation sites and

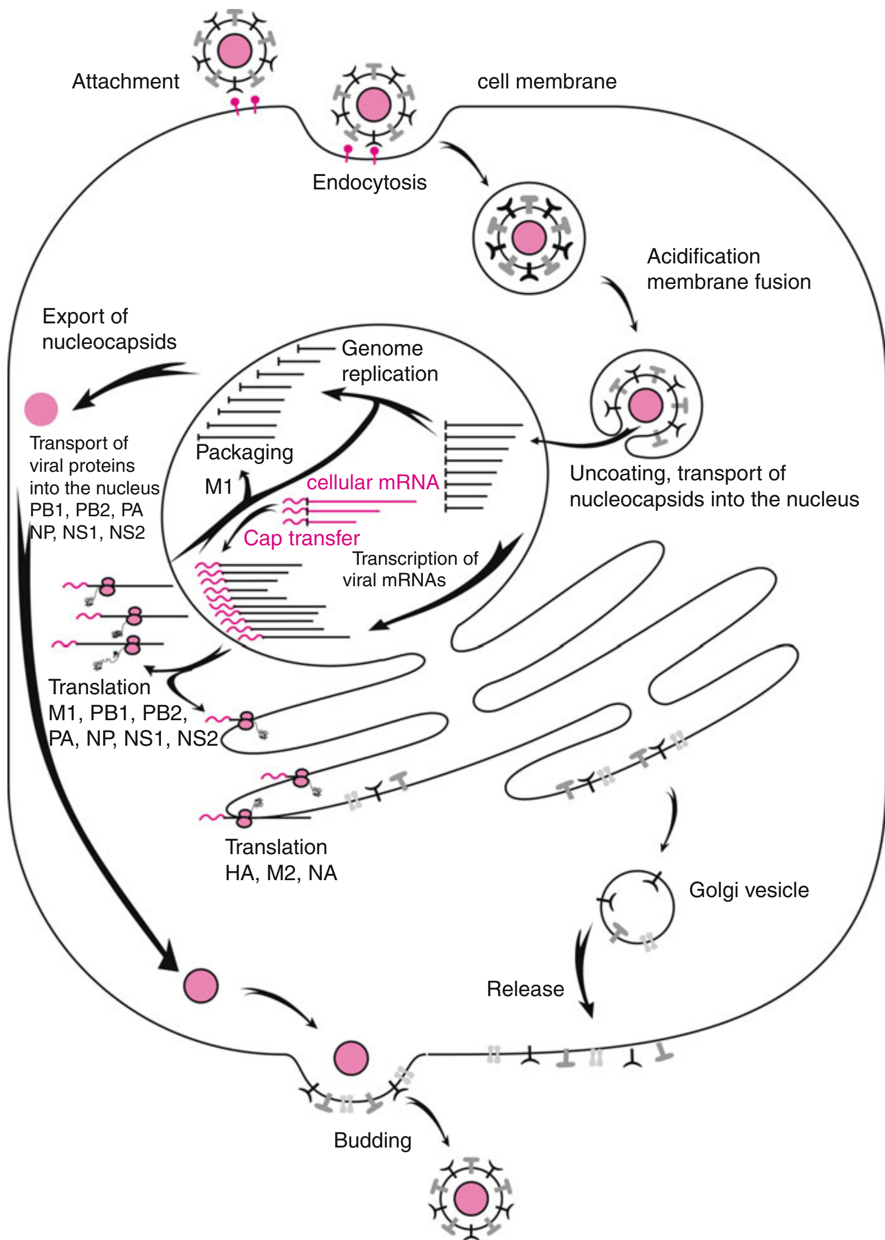


Fig. 16.10 Replication cycle of influenza viruses

form double-stranded structures along with the complementary 5′ termini. The viral-RNA-dependent RNA polymerase cannot autonomously initiate the synthesis of the mRNAs nor are proteins PB1, PB2 and PA able to methylate or modify

mRNA molecules with 5′-cap structures. Therefore, orthomyxoviruses have developed a mechanism to initiate transcription that allows them to use the 5′-cap structures from cellular mRNA molecules. For this purpose, PB2 proteins that are associated with the 3′ ends of genome segments as components of the nucleocapsids bind to the 5′-cap groups of cellular mRNA molecules and attach them to the 3′ ends of viral RNA segments. The 3′-terminal nucleotide is always a uridine. An adenine residue within the first ten to 13 nucleotides of cellular mRNAs hybridizes with the uridine. An inherent endonuclease activity of the PA protein cleaves the cellular mRNA after the adenine residue, producing a free 3′-OH end that serves as a primer for the subsequent polymerization step. This 5′-cap-stealing mechanism (also known as cap-snatching) has the additional advantage for the virus that it interrupts the cell-specific transcription and translation, switching host metabolism to the requirements of viral infection. Viral genes are almost exclusively transcribed and the corresponding proteins synthesized.

PB1, PB2 and PA, the three proteins of the polymerase complex, are involved in mRNA elongation. The transcription is terminated about 15–20 nucleotides before the 5′ end of the genome segments, namely in the transition region in which the single-stranded RNA sequences transform into the panhandle-like double-stranded regions. These are probably a physical barrier for the enzyme complex and slow down polymerization. A uridine-rich sequence, which is conserved in all mRNA species, resides in this region, and serves as a regulatory signal for polyadenylation of transcripts. Transcripts that are synthesized from the small RNA segment are partially spliced. The NS1 protein seems to be involved in this process as a splisosome cofactor. However, the main function of NS1 is to counteract antiviral defence mechanisms in infected cells (Sect. 16.3.3). Among other things, it specifically prevents polyadenylation of cellular transcripts. Viral transcripts are not affected because they are polyadenylated by the pertinent activity of the complex composed of PB1, PB2 and PA proteins.

During export of viral mRNAs from the nucleus, cellular proteins cooperate with viral components (NS2/NEP protein). Translation of the membrane-associated proteins (HA or HEF, NA and M2) occurs at the membrane of the rough endoplasmic reticulum. After translocation of the amino acid chains, a signalase removes cotranslationally the amino-terminal signal peptides from HA proteins. The modified proteins are transported to the cell surface via the Golgi apparatus and *trans*-Golgi network. They assemble into trimeric (HA, HEF) or tetrameric (NA, M2) complexes and become glycosylated. The HA and M2 proteins are also modified by attaching palmitic acid, and the HA protein can be subsequently cleaved by cellular proteases into subunits HA_1 and HA_2. The proton channel M2 protein regulates the pH level in the Golgi vesicles, inhibiting thereby the premature induction of the fusogenic activity of HA complexes in this subcellular compartment.

The proteins PB1, PB2, PA, NP, NS1, NS2/NEP and M1 possess nuclear transport signalling sequences. They develop their different activities during the succeeding replication of the genome segments. To switch from transcription to replication mode, free newly synthesized NP proteins must be enriched in the nucleus. It is thought that NP proteins interact with the components of the

polymerase complex, modifying it functionally and thus initiating replication. In addition, the initiation of replicative RNA synthesis seems to be primer-dependent. The dinucleotides pppApG are sufficient for initiation in vitro; they hybridize with the 3′ ends of genome segments, and provide the required free 3′-OH ends from where complete complementary strands are synthesized by using the PB1, PB2 and PA proteins under dissolution of the panhandle-like structures at end of the genomes. NP proteins attach to these antigenomes, which are used, in an analogous process, as templates for the synthesis of new viral negative-sense RNA molecules, which associate with the NP, PB1, PB2 and PA proteins into nucleocapsids (vRNPs). In the following step, the matrix protein M1 binds to them, and these complexes are transported from the nucleus to the cytoplasm, and thence to the sites where increased amounts of HA, NA and M2 proteins are embedded in the cell membrane. This process is mediated by the nuclear export function of NS2/NEP proteins. It is believed that nucleocapsids bind to the cytoplasmic sequences of HA_2 proteins by their associated M1 proteins, where the initial budding structures are formed. The membrane invaginates and surrounds the nucleocapsids, which are released from the surface by budding. Terminal sialic acid residues are removed from cellular and viral surface proteins by the neuraminidase activity of the NA protein. This prevents the released virus particles from interacting with each other or with membrane components of infection-damaged cells.

There is no mechanism ensuring that the appropriate combinations of eight or seven genome segments are packaged in each virus particle. Such a selection is difficult to imagine. Instead, about 11–13 nucleocapsid segments are packaged in each virus, regardless of their identity. Therefore, only a subset of newly produced virions are infectious. Hence, propagation of influenza viruses in cell culture results in just about 10 % of the offspring viruses being infectious.

16.3.5 Human and Animal Pathogenic Orthomyxoviruses

16.3.5.1 Influenza Viruses

Epidemiology and Transmission

Influenza viruses are primarily transmitted by virus-containing aerosols and droplets. They cause genuine flu or influenza, a severe acute disease of the respiratory tract involving the whole organism in humans. Whereas the courses of the diseases caused by influenza A viruses and influenza B viruses are largely similar, infections with influenza C virus are characterized by only mild symptoms in humans. In addition, influenza A virus infections in poultry as well as in swine and horses play an important role in veterinary medicine. Besides their importance as pathogens in these species, all influenza viruses have a general zoonotic potential. Transmission of influenza viruses from poultry or pigs to humans has been documented and identified in part as causes for the great influenza pandemics. In contrast to influenza A viruses, influenza B viruses and influenza C viruses are of minor importance. Influenza B viruses have

been described as pathogenic in humans, but they play no role in veterinary medicine, although the isolation of influenza B virus from wild seals with respiratory symptoms has recently been reported. Influenza C viruses are occasionally isolated from humans and pigs, but they do not cause serious illnesses.

Influenza A virus infection emerges at irregular intervals as a pandemic in the human population. In the last 120 years, a period that is epidemiologically assessable, there were six major influenza outbreaks, in 1890, 1900, 1918–1919, 1957, 1968 and 1977. Presumably, all had their origin in Southeast Asia and spread from there, e.g. on ships, to Europe and America. In the spring of 2009, the influenza A virus spread globally once again; in this case the pandemic originated in Mexico. The respective pathogens, known as subtypes, caused diseases of differing severity. Whereas deaths occur especially in elderly or debilitated people during seasonal epidemics, people aged between 20 and 40 years were greatly affected during the Spanish flu of 1918–1919. Influenza differs from other viral diseases also by the peculiarity that the acquired immunity, which is formed during infection, only effectively protects against subsequent infections with the same virus subtype. Hence, the same people can be infected several times with various “new” influenza virus subtypes despite the existence of neutralizing antibodies against the “old” virus variants.

The isolation of the first human influenza A virus by Wilson Smith, Christopher Andrews and Patrick Laidlaw in 1933 and the subsequent, partially retrospectively conducted seroepidemiological studies showed that influenza A viruses of successive pandemics differ in the recognition of their surface proteins HA and NA by antibodies (antigenicity). The influenza A virus that was isolated in 1933 was a variant of the virus which caused the pandemic of 1918–1919. It has been classified into the subtype H1N1. The influenza virus of the pandemic of 1957 caused the production of antibodies with different specificities against haemagglutinin and neuraminidase; therefore, it was denominated subtype H2N2. Antibodies from people who had been infected with the virus of 1957 could not neutralize the influenza A virus of 1918 and vice versa. The influenza A virus that caused the epidemic of 1968 had a haemagglutinin H3 different from that in the preceding subtypes; however, it exhibited the same reactivity with regard to neuraminidase. Even though the actual situation concerning the prevailing subtypes at the turn of the twentieth century cannot be conclusively elucidated, there is evidence that the same HA subtype, namely H3, emerged in the flu pandemic of 1900, but in connection with neuraminidase N8 or N2 (Table 16.10). Minor epidemics occurred in the period between the pandemics. The viruses apparently disappeared from the population, which had generated a protective immunity against the respective virus. Some time later, they emerged again in a slightly altered form and triggered new seasonal frequent epidemics generally during the winter months. The variability concerned especially the viral surface proteins HA and NA, which induce the production of neutralizing antibodies. Currently, the virus subtypes H1N1 (seasonal and new viruses) and H3N2 are circulating in the human population.

Table 16.10 Epidemiology of human influenza A viruses

Year		Virus subtype	Presumed original host	Exchanged segments
1890		H2N2?	?	No serological investigations possible
1900		H3N8/ H3N2?	?	No serological investigations possible
Development of pandemic influenza A viruses by forming reassortants				
1918 – 1919 Spanish flu		H1N1	Poultry	Eight segments: adaptation of an avian virus to swine and/or humans
1957 Asian flu	↓ Five segments conserved	H2N2	Poultry	Three segments (double reassortant): PB1, HA, NA
1968 Hong Kong flu	↓ Six segments conserved	H3N2	Poultry	Two segments (double reassortant): PB1, HA
2009 New flu (swine flu)	↓ One segment conserved	H1N1	Swine, poultry	Seven segments (quadruple reassortant): HA, NA, NP, M, NS, PB2, PA
Outbreaks of influenza A virus infections in humans without reassortant formation				
1977 Russian flu		H1N1	–	Identical to the H1N1 subtype of Spanish flu (release of a laboratory strain of Spanish flu?)
Since 1997		H5N1	Poultry	Human infection with an avian virus

The amino acid sequences of the different HA proteins of the various influenza virus subtypes differ considerably. The homology among the HA_1 subunits is only 35 %. The HA_2 subunits and the neuraminidases have a homology of approximately 53 and 56 %, respectively. The modifications of influenza A virus subtypes of successive pandemics are principally based on the exchange of genome segments between different virus types (reassortment). Thus, virus subtypes gain new RNA segments with the corresponding genes. Besides humans, influenza A viruses are widespread in various birds (gulls, wild and domestic ducks, geese, swans) and mammals (pigs, horses). Considering all hosts, there are to date 16 and nine different subtypes of HA and NA proteins, respectively. All 16 subtypes of HA proteins (H1 to H16) have been found in the different avian influenza viruses (Table 16.8). Subtypes H1, H2 and H3 have been identified in human pathogenic isolates, viruses with H3 and H7 versions infect horses, and at least four of the HA proteins (H1, H2, H3 and H9) have been isolated from infected swine. There are sequences that are specific for the surface proteins of avian, equine, porcine and human influenza viruses, and reflect the host specificity. These include the amino acid positions 226 and 228 of the HA protein, which are crucial for the choice of HA proteins to bind preferentially to sialic acid residues linked with a neighbouring galactose either by an α-(2,3) or an α-(2,6) glycosidic bond. Human pathogenic viruses prefer the α-(2,6) glycosidic bond.

Animal pathogenic subtypes behave differently: avian and equine influenza viruses have a high affinity for α-(2,3) glycosidically linked sialic acid residues, swine infectious subtypes are able to bind to both types. The different subtypes have among themselves a much greater variability than the proteins of the same subtype from different hosts. Apart from characteristic amino acid sequences and the cleavage pattern of haemagglutinins, especially the PB1 and PB2 proteins, as well as the NP, M1 and M2 proteins, mediate host specificity. They are conserved in the different influenza viruses of various species. For example, it has been described that mutations in the amino acid sequence of PB1 (aspartic acid to asparagine at position 701) and NP (asparagine to lysine at position 319) in a poultry influenza A virus (H7N7) facilitate the interaction with importin α_1 in mammalian cells. As a consequence, these mutant proteins can be efficiently transported into the nucleus, enabling an avian influenza A virus to adapt to mammalian cells.

Similar to humans, influenza A viruses cause infections of the respiratory tract in pigs and horses. These are associated with high morbidity, but low mortality in those animals. Viruses of the subtype H1N1 especially cause diseases in swine. Influenza viruses of subtype H3N2 are also infectious for pigs, but are only rarely associated with disease. Equine influenza (also known as Hoppegarten cough) is caused primarily by infections with subtypes H7N7 and H3N8 (Table 16.8). Whereas only H7N7 strains were isolated from sick horses until 1979, H3N8 viruses have regularly been found since that time. Recently, infections with H3N8 viruses have been described for racing dogs in the USA. Genetic analysis of these virus isolates suggests a direct transmission from infected horses to dogs.

Influenza viruses are globally spread among water birds (e.g. ducks, geese, gulls, loons). A permanent dissemination and mixing of virus subtypes is caused by bird migration, which cannot be controlled by humans. In contrast to humans and mammals, influenza viruses multiply in the intestinal tract of birds. Therefore, they are excreted with the faeces of birds into the water. The viruses remain infectious for days and weeks, particularly in cold waters. Infections are generally subclinical in wild birds. However, certain subtypes of influenza viruses cause, especially in domestic poultry, systemic infections and severe disease with high mortality (up to 100 %). The highly virulent strains of serotypes H5N1, H5N2 and H7N1 were known as fowl plague virus for a long time. A severe outbreak of avian influenza A virus subtype H5N1 occurred in Scotland in 1959; sine then, more than 20 outbreaks of this highly pathogenic influenza A virus (HPAI) have been described in poultry, and they sometimes lasted several years, caused immense economic damage (Table 16.11) and led to culling of several million animals.

Initially sporadic human infections with the highly virulent poultry influenza virus of subtype H5N1, the virus of avian influenza, were observed particularly in Southeast Asia some years ago. The first documented outbreak occurred in Hong Kong in May 1997: 18 of those viral infections were documented in humans within 8 months, six of which had a fatal outcome. In all cases, the virus was transmitted directly from infected birds to people just by close contact.

Since 2003, influenza A virus subtype H5N1 has epidemically spread from poultry markets into chicken and turkey farms in many countries of Asia.

Table 16.11 Worldwide outbreaks of avian influenza caused by highly pathogenic influenza A viruses in the last 50 years

Year	Continent/country/region	Influenza A virus subtype	Poultry affected
1959	Europe/UK/Scotland	H5N1	Chickens
1963	Europe/UK/Scotland	H7N3	Turkeys
1966	North America/Canada/Ontario	H5N3	Turkeys
1976	Australia/Victoria	H7N7	Chickens
1979	Europe/UK/England	H7N7	Turkeys
1983–1985	North America/USA/Pennsylvania	H5N2	Chickens, turkeys (17 million animals culled, cost US$ 312 million)
1983	Europe/Ireland	H5N8	Turkeys
1985	Australia/Victoria	H7N7	Chickens
1991	Europe/UK/England	H5N1	Turkeys
1992	Australia/Victoria	H7N3	Chickens
1994	Australia/Queensland	H5N2	Chickens
1994–1995	North America/Mexico	H5N2	Chickens (two million vaccinations administered)
1994	Asia/Pakistan	H7N3	Chickens
1994	Australia/New South Wales	H7N4	Chickens
1997	Asia/Hong Kong	H5N1	Chickens (all fowl killed)
1997	Europe/Italy	H5N2	Chickens
1999–2000	Europe/Italy	H7N1	Turkeys (14 million animals killed, cost US$ 620 million)
2002	Asia/Hong Kong	H5N1	Chickens
2002	South America/Chile	H7N3	Chickens
2003	Europe/Netherlands/Belgium/Germany	H7N7	Chickens (30 million animals culled)
Since 2003	Asia/Europe	H5N1	Chickens, ducks, turkeys

Many wild birds have also been infected during that epidemic. Owing to bird migration, the virus has been further disseminated into the countries of Central and Western Asia. This resulted in the culling of large numbers of livestock, including billions of poultry, and the imposition of import bans for birds, poultry and poultry products from the affected countries. In the autumn of 2005, migratory birds carried the H5N1 virus to the countries of eastern and southern Europe. In central Europe (Germany and Austria), the first infected birds were found in February 2006 and dead predators (raptorial birds, stone martens and cats) were discovered. During the past few years, H5N1 viruses have repeatedly been detected also in domestic poultry in Europe. In all cases, farms that kept waterfowl (ducks, geese) were the sites involved. In addition to several European countries, poultry was also infected on the African continent, especially in Egypt. Transmissions to humans have occurred as well, and have been associated with several deaths.

According to the World Health Organization, a total of 608 infections had been described in humans as a consequence of the H5N1 epidemic in poultry as of August 2012, and these led to 359 deaths (a mortality rate of 59 %). The affected regions include Southeast Asia (Indonesia, Vietnam, Thailand, Cambodia, Bangladesh, Laos, Myanmar and China), the Middle East, Central Asia and Africa (Turkey, Egypt, Azerbaijan, Iraq, Djibouti, Nigeria and Pakistan). Even in these cases, the affected individuals were people who frequently had intensive contact with chickens, turkeys or ducks, e.g. farmers, poultry farmers and traders. Transmissions of H5N1 virus from person to person in a few cases have been estimated to be possible (e.g. in two members of a family in Thailand in 2004), or even probable, as in the case of eight members of a family in Indonesia.

Genetics and Epidemiology

Influenza viruses have the ability to recombine individual regions of their genome, and thus to transgress host specificity. In this manner, they obtain proteins that can confer them with completely new serological properties. The exchange of individual genome segments is known as antigenic shift. A prerequisite for this process is the simultaneous coexistence of virus strains with different HA and NA subtypes in an organism and in the same cells. These conditions appear to be met particularly often in Southeast Asia, especially China, because most influenza pandemics originated from there. In such countries, mainly in rural regions, humans live very closely with ducks and pigs. The following scenario is conceivable. In contrast to mammals, influenza A viruses reproduce in the epithelial cells of the avian intestine, and thus birds excrete them in large quantities in the faeces without becoming ill. In their haemagglutinin subtypes (e.g., H5, H7), the viruses exhibit a specificity for α-(2,3) glycosidically bound *N*-acetylneuraminic acid residues, and in highly pathogenic strains multiple basic amino acids in front of the cleavage site. As this sequence is well recognized by intracellular proteases (Sect. 16.3.3), these pathogenic viruses are infectious even on their release from infected cells and are able to spread among birds. Pigs can be infected with some avian influenza virus types. If pigs drink contaminated water, an infection will follow in which the virus proliferates and adapts to this new host by mutations (antigenic drift), thus gaining the ability to specifically bind to α-(2,6) glycosidically bound *N*-acetylneuraminic acid residues and to spread within the swine population, resulting in an infection of the respiratory tract. In the course of this, particularly viral enzymes have to be adapted and optimized to changed temperature conditions. Normally, they are active at 37–38 °C in systemic avian infections, whereas a temperature of only approximately 33 °C prevails in the respiratory tract of mammals.

Swine are also susceptible to infections with human influenza virus types, so a pig can be productively infected with influenza viruses of two different hosts at the same time. If such an infection occurs in the same cell, virus reassortants can arise during morphogenesis at the end of the replication cycle, and they contain a mixture of the different genome segments. In this manner, such a “successful” subtype can sometimes emerge, which is then transmitted from swine to humans, triggering a productive infection in them, and resulting in its further dissemination

among the human population. Subsequently, the influenza virus reassortant is capable of inducing a new influenza virus pandemic because the affected population does not initially possess an immune protection.

It is unclear why only three of the 16 different naturally occurring HA subtypes of influenza A viruses have been found in influenza pandemics among the human population. It is assumed that reassortants arise in other cases for which the human organism is not very susceptible. Apart from these drastic changes that are generated by antigenic shift and lead to new pandemics, surface proteins of influenza viruses also undergo significant alterations during a pandemic and thereafter. These variants especially affect the regions of HA and NA proteins that are recognized by neutralizing antibodies. In the HA protein, these regions are particularly localized in amino acid sequences that surround the receptor binding site, which is hidden in a cavity (Fig. 16.7b, c). They are based on point mutations in the corresponding gene sequences. The RNA-dependent RNA polymerase of influenza viruses has a high error rate when incorporating complementary nucleotides during the replication process and synthesizes incorrect nucleotides into nascent RNA strands with a frequency of approximately 10^{-3}, and these are randomly distributed throughout the genome. The antibodies that are produced during infection exert a selection pressure. Thereby, mutant viruses are selected during an epidemic in which the surface-exposed regions of the proteins are altered, which induces the generation of a neutralizing immune response. Hence, the virus can persist in a population for long periods. This mechanism of changing antigenic protein regions (epitopes) by mutations is termed "antigenic drift". Whereas antigenic mutations occur frequently in human pathogenic viruses, the influenza viruses from horses are relatively stable and are barely changed by antigenic drift.

Emergence of Human Influenza Virus Strains

The virus of the Asian flu of 1957 differed from that of the pandemic of 1918–1919 by a total of three genome segments: PB1, HA and NA. Comparative analyses revealed that these segments originated from a duck influenza A virus. The remaining corresponded to those of the Spanish influenza virus, which were then taken over into the new reassortant (Table 16.10). Two other segments were exchanged in the Hong Kong influenza virus of 1968, the segments encoding haemagglutinin HA and the PB1 protein, which differed from the Asian flu virus. Even in this case, it seemed that they originated from a duck influenza A virus. The NA segment was not exchanged in this reassortant. The H3N2 virus of Hong Kong flu still circulates along with the H1N1 viruses of Russian flu and, meanwhile, of the new influenza in the human population today, and can – with changes caused by antigenic drift – still be isolated from influenza patients today.

Two hypotheses have been proposed to explain the emergence of the virus of the Spanish flu of 1918. One hypothesis postulates that it is not a reassortant, but is a duck influenza virus that was transmitted to swine in the USA at the beginning of the twentieth century, which retaining all eight segments successfully adapted to the new host by continuous changes accumulating successive point mutations.

Subsequently, this virus seems to have circulated between humans and pigs until a mutant with a high pathogenic potential emerged, which, emanating from Fort Dix in the USA, was transmitted to Europe by American soldiers at the end of World War II, where it caused the severe and devastating Spanish flu pandemic (1918–1919). The other hypothesis presumes the emergence of this virus type in Guangdong province in southern China. A H1N1 virus subtype had apparently been circulating in the Chinese population since 1907 and adapted to humans. Accordingly, the H1N1 virus was been transmitted to Europe by Chinese workers who were employed by the US military to build camps in France and Spain. Victims have been exhumed who died from influenza virus infection in 1918, and were buried in Alaska and Spitsbergen in regions with permafrost soil. The viral genome segments were amplified and sequenced from biopsy material of the corpses by using PCR. Formalin-fixed tissue samples which originated from organs of patients who died of Spanish flu in 1918 were also included. As expected, sequence analysis of the HA gene identified the H1 subtype with amino acid variations, which suggests adaptation of the virus to mammals. However, the adaptation to humans as a host by antigenic drift was not well advanced at the time of death of the patients. The H1N1 virus subtype and its variants (modified by antigenic drift) could be detected in the population until 1956. Then it apparently disappeared for 21 years, until it re-emerged as the pathogen of Russian flu in northern China in 1977. This virus variant corresponded exactly to an isolate that had been obtained from a patient in 1950. The origin of the virus of 1977 is still mysterious. The total identity with the virus of 1950 (a variation by antigenic drift was not observed) strongly suggests that this virus survived in a "frozen" state from 1950 to 1977. There are speculations that it might be an accidentally released laboratory strain from a former Soviet institute. On the other hand, in addition to antigenic shift and drift, a third mechanism seems to be responsible for the occurrence of pandemics: It has been observed that human influenza A virus subtypes are able to re-emerge almost unchanged after prolonged time periods, causing severe pandemics. This generally happens after a period of about 60–68 years ("68 rule") and is probably determined by the gradual disappearance of specific immunity in the population. Thus, the pandemics of 1900 and 1968 as well as those of 1918 and 1977 could have been caused by H3N2 and H1N1, respectively.

Since spring 2009, a new pandemic has developed in humans which is caused by influenza A virus of the subtype H1N1. This novel H1N1 pandemic originated in Mexico, and hence has been referred to as "Mexican flu", but is now termed "new flu" or "swine flu" – see the box entitled "The New flu (Mexican Flu, Swine Flu)". Phylogenetic analyses showed that it is a reassortant that has emerged from human, porcine and avian influenza A viruses. Segment 2 remained from the H3N2 virus of the Hong Kong flu that was also circulating up to the end of 2009. It directs the synthesis of the PB1 protein (Table 16.10). Segments 1 and 3 (coding for PB2 and PA, respectively) are specific to avian influenza A viruses, and the remaining segments have been derived from swine influenza A virus isolates. Presumably, a mixture of the viral genome segments occurred in swine on the American continent, and respective reassortants were isolated from pigs in Argentina and

Canada before 2009. Subsequently, the transmission to humans occurred in Central America in April 2009. Since then, this novel H1N1 influenza A virus has spread throughout all continents and was declared a new flu pandemic by the World Health Organization in June 2009. The symptoms associated with these new H1N1 infections have been relatively mild so far; nevertheless, more than 18,000 deaths connected to the new H1N1 flu were recorded by the World Health Organization during the pandemic that lasted until August 2010. In Hong Kong in 1997, 18 people were infected with an influenza virus which had not been previously described as a human pathogen. It was a highly pathogenic avian influenza virus of the subtype H5N1. Thirty per cent of infected individuals died, but the infection did not spread in the population. As previously mentioned, the reason could be the specificity of the avian virus for α-(2,3) glycosidically bound sialic acid. To prevent the danger of a new pandemic, it was decided to kill all poultry in Hong Kong. Despite this measure, influenza A viruses of subtype H5N1 have spread in poultry in Southeast Asia and later via eastern Europe to central Europe and Africa since 2003.

The mechanisms of genetic rearrangement and the resulting emergence of new influenza virus subtypes described here have been predominantly found in influenza A viruses and to a lesser extent in influenza C viruses. To what extent they contribute to the emergence of genotypes with different pathogenicity in influenza C viruses is unclear. The direct reassortment between two human pathogenic influenza C virus strains in infected individuals has been described in individual cases. Influenza B viruses, which hitherto have only be detected in humans, do not generate reassortants.

The Nomenclature System for Influenza Virus Subtypes

The nomenclature of the various human and animal pathogenic influenza virus strains uses the following scheme. In addition to the virus type (influenza A virus, influenza B virus or influenza C virus), the following data are indicated: the host from which the pathogen was isolated, the geographical location of isolation, the number of the isolate, the year and the subtypes of HA and NA proteins. For example, the denomination of one of the first isolated porcine influenza A viruses is as follows: A/Swine/Iowa/15/30(H1N1). It was isolated as the 15th virus subtype H1N1 in Iowa in 1930. If an isolate has been obtained from humans, the host is not indicated. A/HK/1/68(H3N2) was, accordingly, the first virus strain of subtype H3N2 that was detected in humans in Hong Kong in 1968. The reference to the HA or NA subtypes is omitted in influenza B viruses and influenza C viruses.

The New Flu (Mexican Flu, Swine Flu)

A suspicious human influenza disease was reported in Mexico and the USA at the end of April 2009, and whose consequences included deaths in Mexico.

Infection was ascertained to be caused by influenza A virus subtype H1N1, which exhibits similarities with swine influenza viruses. However, further investigations of the virus revealed that it is a novel pathogen: its genome displays RNA segments of porcine influenza A viruses, containing a mixture of Eurasian and American swine virus variants. Owing to the worldwide dissemination of the virus in humans within a few months, the World Health Organization proclaimed the highest level of pandemic alert on 11 June 2009, which lasted until 10 August 2010. Officially, the novel pandemic virus was named new influenza virus. However, this virus had hitherto been named differently. Given the fact that the first cases were reported from Mexico, the disease was initially designated "Mexican flu". Because the Mexican government protested, fearing loss of trade and tourism, it is now officially called pandemic influenza A (H1N1) 2009. However, the daily press mainly uses the term "swine flu", which is very imprecise. Furthermore, the initially infections in Mexico and the USA were not directly linked to contact with infected pigs according to information from the affected countries. The virus was probably transmitted to humans some time ago, rapidly disseminating among the human population. Since this new influenza (swine flu) has mainly manifested itself with mild symptoms so far, infections may have remained undetected at the inception of the outbreak.

In most countries, federal and state governments have established pandemic plans and can introduce appropriate measures and make recommendations So far, therapy with neuraminidase inhibitors has shown good effect, especially in severe individual cases of patients with chronic heart and circulatory diseases. The first vaccines that specifically protect against infections with this pathogen were approved for humans in October 2009. Since 2010, antigen preparations of the new H1N1 influenza have been introduced into influenza vaccinations. The real benefit of a vaccination is controversially discussed.

The Current Handling of Avian Flu in Central Europe

Since 1997, more than 200 influenza A virus infections with subtype H5N1 have been observed in association with an unusually high mortality in humans during the avian influenza epidemic in Southeast Asia. The highly human-virulent H5N1 virus has stirred up concern, especially in western industrialized countries, that it may further mutate via antigenic drift, thus progressively adapting to human hosts. The risk that the "avian flu" virus might be the basis for a new highly virulent pandemic virus resulted in an import ban for live and dead poultry from endemic countries and compulsory livestock housing for poultry during bird migration in the autumn. As part of pandemic planning by the governments of the countries, large amounts

of neuraminidase inhibitors were stored, sufficient to treat at least 10 % of the population. They should be used in emergency cases to control the infection and stop its dissemination. These antiviral drugs are also kept in a large number of private households for emergencies.

Given the fact that the drug is suitable only for short-term prophylaxis during a pandemic, its effect is only really useful in treating the early stage of infection, and its use requires the positive diagnostic detection of influenza infection. This approach needs to be discussed very critically. The incorrect use of neuraminidase inhibitors entails a high probability that resistant mutants might emerge and spread by the resulting selection pressure, similar to other treatable viral infections. Indeed, H5N1 isolates and other influenza virus variants have been described from autumn 2005 whose infections could not be influenced with neuraminidase inhibitors. Therefore, it is to be feared that the indiscriminate use without medical indication may lead to this antiviral chemotherapy becoming ineffective in the long term, and it not being possible to use it in emergency cases.

Clinical Features

The genuine flu is an acute respiratory illness that is associated with very severe general symptoms, and thereby differs significantly from the common cold. Depending on the age of the affected person and possibly underlying illnesses, it can lead to severe complications, which – albeit rarely – can lead to death within a few days. The incubation period is short and lasts between 1 and 5 days. Particularly in adults, the infection can be inapparent, i.e. without symptoms. The main symptom is a sudden onset of high temperature (up to 41 °C) and pronounced malaise. Further symptoms are sore throat, non-productive cough and severe headache and joint pain. Symptoms usually last 3–7 days. In children, the symptoms are similar (especially high temperature, sore throat and cough). However, these can be accompanied by a viral ear infection, severe abdominal pain, diarrhoea and vomiting, as well as pseudo-croup and bronchiolitis in young children. Risk groups are people with existing chronic diseases, who may suffer from severe exacerbations of the underlying disease, e.g. asthma, chronic bronchitis, cystic fibrosis, diabetes mellitus and heart and circulatory diseases. Resulting complications include acute bronchitis, pseudo-croup (infants), primary viral pneumonia (lung infection), myocarditis and pericarditis, as well as, rarely, neurological, muscle and renal problems. The primary viral interstitial pneumonia (often haemorrhagic) also occurs frequently in elderly patients (over 65 years) and can last up to 2 weeks. In addition, pneumonia can develop secondarily by bacterial superinfection, including *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Haemophilus influenzae*, the latter gave the disease its name. In immunocompromised individuals, the virus is excreted for considerably longer, resulting in a high risk of nosocomial transmission. That means that other patients in hospitals and medical practices, for example, may be infected. Another risk group is pregnant

women, who can not only transmit the virus to the fetus, but who also have an increased risk of death. Moreover, severe diseases are also observed in people without underlying illnesses after primary contact with a pandemic virus.

Influenza B virus infections are very similar. However, fatal diseases with primary viral pneumonia are rare; contrarily, secondary bacterial pneumonia is frequent. In children, influenza viruses cause Reye syndrome, which is associated with massive brain and liver damage. However, it only occurs when aspirin is used for the treatment of influenza.

Human infections with highly pathogenic variants of the avian H5N1 virus develop differently. These begin with the common flu symptoms (see above), but then develop into a severe respiratory disease, such as viral pneumonia or acute respiratory distress syndrome, and frequently lead to life-threatening complications culminating in multiorgan failure. The severe systemic diseases may affect the liver, the digestive tract, the bone marrow or the kidneys. Bacterial superinfections do not play a role in H5N1 infections. The mortality is significantly above 50 %.

Porcine and equine influenza resembles that of humans. It manifests itself as a highly acute febrile disease that is associated with respiratory symptoms and rapidly spreads within affected livestock. The morbidity is high, but the mortality is very low.

In fowls, the clinical picture depends on the virus type and the poultry or bird species. Turkeys and chickens are extremely susceptible to the highly pathogenic H5 and H7 viruses, and develop an acute, dramatic, systemic disease pattern with high lethality. Other species are also susceptible, especially swans and various grebes are regularly infected. In the classic waterfowl (ducks, geese), the infection is generally mild. In addition to the highly pathogenic influenza viruses, there are many low-pathogenicity strains that induce a mild disease pattern. Since these viruses may generate in principle highly pathogenic avian influenza viruses by mutating the cleavage site of the HA_0 protein at any time, these infections are also combated in Europe.

The Host Tropism of Influenza Viruses is Based on Species- – and Cell-Specific Differences in the Glycosylation Pattern

Species-specific glycosylation patterns of cellular proteins are responsible for the differences in the specificity of receptor binding of different influenza virus subtypes. Therefore, mucins (▶ Sect. 15.3.5), highly glycosylated proteins in the protective mucus of the human lung epithelium, contain principally sialic acids as terminal sugar residues linked by an α-(2,3) glycosidic bond. If influenza viruses have HA subtypes with this specificity, then they bind to the sugar modifications in the mucus layer and, therefore, do not reach their host cells, the epithelial cells of the lung, where α-(2,6) glycosidic bonds predominate. Consequently, a selection for viruses with HA types (H1 to H3) occurs in the human pathogenic subtypes, which preferentially attach to sialic acids linked by α-(2,6) glycosidic bonds. In birds and horses, the situation is the converse: α-(2,6) glycosidically bound sialic acid is very frequent in the mucous regions of the avian intestine. This is also the prevailing protein

modification in the respiratory tract of horses, whereas α-(2,6) glycosidic bonds predominate on the cell surfaces. Both glycosylation types are found in swine; thus, pigs assume a virtually intermediate position between humans and birds or horses. However, unlike human pathogenic virus subtypes, the selection pressure acts differently on avian viruses, whose HA proteins recognize α-(2,6)-linked sialic acids with high affinity. Probably, this was the reason why the H5N1 influenza virus subtype, which has been found in Hong Kong and Asian countries since 1997, could not spread in the human population. The virus was transmitted directly from birds to humans without previous adaptation to mammal-specific traits. It has the H5 subtype, and thus the receptor specificity for sialic acids linked by α-(2,3) glycosidic bonds. Just 18 people have been infected with this virus, which originally emerged as an avian influenza virus in poultry in Hong Kong, and has infected ducks, turkeys and chickens. The H5 type has a polybasic sequence in front of the cleavage site, which is typical for avian influenza viruses and facilitates systemic infections (Sect. 16.1.3).

Pathogenesis

In humans and mammals, influenza viruses enter the organism by droplet infection, and infect cells by binding of the HA protein to terminal sialic acid residues on the epithelial cells of the respiratory tract. The linkage of sialic acid by an α-(2,6) glycosidic bond is predominant in this organ region in humans. Whereas α-(2,3) glycosidic bonds do not exist in the human upper respiratory tract, they are present in lower lung regions. These biochemical differences in the human respiratory tract are one reason why transmissions of avian influenza viruses (H5, H7) to humans very rarely lead to infections. The preference for α-(2,6) glycosidic bonds does not allow an efficient human-to-human transmission of avian virus types. However, there is a danger that the virus may change by mutations in the haemagglutinin H5 coding segment, thus adapting to humans as a host organism. If the hitherto high human pathogenicity with a death rate of more than 50 % is maintained in such an adaptation process, then there will be a risk of the emergence of a new severe pandemic caused by influenza viruses (H5N1) in the human population.

The virus spreads from the upper respiratory tract into the lower airways; viraemic phases, in which the virus is present in the blood, are rare in human infections with viruses of subtypes H1, H2 and H3. Cell damage can be observed in all ciliated epithelia and mucus-producing layers of the respiratory tract, and a thickened, hyalinized basal layer is exposed in association with submucous, oedematous swelling. Infiltrates of neutrophils and mononuclear cells are found in these regions. If a primary, interstitial pneumonia develops, then the virus is transmitted to the cells of the lung parenchyma. There is severe swelling of the alveolar walls, whose epithelium is frequently completely removed by cell destruction. As a result of necrosis, bleeding and fissures occur in the walls of alveoli and bronchioles. Particularly mononuclear cells migrate into these

regions. By means of the NS1 protein, influenza viruses possess the ability to largely neutralize immune responses mediated by interferon-α. It is also known that the PB1-F2 protein can preferentially induce apoptosis in alveolar macrophages and other immunologically active cells. However, it has not yet been finally resolved whether these properties facilitate bacterial superinfections, thus influencing the pathogenesis of influenza virus infection in vivo. In contrast to the ways of evading the immune response, the viruses also have mechanisms to induce it in a particular way: recent work has demonstrated that the single-stranded RNA segments of influenza viruses act as immunological recognition molecules (pathogen associated molecular patterns) and as ligands for Toll-like receptors 7 and 8. These effects are signals for the nonspecific immune system that a viral infection is occurring in the organism.

In bacterial pulmonary inflammation, proteases from *Staphylococcus aureus* and other pathogens promote the cleavage of the HA_0 protein, thus increasing the infectivity of the influenza virus; therefore bacterial co-infections can synergistically contribute to the development of pneumonia. This also explains the good therapeutic effect of antibiotics in the treatment of influenza.

Innate Defence Against Influenza Viruses and Other Negative-Sense RNA Viruses

The host response to influenza viruses is based on an intracellular defence protein whose synthesis is induced early during infection by interferon-α and interferon-β, in addition to the induction of 2′-5′-oligoadenylate and protein kinase R synthesis (▶ Chap. 8). The first member of these so-called Mx proteins ("Mx" for "myxovirus resistance") was originally found in mice that showed a high level of interferon-mediated resistance to experimental influenza virus infection. Subsequently, similar proteins have also been identified in humans and other vertebrates. Mx proteins belong to the superfamily of large GTPases (80–100 kDa). These are probably mechanochemical enzymes that, like dynamin, are involved in intracellular transport processes. The human MxA protein has an antiviral activity against influenza viruses, thogotoviruses, a number of paramyxoviruses (e.g. measles virus) and members of bunyaviruses (hantaviruses, Rift Valley fever virus, La Crosse virus; Sect. 16.2). The exact mode of action is still not completely understood. The MxA GTPase seems to recognize the nucleocapsid or nucleocapsid-like structures of the viruses mentioned, rendering them harmless by impairing their intracellular transport and depositing them in complexes.

Immune Response and Diagnosis

IgM, IgA and IgG antibodies are produced during the course of infection. Influenza viruses induce a lasting immune response, which confers a relatively efficient protection against re-infections with the same virus subtype. The neutralizing IgG and IgA antibodies are directed against HA proteins, especially against five

epitopes that are located on the surface of the protein near the receptor binding site. Antibodies against neuraminidase can also restrict the spread of infection in the organism. The fact that during the recurrence of influenza virus H1N1 in the Russian flu of 1977 older people were largely protected who had first been infected with this virus type during the pandemic of 1918–1919 indicates the effectivity of the long-lasting, virus subtype-specific protection.

The elimination of the virus from the organism is done particularly by cytotoxic T cells, which recognize oligopeptides of the NP protein in combination with MHC class I proteins. Since the NP protein is relatively highly conserved in all influenza A viruses, after the initial infection there are $CD8^+$ memory cells which can be rapidly reactivated in subsequent infections, thus contributing to rapid elimination of virus-infected cells. In addition to the NP protein, T-cell epitopes are also situated in the sequences of the M, NS, and polymerase proteins. Virus-specific $CD4^+$ T-helper cells are not involved in direct elimination of infected cells, but are important for induction and enhancement of the humoral immune response and antibody production. In addition, they secrete interferons and other cytokines, as well as activated macrophages and natural killer cells, which are already present early during the infection. These promote the migration of additional T cells and macrophages into infected tissue; interferon-γ is probably responsible for the increased synthesis of MHC class I proteins, which in complex with viral peptides on the cell surface stimulates the recognition by cytotoxic T cells. Influenza viruses also induce the synthesis of interferon-α and interferon-β, but this process is antagonized by the activity of the viral NS1 protein. It inhibits the induction of interferon-α and interferon-β gene expression by binding to the double-stranded RNA segments that arise during replication.

The diagnosis of acute infections in humans and animals is performed by determining IgM and IgA antibodies in the serum, by isolation of the virus from pharyngeal lavage and nasal swab samples, and by detecting viral proteins by several rapid tests. Another possibility is the detection of viral nucleic acid by RT-PCR in materials from the respiratory tract. Care must be taken in the PCR test as all subtypes of influenza A viruses, including the H5N1 subtype, and influenza B viruses must be detectable. IgG antibodies evidence of a past infection; IgA_1 antibodies protect against reinfections, especially when secreted in the nasal mucosa. Influenza viruses can be cultivated in a number of primary and immortalized kidney cell lines (Madin–Darby bovine kidney or Madin–Darby canine kidney cells) from different hosts (dogs, monkeys, calves, hamsters), in which they are able to develop a cytopathic effect. Infectious viruses can be produced only if the cells synthesize sufficient amounts of trypsin-like enzymes that can cleave the HA_0 protein. Apart from those systems, cultivation of viruses is also possible in embryonated chicken eggs. This method is still used for vaccine production.

Therapy and Prophylaxis

Amantadine and rimantadine can be applied therapeutically for prophylaxis and treatment of influenza A virus infections, whereas influenza B viruses and influenza C viruses are not sensitive to these compounds. Both drugs are tricyclic, primary

amines (▶ Fig. 9.1) which inhibit viral replication at the level of particle penetration and uncoating of the nucleocapsid into the cytoplasm. The target is the A/M2 protein, which forms proton channels in the viral envelope. Mutations in the sequences encoding the hydrophobic transmembrane domain appear rapidly during treatment. Especially, amino acid substitutions at position 26, 27, 30, 31 or 34 lead to resistant virus strains. Therefore, the use of amantadine is restricted to high-risk groups (such as people in nursing homes). In addition, inhibitors of viral neuraminidase (zanamivir, oseltamivir) are currently in use. These are applied within 48 h after an infection (if possible a proven one) with influenza viruses in order to contain viral dissemination in the organism in an early stage of infection (▶ Chap. 9). Hence, neuraminidase inhibitors do not prevent infections, but attenuate them; they are not aimed at long-term, prophylactic applications. An exception that justifies the administration of neuraminidase inhibitors for a long time is their use in outbreak-control vaccinations in connection with possible pandemics; in such cases, it should be attempted to prevent transmissions to people who are primary and potential contacts. Resistant virus mutants have been isolated from patients treated with neuraminidase inhibitors who were predominantly immunosuppressed transplant recipients. During the winter epidemic of 2008–2009, it became apparent that the vast majority of influenza virus isolates had become resistant to neuraminidase inhibitors. The mutations responsible cause amino acid substitutions in the neuraminidase active centre and affect preferentially positions 119, 274 and 292 (E119V, E119D, H274Y, R292K). Mutants of subtype H5N1, whose neuraminidases have become resistant to these inhibitors by replacing the amino acid histidine with tyrosine at position 274, emerged for the first time in autumn 2005. Furthermore, it has been shown that mutations in the gene encoding the HA_1 protein also contribute to the emergence of virus variants resistant against neuraminidase inhibitors. Substitutions, for instance, at amino acid position 226 (valine with isoleucine) result in haemagglutinin binding with reduced affinity to the receptor, thus rendering dispensable the neuraminidase function.

Vaccines against infections with influenza A viruses and influenza B viruses are available. These are based on inactivated viruses which were previously propagated in embryonated chicken eggs or cell cultures. Owing to the high variability of influenza viruses and the low immunogenicity of the vaccines, these have to be adapted to the currently circulating virus subtypes or subtype variants every year. Accordingly, guidelines for the composition of the new seasonal vaccines based on the recommendations of the World Health Organization are established every year by the respective national health authorities. The vaccine against seasonal influenza for 2012–2013 that is used in the countries of the northern hemisphere contains the strain composition of currently circulating variants of influenza A viruses H1N1 (A/California/7/2009) and H3N2 (A/Victoria/361/2011) and influenza B virus (B/Wisconsin/1/2010). The composition for the countries in the southern hemisphere is adapted on the basis of the virus subtypes that are present in these regions. There, the seasonal vaccine for 2012 was composed of the virus variants A/California/7/2009/H1N1, A/Perth/16/2009/H3N2 and B/Brisbane/60/2008. In the case of a new pandemic outbreak, the vaccine will be adapted to the new virus subtype as soon as possible.

During the last H1N1 pandemic it was shown that despite the molecular biological methods which allowed the rapid and efficient characterization of the novel pandemic virus, technical and logistical problems impede the provision of a new vaccine for billions of people, and are difficult to manage.

Besides these inactivated vaccines, a live, attenuated, cold-adapted vaccine is in use in some countries (e.g. the USA), but has, however, not been approved for use in Europe yet. This vaccine can be applied in the respiratory tract, generating a local immunity.

Vaccines are available against animal influenza viruses which can theoretically be used to protect poultry. However, a fundamental vaccination of poultry has not been implemented in countries of the European Union. Exceptions are zoo animals and special purposes. However, these exceptions are discussed very controversially, as H5N1 infections still occur. Immunizations with inactivated bivalent vaccines (H1N1 and H3N2) are routinely performed in swine. Furthermore, there are also inactivated vaccines against equine flu. These comprise inactivated virus subtypes H7N7 and H3N8. The duration of immunity is short, and booster vaccinations are required in semiannual or annual intervals.

Controlling H5N1 Infectious Animal Diseases

Animal disease control is harmonized within the member states of the European Union. That means that specific animal diseases are combated with the same measures in all member states. In the case of avian flu caused by highly pathogenic avian influenza virus subtypes H5 and H7, the European Union has a non-vaccination policy and fundamentally prohibits vaccination of poultry. Exceptions are possible in principle, but they result in rigorous trade restrictions. The control of an outbreak of avian flu in a livestock is achieved by killing all birds in the stock concerned and in poultry flocks within a radius of 3 km (restricted area). In the case of detection of H5N1 influenza viruses in wild birds, as a rule, a restricted area is established in which the poultry must be housed and cats and dogs must not run free.

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The members of the families *Reoviridae* and *Birnaviridae* possess a double-stranded, segmented RNA genome. Similar molecular properties are found in plant viruses, such as partitiviruses. The genome of birnaviruses, which are not pathogenic for humans, has two genome segments. In contrast, the genomes of reoviruses encompass 9–12 RNA segments. The viruses have a worldwide distribution and cause, to some extent, severe diseases in humans and animals.

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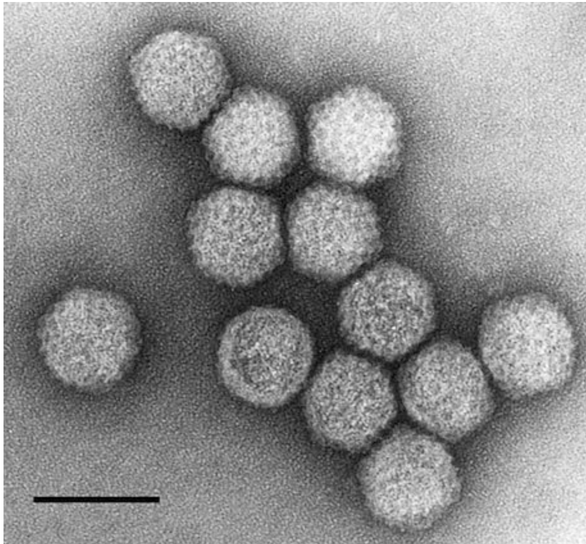
17.1 Birnaviruses



Birnaviruses have a bisegmented, double-stranded RNA genome. The name of the virus family (from "bi-RNA") indicates that molecular characteristic. Birnaviruses have been described as pathogens in birds, fish and crabs. Birnavirus infections of mammals or humans have not been described.

17.1.1 Classification and Characteristic Prototypes

The birnavirus family is subdivided into three genera (Table 17.1), but only members of the genera *Avibirnavirus* and *Aquabirnavirus* have veterinary relevance. Infectious bursal disease virus (IBDV; also known as Gumboro virus) of chickens, a member of the genus *Avibirnavirus*, and infectious pancreatic necrosis virus (IPNV) of salmonids, a member of the genus *Aquabirnavirus*, are considered as archetypes of the family. Blotched snakehead virus was isolated from a cell line derived from a tropical freshwater fish (*Channa lucius*), and has recently been confirmed as a birnavirus. Because of some molecular peculiarities, it has been classified into a separate genus, *Blosnavirus*.

17.1.2 Structure

17.1.2.1 Virus Particle

Birnavirus particles are non-enveloped icosahedrons with a diameter of approximately 60 nm (Fig. 17.1). The virus particle is composed of the structural proteins VP2 and VP3 ("VP" for "viral protein") and contains two double-stranded RNA

Table 17.1 Characteristic birnavirus prototypes

Genus	Animal virus
Avibirnavirus	Avian infectious bursal disease virus (Gumboro virus)
Aquabirnavirus	Infectious pancreatic necrosis virus of salmonid fishes
Entomobirnavirus	Birnavirus of insects (*Drosophila* X virus)
Blosnavirus	Blotched snakehead virus

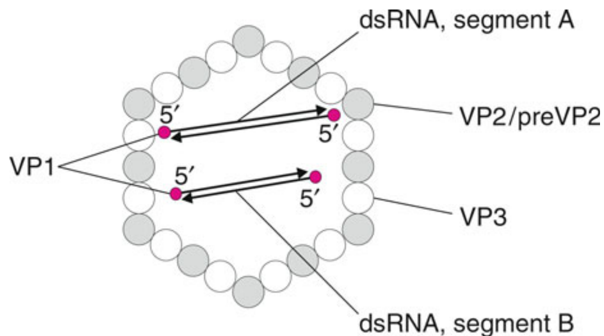


Fig. 17.1 A birnavirus particle. The genome is composed of two double-stranded RNA (*dsRNA*) segments. The VP1 protein, which also exhibits RNA-dependent RNA polymerase activity, is covalently bound with the 5′ ends of the genome. Both segments are enclosed in an icosahedral capsid consisting of viral proteins VP2/preVP2 and VP3

segments, which comprise in total about 6,000 base pairs. Like reoviruses, the symmetry of the icosahedron is a T = 13 structure, which is formed by a quasi-equivalent arrangement of VP2 trimers and groups of four VP2 trimers (G4).

17.1.2.2 Genome Organization and Structure

The two double-stranded RNA genome segments of birnaviruses (segment A, 3,200 base pairs; segment B, 2,800 base pairs) have a total of three open reading frames, to which the corresponding encoded proteins have been assigned (Fig. 17.2). Two open reading frames are encoded by the larger segment A (ORF1 und ORF2), and one is located on segment B (ORF3). The VP1 protein is covalently linked with the 5′ termini of both segments. The intermolecular interaction of VP1 proteins at both ends gives rise to a quasi-circular structure of RNA segments. Homologous palindromic sequences, which form loop structures and are important for replication, are located at the ends of adjacent regions of the genome. In IPNV, segment A comprises 3,214 base pairs and contains two open reading frames (Fig. 17.2). ORF2 encodes a precursor protein comprising proteins VP2, VP3 and VP4. It is probably cleaved by the protease activity of VP4. VP2 and VP3 constitute the capsid proteins, and VP2 contains epitopes that are recognized by neutralizing antibodies. Segment A contains a second open reading frame (ORF1) that, in

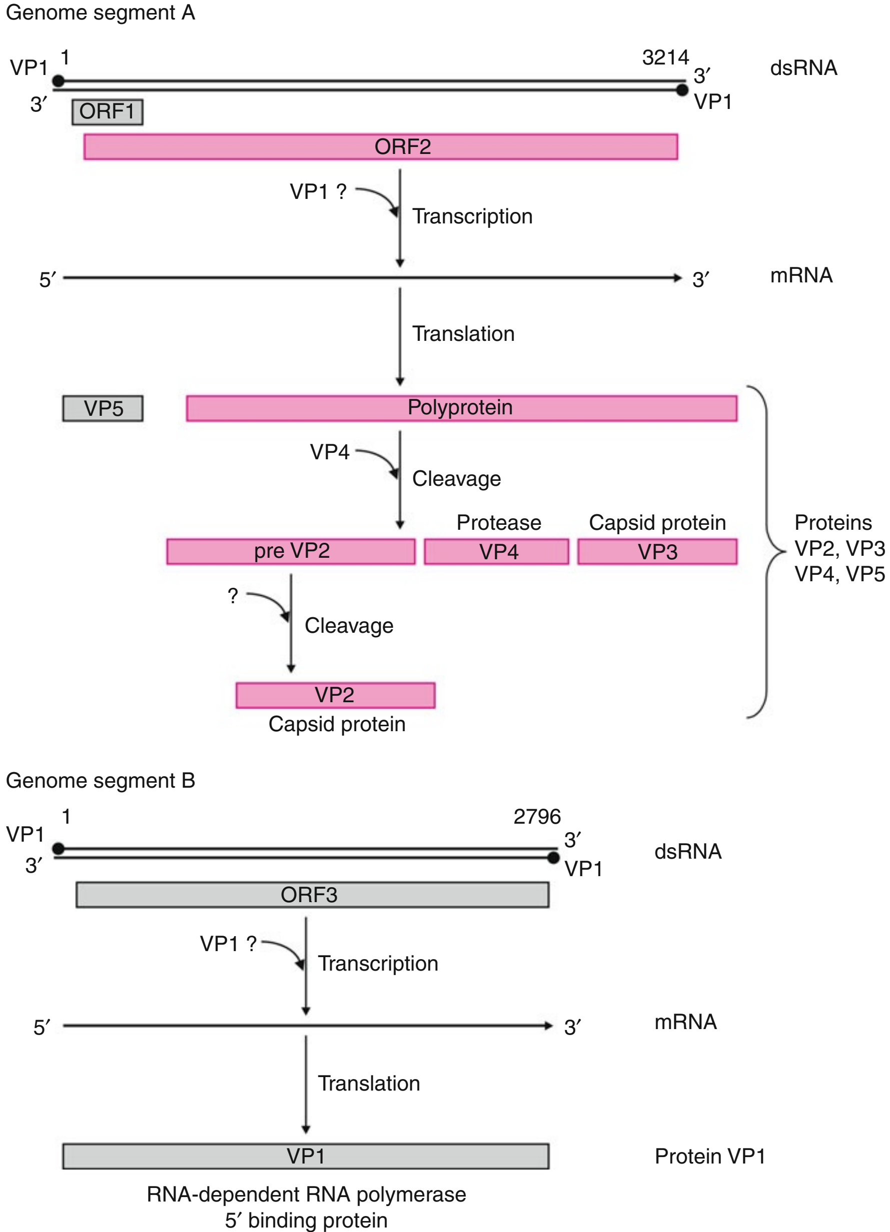


Fig. 17.2 Genome organization and gene expression of birnaviruses represented by infectious bursal disease virus of chickens. The genome is made up of two double-stranded RNA (*dsRNA*) segments. One molecule of protein VP1 is covalently linked with the ends of the 5′ segment. The transcription of segments is probably performed using the RNA-dependent RNA polymerase activity of the VP1 protein, whereby only one strand is used as a template for messenger RNA (*mRNA*) synthesis. Genome segment A contains two open reading frames (ORF1 and ORF2).

IBDV, encodes a VP5 protein of about 150 amino acids. Genome segment B of IPNV has a size of 2,796 base pairs. It encodes the VP1 protein that is covalently linked with the termini of RNA segments, thus stabilizing the quasi-circular shape of the genome segments. In addition, it has RNA-dependent RNA polymerase activity.

Whereas ORF1 of IBDV and IPNV, which resides on segment A and encodes the VP5 protein, is located in 5′ orientation to ORF2, ORF1 of *Drosophila* X virus is located in the opposite orientation. The peculiarity of blotched snakehead virus resides in a protein of 71 amino acids, encoded in segment A between the sequences encoding proteins VP2 and VP4. It is synthesized as a part of the precursor protein.

17.1.3 Viral Proteins

17.1.3.1 Structural Proteins

VP2 is the main capsid protein of birnaviruses. It is synthesized as a component of the precursor polyprotein and is released by proteolytic cleavage by the protease VP4. VP2 harbours the epitopes that are important for neutralization of the virus. The second structural protein, VP3, is also a constituent of the polyprotein, but its sequence does not contain neutralizing epitopes. It interacts with both the VP1 protein, which is covalently linked with the 5′ termini of RNA segments, and the VP2 protein. The VP1 protein constitutes the RNA-dependent RNA polymerase, which is encoded by the B segment. It also has a guanylyltransferase activity that is necessary for modifying the 5′ ends of viral messenger RNAs (mRNAs) with 5′-cap structures.

17.1.3.2 Non-Structural Proteins

VP4 is probably the viral protease that cleaves the structural proteins from the polyprotein. The ORF1 of segment A encodes VP5, whose function is unknown. An overview of the characteristic properties of birnavirus proteins is given in Table 17.2.

17.1.4 Replication

Little is known about birnavirus replication; it occurs exclusively in the cytoplasm of infected cells. The receptors of birnaviruses are largely unknown. Nonetheless, binding of virus particles to integrin $\alpha_4\beta_1$ has recently been described in the case of IBDV of chickens. Like in reoviruses, also only one strand of double-stranded RNA

Fig. 17.2 (continued) ORF2 encodes a polyprotein containing the protein components preVP2 (capsid protein), VP4 (protease) and VP3 (capsid protein). The cleavage of the precursor protein is performed by the autocatalytic activity of the protease VP4. The VP2 protein is generated from preVP2 by cleavage of the amino-terminal moiety by a still unknown protease. The small ORF1 encodes VP5, a protein of unknown function. Genome segment B contains ORF3, which encodes the VP1 protein

Table 17.2 Properties and functions of birnavirus proteins

Protein	Molecular mass	Function
VP1	90 kDa (IBDV) 94 kDa (IPNV)	Covalently linked to the 5′ ends of RNA segments; presumably RNA-dependent RNA polymerase
VP2	60 kDa (IPNV) 40 kDa (IBDV)	Main capsid protein; induction of neutralizing antibodies
VP3	32 kDa (IBDV) 30 kDa (IPNV)	Capsid protein
VP4/NS	29 kDa	Protease; cleavage of polyprotein into VP2, VP3 and VP4
VP5	17 kDa	Non-structural protein ORF2 in segment A
X	71 aa (BSNV)	Function unknown

aa amino acids, *BSNV* blotched snakehead virus, *IBDV* infectious bursal disease virus, *IPNV* infectious pancreatic necrosis virus

strands codes for proteins in birnaviruses. Structural proteins are synthesized as a precursor polyprotein, which is cleaved by the protease VP4 to yield preVP2 and VP3 protein subunits. Subsequently, preVP2 is further processed by an unknown mechanism in which the amino-terminal moiety is removed. The RNA-dependent RNA polymerase activity resides in the VP1 protein. The fact that the VP1 protein is covalently linked to the 5′ ends of RNA strands, suggests that a VP1-dependent initiation mechanism is necessary for the synthesis of new RNA strands. Furthermore, little is known concerning the morphogenesis of birnaviruses. Recombinant production of preVP2, but not of VP2, leads to the formation of tubular structures; contrarily, virus-like particles are only produced when the entire precursor protein is synthesized.

17.1.5 Animal Pathogenic Birnaviruses

No human pathogenic viruses have been described within the family *Birnaviridae*. However, two family members have veterinary importance: IBDV of chickens and IPNV of salmonids.

17.1.5.1 IBDV of Chickens (Gumboro Virus)

Epidemiology and Transmission

The vernacular name “infectious bursal disease virus of chickens” is named after the location, Gumboro (Delaware, USA), where the first detected outbreak of infectious bursal disease occurred in a poultry flock. On the one hand, IBDV has great veterinary relevance as the causative agent of infectious bursal disease of chickens, but on the other hand, it has also become very important in basic immunological research because of its pronounced tropism for B lymphocytes, which are destroyed by the infection. The virus has a worldwide distribution, and is highly contagious and very stable in the environment. This leads to its rapid spread

within chicken livestock and between flocks. Infected animals excrete the virus in the faeces for up to 2 weeks. It is transmitted directly from animal to animal, or indirectly through drinking water or litter.

The virulence of different IBDV strains differs considerably. The very virulent strains (vvIBDV) are of major veterinary importance. Their sudden appearance in the 1980s and their rapid spread are now attributed to a reassortment between different IBDV strains. In that process, the A segment (including the capsid protein VP2) of the old IBDV strain has been replaced by a phylogenetically new IBDV type, possibly from a wild bird. The amino acids at positions 284 and 253 of VP2 seem to be decisive in determining the virulence of vvIBDV strains; they possibly cause differences in the replication efficiency of B cells. By contrast, the influence of the B segment on the particular epidemiology of these strains cannot yet be explained.

IPNV of Salmonids Has a Worldwide Distribution Today

IPNV, a member of the genus *Aquabirnavirus*, was originally isolated from trout in the USA around 1950. It infects various bony fishes, and is disseminated throughout the world today. There are different distinguishable serotypes. The virus is spread by faeces, urine, eggs and sperm, and its transmission to fry is epidemiologically important. The virus usually persists lifelong in the fishes. IPNV is also spread by fish-eating birds such as herons, ospreys and kingfishers, which excrete them in the faeces. Sick fishes exhibit a dark colour, exophthalmos (bulging eyes) and movement disorders. Histologically, focal necroses are detectable in the pancreas. Large amounts of virus are detectable in the kidneys, from which the pathogen can be isolated. They can be cultivated in chinoock salmon embryo or rainbow trout gonad cells. Infectious pancreatic necrosis is a notifiable disease in most countries worldwide. Vaccines are available (modified live virus vaccines, inactivated vaccines, and recombinant vaccines based on VP2 proteins); they are applied in some European countries such as Norway.

Clinical Features

In a susceptible herd, the virus spreads in an acute epidemic wave. The symptoms are not very characteristic, and include anorexia, diarrhoea, tremors and ruffled feathers. The symptoms occur mainly in young animals aged from 3 to 10 weeks. In birds older than 6 weeks, the infection is usually asymptomatic, but more virulent IBDV strains cause diseases in them too. Depending on the virulence of the IBDV strain, the mortality can be up to 80 %, and the morbidity usually reaches 100 %.

Pathogenesis

The characteristic feature of infectious bursal disease is the exclusive infection of the cells of the bursa of Fabricius, which is the production site of B lymphocytes in chickens. The lymph follicles become atrophied as a result of lytic infection of B cells; therefore, surviving animals have virtually no B lymphocytes and develop

a deficiency of the humoral immune response. Highly virulent virus strains are also able to infect cells of other lymphoid tissues, such as the thymus, spleen and bone marrow, and to multiply in them.

After oral ingestion and initial proliferation in the macrophages of the gastric and intestinal mucosa, the virus reaches the liver, and finally the bursa of Fabricius via infected macrophages, where it proliferates in B lymphocytes, producing large amounts of progeny viruses. The viruses infect only differentiating B lymphocytes in defined stages of cell maturation. In the course of infection, virtually all B lymphocytes become infected in the bursa of Fabricius, whence the virus reaches other lymphatic organs during a secondary viraemia. As a result of B lymphocyte depletion (viral bursectomy), the birds have an increased susceptibility to various other infectious pathogens, such as salmonellas.

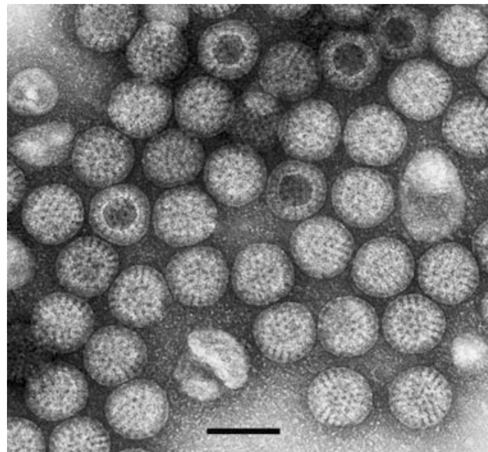
Immune Response and Diagnosis

Viral proteins can be detected by immunofluorescence in smear preparations of the bursa of Fabricius. Alternatively, the virus can be cultivated and isolated in embryonated chicken eggs or in cell cultures. Since most virulent IBDV strains are difficult to cultivate in vitro, the use of incubated chicken eggs is preferable for isolation of the virus. Reverse transcription PCR is increasingly being used for detection of viral RNA segments.

Control and Prophylaxis

Several vaccines based on attenuated viruses are available. They are administered to animals in drinking water. Inactivated vaccines are also available, and are administered parenterally. Both vaccines induce high titres of neutralizing antibodies that efficiently protect against infection.

17.2 Reoviruses



Reoviruses are found worldwide in various mammals, including humans, monkeys, dogs, mice, sheep and cattle. They have also been isolated from fish, reptiles, insects, fungi and algae. Three genera of the family *Reoviridae* encompass plant pathogenic viruses. The name "reovirus" was assigned to this virus family by Albert Sabin in 1959. This name is derived from the term "*r*espiratory *e*nteric *o*rphan *virus*", which indicates that these viruses infect the respiratory and digestive tracts, but no disease could be associated with the virus at that time. The particles have high stability, and thus can persist in the environment for a long time, e.g. in rivers, lakes and effluents. Most reoviruses infect the respiratory or gastrointestinal tracts, and infections are usually asymptomatic. Rotaviruses are human pathogenic reoviruses, and they mainly cause infectious gastroenteritis in young children, i.e. vomiting and diarrhoea. Annually, 800,000–1,000,000 deaths are attributed to rotavirus infections worldwide, which occur almost exclusively in children in developing countries. Reoviruses of veterinary importance are found in the genera *Rotavirus* and *Orbivirus*; they are discussed in detail in the following sections. Animal pathogenic viruses of the genera *Orthoreovirus* and *Coltivirus* are of minor importance.

17.2.1 Classification and Characteristic Prototypes

The family *Reoviridae* is divided into several genera. Each genus is characterized by specific features of particle morphology, the number of structural proteins and genome segments, and by the host tropism (Table 17.3). The further division into serogroups is based on the immunological recognition of capsid proteins.

17.2.2 Structure

17.2.2.1 Virus Particle

Infectious particles of reoviruses are icosahedral, and have a diameter of 60–85 nm, and even 95 nm in the recently described mimoreovirus. The particles are composed of up to three concentric protein layers. With regard to their structure, reoviruses can be divided into two groups, those with 12 "turret-like" protein protrusions on the fivefold symmetry axes of the vertices of the icosahedron, such as orthoreoviruses and cypoviruses, and the "turret-less" rotaviruses or orbiviruses that display a much less structured, smoother surface containing 60 small protrusions on the side lines of the icosahedron, the twofold symmetry axes.

Rotaviruses are spherical particles with a diameter of 70–80 nm. They have an inner capsid (55-nm diameter) which surrounds an inner core structure, and is enclosed by a second outer capsid. The virions have no envelope (Fig. 17.3). In cryo-electron microscopy images, the particles exhibit a well-formed, wheel-like structure with several "spokes" that result from the mutual interaction between both capsids, from which the name "rotavirus" is derived (Latin *rota*, "wheel"). The 60 protein protrusions (10 nm) on the particle surface are made up of VP4 molecules

Table 17.3 Characteristic prototypes of reoviruses

Genus[a]	Human virus	Animal virus	Plant virus
***Orthoreovirus* (10)**	Reovirus serotypes GT1–GT3	Murine reoviruses 1–3 Avian reoviruses Baboon reovirus	
***Orbivirus* (10)**	Orungo virus Kemerovo virus	Ovine bluetongue virus African horse sickness virus Equine encephalosis virus	
***Rotavirus* (11)**	Rotavirus serogroups A–C	Rotavirus serogroups A–F	
***Coltivirus* (12)**	Colorado tick fever virus Eyach virus	Colorado tick fever virus Eyach virus	
***Seadornavirus* (12)**	Banna virus Kadipiro virus	Banna virus Kadipiro virus	
***Aquareovirus* (11)**		Golden shiner virus Crab reovirus P, W2 (fish, crustaceans, snails)	
***Cypovirus* (10)**		Cytoplasmic polyhedrosis virus (insects)	
***Dinovernavirus* (9)**		*Aedes pseudoscutellaris* reovirus (insects)	
***Idnoreovirus* (10)**		*Hyposoter exiduae r*eovirus (insects)	
***Mycoreovirus* (11)**		Mycoreovirus 1–3 (fungi)	
***Mimoreovirus* (11)**			*Micromonas pusilla* reovirus (algae)
***Phytoreovirus* (12)**			Plant reoviruses (subgroup I) Rice dwarf virus
***Fijivirus* (10)**			Plant reoviruses (subgroup II) Fiji disease virus
***Oryzavirus* (10)**			Plant reoviruses Rice ragged stunt virus

[a]The numbers in *parentheses* refer to the number of genome segments which are contained in the virus particles of the various genera.

with a molecular mass of 86 kDa; the VP7 protein (34 kDa) is another component of the outer capsid. The protein layer of the outer capsid is interspersed by 132 channels. They connect the outer capsid with the inner capsid. At the vertices of the icosahedron, they lead into the pores of the inner capsid, which are formed by VP6 proteins (44 kDa), and reach to the core particle. The latter is constituted of VP2 proteins (102 kDa), which form a protein layer, the core shell. The double-stranded RNA segments reside inside the core, and are associated with VP1

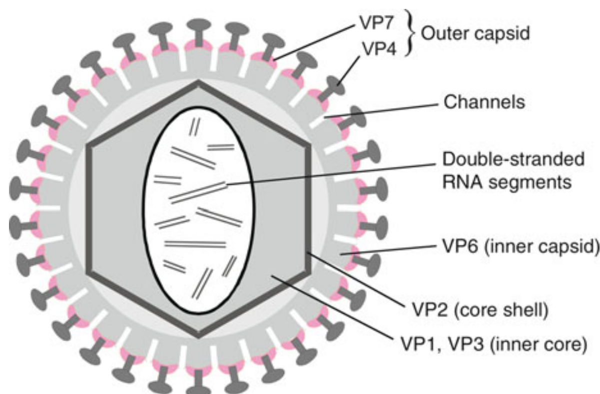


Fig. 17.3 Structure of a rotavirus particle. The virions are composed of three layers. The inner core is made up of double-stranded RNA segments and proteins VP1 and VP3, which interact with VP2 proteins. The latter assemble into a protein layer that constitutes the core shell. The VP6 proteins form the inner capsid, and VP4 as well as VP7 shape the outer capsid. The layers of the inner and outer capsids are interspersed by channels, which reach into the inner core

(125 kDa) and VP3 (88 kDa) proteins. VP1 is the RNA-dependent RNA polymerase; VP3 has methyltransferase/guanylyltransferase activity and is responsible for the modification of viral transcripts with 5′-cap structures. Ions can diffuse through the channels into the interior of the virus. Even genome segments and newly synthesized transcripts are probably transported in both directions through the channels during morphogenesis or prior to translation.

17.2.2.2 Genome Organization and Structure

The genome of reoviruses consists of nine to 12 double-stranded RNA segments. The coding capacity differs considerably among members of the different genera, ranging from 18,200 base pairs in rotaviruses, up to 30,500 base pairs in cypoviruses. In the case of rotaviruses, there are 11 RNA segments, which are arranged according to their length (Table 17.4). The rotavirus subgroup A genome has a total of about 18,200–19,000 base pairs. The 5′ termini bear 5′-cap structures – $m^7GpppG^{(m)}GPy$. Every non-polyadenylated 3′ end contains cytidine residues. Short conserved regions of seven to ten base pairs are located at both ends of the segments. It is thought that they are important for initiation of transcription and replication, as well as for packaging of the viral genetic information into the particles. Each of the 11 RNA segments is transcribed into mRNA during replication. With the exception of the smallest segment, 11, each RNA segment encodes one viral protein; thus the ten major segments are responsible for the synthesis of the six structural proteins (VP1, VP2, VP3, VP4, VP6, VP7) and four (NSP1–NSP4) of the six non-structural proteins. Segment 11 harbours two open reading frames encoding non-structural proteins NSP5 and NSP6 (Table 17.4).

Table 17.4 Genome segments of human rotaviruses and their respective encoded proteins

Segment no.	Length (base pairs)	Protein	Molecular mass (kDa)	Localization	Function
1	3,266–3,302	VP1	125	Inner core	Slightly basic, RNA-dependent RNA polymerase
2	2,687–2,690	VP2	102	Core shell	RNA binding, myristoylated
3	2,591	VP3	88	Inner core	Basic, methyltransferase/ guanylyltransferase (capping enzyme)
4	2,350–2,364	VP4	86	Outer capsid	Surface protein (P antigen), haemagglutinin, virus attachment, induces formation of neutralizing antibodies, is cleaved into VP8* (28 kDa) and VP5* (60 kDa).
					VP8*: interaction with sialic acid, VP5*: fusion, penetration, binding to integrin $\alpha_2\beta_1$
5	1,581–1,611	NSP1	55		Zinc finger protein, RNA binding, interacts with IRF-3 and causes its degradation, is not essential for replication
6	1,356	VP6	44	Inner capsid	Trimeric, myristoylated? defines membership in serogroups A–G
7	1,075–1,104	NSP3	34		Basic, active as homodimer, binds to 3′ ends of RNA and to eIF4G
8	1,059–1,062	NSP2	36		Octamer, basic, ssRNA-binding nucleotidyl hydrolase, RNA helicase, interaction with NSP5 causes formation of viroplasm-like structures
9	1,062	VP7	34/37	Outer capsid	Glycosylated (G antigen), surface protein, induces formation of neutralizing antibodies, interaction with integrins $\alpha_x\beta_2$ and $\alpha_v\ \beta_3$, and Hsc70
10	750–751	NSP4	28		Glycosylated, incorporated in the ER membrane, interacts with VP6 of the inner capsids during morphogenesis, proteolytic cleavage causes the formation of a 7-kDa secreted product which acts as an enterotoxin
11	663–667	NSP5	32–34		Dimer, phosphorylated, O-glycosylated, causes interaction with ssRNA and dsRNA, interacts with NSP2, causes formation of viroplasm-like structures
		NSP6	11		Missing in rotavirus serogroup C and some members of serogroup A, interaction with NSP5

dsRNA double-stranded RNA, *ER* endoplasmic reticulum *IRF-3* interferon regulatory factor 3, *ssRNA* single-stranded RNA

Possibly, a bicistronic mRNA also exists in segment 9 of rotaviruses. The open reading frames are flanked by short non-coding sequences. Their length ranges between 9–46 and 17–182 nucleotides at the 5′ and 3′ ends of mRNAs, respectively. In other reoviruses, there are some segments that encode two or three proteins.

17.2.3 Viral Proteins

17.2.3.1 Structural Proteins

Outer Capsid

Infectious particles of rotaviruses contain 120 molecules of VP4 (86 kDa). VP4 is a non-glycosylated component of the outer capsid, is present as a homodimer and forms the protein projections that can be found at the twofold symmetry axes of the icosahedron; as “P antigen” (proteolytically cleaved antigen), it characterizes the P serotype of the different virus isolates. So far, 28 different P serotypes have been characterized in rotaviruses worldwide. The VP4 protein is responsible for binding to cellular receptors on the cell surface, for penetration of the virus, and for its infectivity and haemagglutination properties. Most of the neutralizing antibodies are directed against VP4. A trypsin-like protease cleaves the VP4 protein between amino acids at positions 247 and 248 into an amino-terminal part, VP8* (28 kDa), and a carboxy-terminal moiety, VP5* (60 kDa). Both cleavage products are components of the virions; this cleavage reaction facilitates penetration of the virus into the cell, and enhances the infectivity of the virus. The proteolytic cleavage is also important when rotaviruses are cultivated in cell culture. Amino acid residues 93–208 of VP8* are responsible for the haemagglutination activity of rotaviruses. There is high variability in the VP8* sequence of various rotavirus types, and virus-type-specific neutralizing antibodies are mainly directed against VP8*. VP8* binds to sialic acid on the cell surface, and induces the initial interaction of virus particles with the cell. in a second interaction step, VP5* binds to various integrins, such as $\alpha_2\beta_1$. Furthermore, VP5* is responsible for penetration of the virus particles into the cytoplasm, and thus for the infectivity of the virus. The cleavage reaction induces dimerization of VP5*, which in turn stabilizes the icosahedral structure. Proteolysis probably activates a fusogenic protein domain, which, however, is not located at the amino terminus of the VP5* cleavage product. The fusogenic activity of the F and HA proteins of paramyxoviruses and influenza viruses resides in the amino-terminal hydrophobic regions of the F_1 and HA_2 polypeptides, respectively (▶ Sects. 15.3 and ▶ 16.3). Since VP5* has many polar amino acids at the amino terminus, an alternative mechanism must be postulated for the fusogenic activity of the rotavirus VP5* protein. In addition, it has also been found that the different virulence of individual virus strains correlates with variations in the amino acid sequence of the VP5* protein. If phenylalanine or leucine is replaced by glutamine at amino acid position 469, then the porcine rotavirus PRV-4F shows increased virulence. Cross-reacting, group-specific immunoglobulins are preferentially directed against the VP5 moiety of VP4.

VP7 (34 kDa) is glycosylated at asparagine residues and is, with a copy number of 780 molecules, the major component of the outer capsid of rotaviruses. The proteins assemble together into 260 trimers and make up the faces and vertices of the icosahedron; trimerization is dependent on the presence of Ca^{2+} ions. The VP7 protein represents the "glycosylated antigen" that defines the G serotype of the various rotavirus isolates. Most neutralizing antibodies are directed against epitopes of this protein. Owing to their specificity, rotaviruses can be assigned to 15 different G serotypes (G1–G15). In addition to VP8* and VP5*, VP7 also appears to be involved in binding of the virus particle to the cell surface: interactions with integrin $\alpha_x\beta_2$ or integrin $\alpha_v\beta_3$ and heat shock cognate protein Hsc70 have been described. VP7 is anchored in the membrane of the endoplasmic reticulum (ER) by an amino-terminal hydrophobic domain as early as during its synthesis. A signalase recognition site is situated adjacent to this domain at position 51. The carboxy-terminal moiety, which was translocated into the lumen, is released from membrane anchoring by proteolytic cleavage, but remains associated with the ER membrane. VP7 is possibly synthesized with two different amino termini because a second start codon is located in the same reading frame 90 nucleotides downstream. Its use gives rise to a truncated protein lacking 30 amino-terminal amino acids, and thus lacking the anchor domain.

Inner Capsid

The VP6 proteins of rotaviruses (44 kDa) form trimers, and each virus particle contains 260 trimeric complexes. They form an intermediate layer between the outer capsid and the viral core. VP6 trimers are oriented in such a way that they are arranged beneath the VP7 complexes; therefore, the channels which traverse the outer and inner capsid are interconnected with each other. The VP6 proteins mutually interact by their amino-terminal domains, and are concurrently associated with the VP2 components of the viral core and the VP7 proteins of the outer capsid. VP6 constitutes the group-specific antigen of rotaviruses and is responsible for the assignment of the different rotaviruses to serogroups A–G. Immunoglobulins directed against this polypeptide serve, independently of the serotype, as a diagnostic clue for rotavirus infections.

Viral Core

The VP2 protein (102 kDa) is the main component of the shell, whereby 120 molecules assemble into 60 dimers, which form the icosahedral viral core. This protein layer interacts with VP6 proteins of the inner capsid. VP2 proteins have RNA-binding properties, which reside within the amino-terminal region (amino acids 1–132). In the case of rotaviruses, but also in orbiviruses and orthoreoviruses, structural proteins VP1 (125 kDa) and VP3 (99 kDa) are located at the 12 vertices inside the VP2 core shell, where they form a heterodimeric complex. On the other hand, VP3 of aquareoviruses and cypoviruses is localized at the vertices of the icosahedron on the outside of the VP2 protein layer. The VP1 protein possesses RNA-dependent RNA polymerase activity, and VP3 is a methyltransferase and guanylyltransferase. It modifies mRNA molecules, which are synthesized during infection, with methylated 5′-cap groups. It is believed that each of the 12 VP1/VP3 complexes is associated with one double-stranded RNA genome segment.

17.2.3.2 Non-Structural Proteins

Rotaviruses encode six non-structural proteins which are involved in viral replication and morphogenesis in infected cells. However, their individual functions are not completely understood. The known functions and molecular characteristics of all rotavirus proteins are summarized in Table 17.4.

The RNA-binding NSP1 protein (55 kDa) is not essential for replication of rotaviruses. With regard to its sequence, it is very variable among the different virus types, with the exception of a conserved cysteine-rich domain in amino acid region 42–72 that displays a similarity to zinc finger motifs. NSP1 influences the virulence of rotaviruses and prevents the activation of the interferon-dependent immune response. After its synthesis in the cytoplasm, it binds interferon regulatory factor 3 (IRF-3) and induces its degradation. There is evidence that this step is proteasome-dependent and is not performed by the proteolytic activity of NSP1. IRF-3 is present in the cytoplasm of all cells, and is an early regulatory protein for induction of the innate immune system. If a cell is infected, IFR-3 is phosphorylated, it dimerizes and is transported into the nucleus, where it functions as a transactional activator of interferon genes (▶ Chap. 8).

The basic NSP2 protein (35 kDa) is highly conserved: analogous proteins have also been described in orthoreoviruses (NS) and in bluetongue virus (NS2), a member of the genus *Orbivirus*. In both cases, its molecular mass is 41 kDa. NSP2 of rotaviruses is synthesized in large amounts in infected cells and forms stable octamers. These complexes are the functionally active form of NSP2 proteins; they have a circular structure with a 35-Å orifice. NSP2 octamers bind to RNA in a sequence-independent fashion. Responsible for this is probably the amino-terminal domain of NSP2, in which a sequence of 24 basic amino acid residues is located. These line four depressions, which are arranged diagonally across the surface of the octamer, and probably represent the interaction sites with the RNA, which, according to this model, wraps around the octamer. A Mg^{2+}-dependent NTPase activity resides in the carboxy-terminal domain of NSP2, in which the histidine residues at positions 110, 221 and 225 form a histidine-triad-like structure, as known from other nucleotidyl hydrolases. In addition, the NSP2 octamer complex exhibits helicase activity: it destabilizes double-stranded RNA molecules. This enzymatic activity is independent of Mg^{2+} ions. The interaction of NSP2 with the amino-terminal domain of NSP5 proteins has the effect that the latter become hyperphosphorylated by unknown viral or cellular kinases and form multimers which stimulate the formation of viroplasma-like structures composed of NSP2 and NSP5. Both non-structural proteins are able to form a kind of molecular motor that promotes packaging of viral mRNAs into core-like replicative intermediates in the cytoplasm of infected cells.

The Zn^{2+}-binding, basic and dimeric NSP3 protein (34 kDa) interacts sequence-specifically with the 3′ ends of viral mRNA molecules via its amino-terminal region, which contains two consecutive zinc finger motifs. In serogroup A rotaviruses, the sequence 5′-GUGACC-3′ is involved. Since mRNA synthesis occurs in the cytoplasm in all reoviruses, the pathogens are not capable of using the nuclear-localized cellular enzymes for modification of their 3′ ends of mRNAs with poly(A) tails.

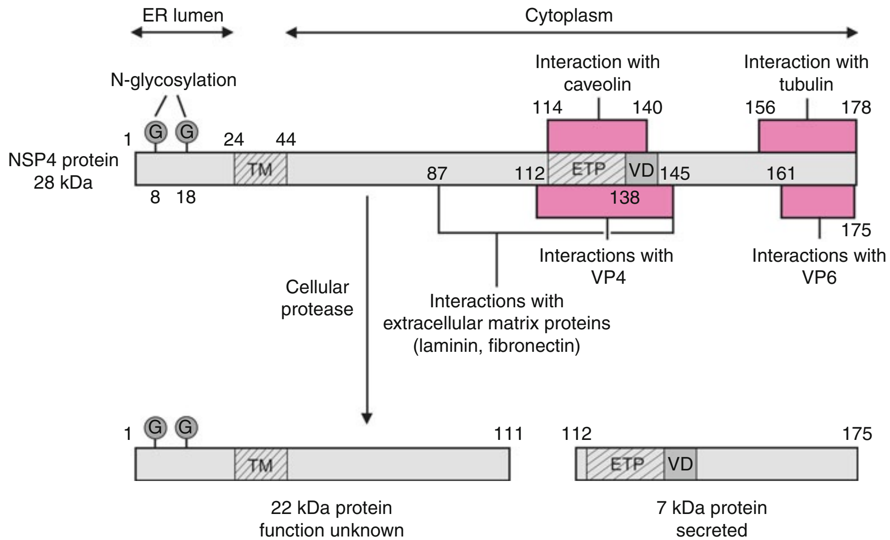


Fig. 17.4 Localization of functionally active domains of the non-structural NSP4 protein. *Numbers* refer to the amino acid positions, starting from the amino terminus. *ER* endoplasmic reticulum, *TM* transmembrane domain, *ETP* enterotoxic peptide, *VD* variable domain (species specificity)

Furthermore, they do not have their own enzymatic functions for polyadenylation; therefore, the 3′ termini of viral transcripts are not polyadenylated. NSP3 binds to the 3′ ends of viral mRNAs and promotes the interaction with the factor eIF4G. This cellular translation initiation factor usually binds to poly(A)-binding protein (PABP) – a cellular protein that recognizes polyadenylated 3′ ends of mRNAs. It is responsible, along with the 5′-cap-binding eIF4E factor, for the transport of capped and polyadenylated cellular mRNAs to the ribosomes, the translation sites. Reoviruses circumvent this cellular mechanism by replacing PABP with NSP3. NSP3 binds to the conserved sequence motif at the 3′ ends of viral transcripts and mediates the interaction with eIF4G. In this process, the binding of eIF4G to the mRNA/NSP3 complex is stronger than that with PABP. Hence, in infected cells, eIF4G preferentially binds to viral transcripts, which are then transported to the ribosomes and translated.

The glycosylated NSP4 (28 kDa) is a multifunctional protein. A hydrophobic sequence motif in the amino-terminal region (amino acids 24–44) anchors NSP4 in the ER membrane. The 23 amino-terminal residues with two recognition sites for N-glycosylation project into the lumen of the ER, whereas the carboxy-terminal amino acids are oriented in the cytoplasm. NSP4 remains associated with the ER; it is neither processed through the Golgi vesicles or the *trans*-Golgi network nor transported to the cell surface. In infected cells, a cleavage product of NSP4 (7 kDa) has been found which corresponds to the carboxy-terminal domains downstream of amino acid residue 112 (Fig. 17.4). It is unknown which

cellular protease is responsible for this cleavage reaction. The cleavage product is transported by a *trans*-Golgi-network-independent mechanism to the cell surface, is secreted and can accumulate as a soluble product on the surface of uninfected cells. Amino acids 114–140 are responsible for the interaction of the 7-kDa cleavage product with caveolin-1, a protein that is localized in caveolae or lipid rafts. The interaction of the NSP4 fragment with caveolin-1 is presumably responsible for the pathogenic processes and the symptoms (diarrhoea) that are associated with the infection, as NSP4 acts as an enterotoxin: if cell cultures or the intestinal mucosa of newborn mice are incubated with NSP4, the 7-kDa cleavage product or peptides derived from amino acids 114–135, then the phosphoinositol-dependent signal transduction pathway becomes activated. This induces the transport of Ca^{2+} ions from the ER and gives rise to an increase of Ca^{2+} concentration in the cytoplasm. This ultimately leads to the release of Cl^- ions and water by the cells, and probably causes the diarrhoea symptoms associated with rotavirus infections.

Besides the interaction with caveolin-1, it has also been described that other sequence segments of the carboxy-terminal cleavage product interact with tubulin of cellular microfilaments as well as with the extracellular matrix (Fig. 17.4). Nevertheless, another function of NSP4 is important for the production of progeny viruses. During viral morphogenesis, this ER-membrane-anchored protein functions as a receptor for the newly formed subvirus particles, which consist of the inner capsid (viral cores complexed with VP6, bilayer particles) and the precursor proteins VP4, which shape the spike-like protrusions in the outer capsid of virions. The interaction sites for VP6 and VP4 have been localized in the carboxy-terminal, cytoplasmic-oriented domain of NSP4 (Fig. 17.4). After accumulation of subviral structures and proteins, budding of particles occurs in the lumen of the ER. As a result, membrane-enveloped precursors of virions are temporarily formed; NSP4 and lipids are detached in the further course, but it is not clear by what mechanism.

The acidic NSP5 is encoded, along with NSP6, by the smallest segment. The μ2 protein of reoviruses (genus *Orthoreovirus*) appears to be a protein analogous to NSP5, whereas such a protein has not yet been identified in orbiviruses. The unmodified NSP5 of rotaviruses has a molecular mass of 26–28 kDa, which increases to 32–34 kDa after O-glycosylation and phosphorylation at specific serine and threonine residues. Dimerization of NSP5 proteins is a prerequisite for phosphorylation. NSP5 binds to both single-stranded RNA and double-stranded RNA, and it interacts with NSP2 through its amino-terminal domain and with NSP6 through its carboxy-terminal domain.

The NSP6 protein (11 kDa) is encoded, along with NSP5, by segment 11 in an alternative reading frame which it is absent in some rotaviruses of serogroup A and in all members of the serogroup C. This means that NSP6 is not absolutely necessary for rotavirus replication. It is produced in small amounts and binds to the carboxy-terminal domain of NSP5. Whether this may regulate the phosphorylation status of NSP5 and affect its function is unclear.

Lipid Rafts and Caveolae Are Defined Structures of the Cytoplasmic Membrane
The term "lipid rafts" characterizes platforms or islands in the cytoplasmic membrane which contain high concentrations of cholesterol and sphingomyelin (gangliosides). The saturated hydrocarbon chains of sphingomyelins facilitate a very tight interaction with cholesterol molecules, and thus form limited, lateral swimming areas; in this regard, lipid rafts resemble model membranes. Membrane-anchored proteins are located in these islands (including integrins and heat shock proteins), which confer the lipid rafts with specific functions, such as in interaction with the extracellular matrix, in signal transduction, and in sorting of proteins to different sides of the cell. Caveolae (a descriptive term used in electron microscopy) are considered a subgroup of lipid rafts: they are monochambered or multichambered invaginations of the membrane which are open to the cell surface and are anchored to the cytoskeleton system of the cell; therefore, they can only slightly move laterally. They can be found on the surface of many cells. They have a size of 50–100 nm, and are caveolin-rich. Caveolin-1, caveolin-2 and caveolin-3 (molecular mass about 21 kDa) are members of a protein family whose presence in the caveolae is necessary for generation of membrane invaginations.

Palmitoylated caveolins are not anchored in the membrane by transmembrane domains, but are associated with the inside of caveolae-forming membrane compartments by carboxy-terminally attached palmitic acid molecules and by a hydrophobic sequence, whereby they line the interior layer of the plasma membrane and the amino and carboxy termini of caveolins are oriented into the cytoplasm. Caveolins assemble into dimers by intermolecular interaction between their amino-terminal domains, which are also linked with filamin, a component of actin filaments. This anchors lipid rafts to the cytoskeleton, and prevents their lateral diffusion in the membrane. Vesicles are formed and released from the invaginations at the membrane inside, and are then transported further to other cell compartments. It is believed that, depending on the cell type, lipid rafts as well as caveolae can have different functions. These include incorporation of surface-bound molecules and complexes (e.g. even virus particles), transendothelial transport of molecules, signal transduction by interaction of specific receptors anchored in the caveolae, and cholesterol uptake and release.

17.2.4 Replication

Reoviruses enter cells in several steps. Sialic acid seems to be involved in attachment of the virus, as treatment of the cell surface with neuraminidase prevents binding of some reovirus and rotavirus types. For example, porcine rotavirus type OSU seems to attach to the ganglioside GM3, a neuraminic acid containing structure on the cell surface. In contrast, binding of most human rotaviruses occurs

even when terminal sialic acid residues have been removed. It is assumed that in such cases viruses possibly bind to internal, non-terminal sialic acid molecules, which are not accessible to the neuraminidase treatment. For example, an interaction with the ganglioside GM1 has been found in human rotavirus types KUN and MO, which are neuraminidase-resistant virus types. The interaction partner for the sialic acid is the rotavirus VP8* protein; however, the binding affinity is low. In a second step, the viruses bind to integrin $\alpha_2\beta_1$ by the VP5* moiety of the protein protrusions on the outer capsid; in viruses of serogroup A, this happens by means of the motif aspartic acid-glycine-glutamic acid at positions 308–310. In a third step, VP7 proteins interact with integrin $\alpha_v\alpha_3$ or integrin $\alpha_x\alpha\beta_2$ or with other membrane proteins (Hsc70). A prerequisite for penetration of the cell-surface-bound virus seems to be that the various cellular interaction partners are organized in lipid rafts (see the box entitled "Lipid Rafts and Caveolae Are Defined Structures of the Cytoplasmic Membrane"). Just how the virus enters the cell has not yet been conclusively resolved. It is assumed that the particles penetrate the cells via clathrin-containing vesicles or – clathrin-independently – by raft-mediated endocytosis. The cleavage of the VP4 protein into VP8* and VP5* promotes penetration of the virus. A hydrophobic fusion domain within VP5* permeabilizes the vesicle membrane, in which the virus resides after entry. At the same time, an ATP-dependent proton pump in the vesicle membrane lowers the concentration of Ca^{2+} ions inside the vesicle. Low concentrations of Ca^{2+} ions, which are necessary for stabilization of the VP7 proteins into the trimeric complexes that form the icosahedral structure of the outer capsid, facilitate shedding of the protein layer of the outer capsid: the triple-layered particle becomes a double-layered particle, and the inner capsid, which contains the viral core and proteins VP1, VP2, VP3 and VP6 as well as the RNA segments reaches the cytoplasm of the cell.

Shedding of the outer layer induces a rearrangement of the inner capsid. Nucleotide triphosphates and other components diffuse into the viral core through the channels. This facilitates transcription of RNA segments by the RNA-dependent RNA polymerase activity of the VP1 protein; VP3 is responsible for capping of mRNAs. Retaining the structure of the inner capsids, both proteins perform the simultaneous transcription of all double-stranded RNA genome segments, and synthesize 5′-capped, non-polyadenylated mRNA molecules. The enzyme complex functions very fast: investigations with orthoreoviruses revealed that approximately 50 nucleotides are synthesized per second. Only the negative strands of the double-stranded genome segments are transcribed; thus, the process is asymmetrical. These mRNAs are complementary to the genome segments in their full length, and are extruded into the cytoplasm for translation through the channels that are formed by VP6 proteins at the vertices of the icosahedrons. When newly synthesized NSP3 proteins become available after the initial translation processes, the interactions of these proteins with the 3′ ends of mRNAs and with the cellular translation initiation factor eIF4G will increase the transport of the non-polyadenylated transcripts to the ribosomes and their translation. The accumulation of viral proteins NSP2 and NSP5 leads to the formation of inclusion bodies in the cytoplasm, which are also referred to as viroplasms.

RNA replication occurs in the viroplasms, as does a second round of transcription and the assembly of VP1, VP2, VP3 and VP6 proteins and RNA molecules into double-layered particles, i.e. the inner capsid structures. The NSP4 and NSP7 proteins are synthesized at the ER membrane, translocate through it and become glycosylated. As an integral membrane protein, NSP4 remains anchored in the ER membrane and forms, together with the VP7 proteins, target structures, to which the two-layered particles attach with their surface-exposed VP6 moieties. The amino-terminal domains of NSP4 proteins, which are oriented to the cytoplasm, serve as interaction partners for VP6. In a budding-like process, the double-layered particles are surrounded by the ER membrane and are released into the ER lumen, whereby they temporarily obtain an envelope consisting of NSP4 and VP7. In the further course, NSP4 proteins and phospholipids of the shell are lost by unknown mechanisms, whereby VP7 proteins consolidate into icosahedrons. How and when VP4 proteins are bound to the surface of the now three-layered particles has also not yet been clarified.

Similar to influenza A viruses, the emergence of reassortants is frequently observed during morphogenesis in vitro. Co-infection of a cell with two virus types differing in each of the 11 genome segments would theoretically yield 2^{11}, i.e. 2,048, different reassortants. Epidemiological studies indicate that generation of reassortants is common in vivo, but most of the theoretically possible combinations are not viable. Rearrangements of RNA segments have also been ascertained in cell cultures. In the course of this, parts of a segment are incorporated into other segments, or are deleted. It is not known whether this process leads to viable virus variants with altered cell specificity or virulence. The activity of the transcriptase complex, which is incorporated in the particles, transforms single-stranded mRNAs into double-stranded molecules during morphogenesis. The nucleotides that are essential for double-stranded RNA synthesis diffuse into the interior of the particles through the channels of VP6 proteins. This process is a conservative replication mode: The parental strands remain unaltered during the process, and the newly synthesized RNA genome segments do not contain any of the original RNA strands. Mature viruses are released by cell lysis. Therefore, many incomplete RNA segments (partially single stranded) can be found in the particles as the infected cell usually dies before definitive completion of RNA duplexes. What mechanism ensures that the new virus particles receive the correct set of genome segments remains to be elucidated. The association of double-stranded RNA molecules with the 12 vertices of the icosahedron in the interior of the viral core allows the packaging of a maximum of 12 segments. For this reason, it is believed that only those reoviruses are viable which have a maximum of 12 genome segments.

17.2.5 Human Pathogenic Reoviruses

17.2.5.1 Rotaviruses

Epidemiology and Transmission

Rotaviruses are ubiquitous and can be found in virtually every species: they infect both animals and humans. Seven serogroups can be distinguished, A–G, on the

basis of serological recognition of the structural protein VP6, the major component of the inner capsid. Human rotaviruses are pathogens that have a worldwide distribution and are associated with diarrhoeal diseases in children. Human pathogenic rotaviruses are found in serogroups A, B and C; most virus isolates from patients can be assigned to serogroup A. The further subdivision into serotypes P (proteolytically cleaved antigen and G (glycosylated antigen) is made on the basis of differences in the outer capsid proteins VP4 and VP7. To date, 16 and 27 different G and P serotypes have been identified in human rotaviruses, respectively. The strains P1A[8]/G1, P1B[4]/G2, P1A[8]/G3, P1A[8]/G4 and P1A[8]/G9 prevail worldwide, and differ by double-stranded RNA genome segments 4 and 9 encoding proteins VP4 and VP7, respectively. However, other regional types can also be found occasionally – G5, G8, and P2A[6]. Up to 42 different P–G combinations have been characterized by using PCR and genome sequencing. In addition, three different alleles of the NSP4 genome segment are known: Wa, KUN and Au-1. Since NSP4 proteins interact with VP6 during viral morphogenesis – a crucial step for viral morphogenesis – not all theoretical combinations of NSP4 alleles are possible.

Human rotaviruses were first isolated in 1973 by Ruth Bishop and colleagues at the Royal Children's Hospital in Melbourne (Australia) from the stool of children who had diarrhoea. Previously, these viruses were known as the cause of diarrhoea only in mice and monkeys. It is now known that rotaviruses are the principal cause of severe gastroenteritis, especially in children during the first 2 years of life. Group A rotaviruses have a worldwide distribution. Infections with group B rotaviruses are particularly found in Asia and on the Indian subcontinent. Group C rotaviruses are found worldwide, but epidemic appearances are rare.

Rotavirus infections occur more frequently in children in regions with temperate climates during the winter months; the pathogens are excreted by infected individuals in the faeces and are transmitted via the faecal-oral route. In infected children, there are more than 10^{12} virus particles per gram of stool; infectious virus particles remain in the environment for long periods because of their remarkable stability. Fewer than 100 particles are sufficient for transmission of infection. Therefore, rotavirus infections can easily spread and develop as epidemics, especially under poor hygiene conditions in children's hospitals, nurseries and kindergartens, but also in old people's homes. During epidemics, new serotypes and virus reassortants are rapidly developed, and these are responsible for the high serological heterogeneity of rotaviruses. Furthermore, zoonotic infections with animal rotaviruses also contribute to the ample heterogeneity, and especially those that are common in domestic animals and livestock. Although rotaviruses are generally deemed to be host-specific, and zoonotic infections caused by them are rare, this aspect should not be underestimated in the formation of new virus variants and serotypes, considering the high frequency of infection in both animals and humans.

As immunoprotection declines with increasing age, apart from children, adults are infected as well. Transmissions from sick children to other family members are usually responsible for this. Moreover, rotavirus infection causes diarrhoea in adults,

especially among travellers in Central America and the Caribbean. Rotaviruses are increasingly recognized of as the causative agent of chronic diarrhoea in immunosuppressed adults (transplant recipients, HIV-infected individuals).

Nomenclature of Rotavirus Strains

Rotaviruses possess 11 double-stranded RNA segments. Reassortants are frequently generated owing to co-infections with different virus strains and transmissions of animal rotaviruses to humans. Therefore, the characterization of individual virus strains in regard to their P (protease-sensitive or proteolytically cleaved antigen) and G (glycosylated antigen or glycoprotein) combinations is especially difficult. P types can be assigned to the VP4 protein and G types can be assigned to the VP7 protein. These proteins are encoded by double-stranded RNA segments 4 and 9, respectively. The determination of G types is usually done serologically; distinguishable variants are denominated with different numbers (G1 to G15). The determination of the at least 28 distinct P types is much more difficult: if a serological characterization is available, the different P antigen serotypes are designated with corresponding number and letter combinations, e.g. P1A. Since serological tests are very complex, in recent years, genotyping has usually been done only by PCR amplification of genome segment 4, followed by sequencing. The genotype assignment is indicated by a number in square brackets, e.g. P[8].

Clinical Features

Approximately half of all diarrhoea cases which need medical treatment in children aged between 6 months and 2 years are caused by rotaviruses. The infections are usually asymptomatic, or have a mild clinical course in neonates and older children. However, it is estimated that in developing countries roughly 800,000 children die of the consequences of rotavirus infections every year. In developed countries, rotavirus infections are often nosocomially acquired, e.g. in paediatric wards. The incubation period lasts only 1–3 days. The disease is characterized by sudden vomiting and severe, watery diarrhoea. Fever and abdominal pain may also occur. The symptoms last, on average, 4–5 days. Approximately 40 % of severely sick children need treatment in hospital. In the prevaccine era, up to 400,000 and 77,000 cases, respectively, were reported in the USA and Germany annually, but it is estimated that the number of unreported cases was more than five times greater. In severe cases, shifts in electrolyte concentrations in blood and lethargic states occur owing to massive dehydration. However, in contrast to the situation in developing countries, fatal courses associated with circulatory failure are rarely observed in industrialized countries. Infected children can transmit the virus to adult family members, in which the infection develops as an asymptomatic or attenuated form. In recent years, there have been growing indications mainly obtained from infections in immune-suppressed children that rotaviruses can be present not only in the intestine, but also in blood and other organs, such as the central nervous system and liver. Which organ manifestations can be inferred from this is currently unclear.

Pathogenesis

Rotaviruses are transmitted orally (smear infection). They infect differentiated enterocytes of the intestinal epithelium – first the upper small intestine and later also the lower small intestine – and multiply in them. Mononuclear cells migrate into the infected tissues. Infected cells swell, generate vacuoles and die. Thereby the villi appear broadened and compressed. Large amounts of viral proteins are detectable as inclusion bodies (viroplasms) in the cytoplasm of infected cells. An increase in Ca^{2+} ion concentration is found in the cells, which is presumably caused by the 7-kDa cleavage product of the NSP4 protein that acts as an enterotoxin. This leads to an increased release of Cl^{-} ions and water. This alteration of ion homeostasis gives rise to further functional damage to enterocytes and the intestinal epithelium, which is manifested as severe diarrhoea. Interaction of the 7-kDa cleavage product with caveolin-1 presumably induces the collapse of the cytoskeleton; additionally, microtubules and microvilli structures disintegrate. Since the 7-kDa cleavage product is secreted by infected cells, it can bind to the surface of uninfected neighbouring cells in the intestinal epithelium, thus triggering the processes mentioned. In addition, the damage to enterocytes impairs the functions of water absorption and nutrient transport systems (e.g. the activity of sucrose-isomaltase is significantly reduced) and also water absorption. The diminished absorption capacity of the intestine gives rise to massive osmotically determined loss of water into the intestinal lumen. This results in severe dehydration, which, if untreated, can end in hypovolaemic shock with circulatory failure.

Immune Response and Diagnosis

In the course of infection, IgM, IgG and IgA antibodies are produced against proteins VP4, VP6, VP7 and NSP4. VP4- and VP7-specific antibodies are neutralizing, recognize the respective virus type and are not cross-reactive. IgA antibodies of the intestinal mucosa are involved in eliminating the virus and confer protection against reinfections. Most children have contact with rotaviruses in their first 3 years, and are protected against re-infections by IgA antibodies. IgA antibodies are also present in the colostrum, and this can explain why breast-fed infants are significantly less affected by rotavirus infections. In rotavirus-infected mice, cytotoxic T cells have been detected in the intestinal mucosa which are directed against epitopes of various structural proteins, and also against the antigenic determinants of the NSP4 protein. It is assumed that they are indispensable for the elimination of the virus.

Direct proof of rotavirus infection is notifiable in most industrialized countries. The diagnosis of an acute infection is done by electron-microscopic detection of virus particles in the stool, or by detecting viral RNA by PCR. The infection is detected in the daily diagnostic routine by antigen detection tests (antigen capture ELISA) or rapid immunochromatographic tests within a few minutes. The commercially available systems detect only viruses of group A. Group-specific antibodies particularly directed against the VP6 protein can be detected by ELISA, and indicate a previous infection. However, this does not play a diagnostic role. Following trypsin treatment to digest the VP4 proteins, group A rotaviruses can be cultivated in monkey kidney cell cultures.

The Development of Vaccines Against Rotavirus Infections Was Beset with Problems

Rotavirus infections cause severe diarrhoea in children. Despite improved therapies by oral or intravenous fluid replacement, more than 800,000 children die of the infection every year, especially in the countries of South Asia as well as Central Africa and southern Africa. Therefore, the development of a vaccine to protect small children from rotavirus infection was given the highest priority of the programmes of international health organizations for a long time (World Health Organization, Global Alliance for Vaccines and Immunizations). A first vaccine was developed on the basis of animal rotaviruses which are non-pathogenic for humans. This tetravalent vaccine was a mixture of live attenuated reassortants which contained the VP7-coding double-stranded RNA segments of human rotavirus types in combination with the remaining segments of a rhesus monkey rotavirus. It was approved for vaccination of infants in the USA in 1998, and was applied to more than 600,000 children with a reliable protective effect. About 1 year later, it became clear that in very rare cases life-threatening intestinal invaginations occurred in some children during the first 2 weeks after vaccination, mostly in those who were older than 3 months at the time of immunization. Although the causal relationship has never been resolved, the vaccine was withdrawn from the market in 1999. The risk of the side effect was calculated to be one case per 30,000–50,000 vaccinated children. On the other hand, extensive use of the vaccine could have saved more than half a million children in developing countries annually – a contradiction that led to fierce discussions.

Therapy and Prophylaxis

There is no antiviral drug against rotaviruses. As a result of the self-limiting course, care must be taken with remedies that inhibit vomiting or diarrhoea. Therefore, treatment is limited to adequate oral or intravenous fluid and electrolyte administration. Currently, worldwide there are two approved vaccines to prevent rotavirus infections in infants. After its initial registration in Mexico, the Dominican Republic and other countries in 2004, a human rotavirus (strain 89-12, type P1A[8]/G1) has been also used for vaccination in Europe. This strain has proven to be attenuated after continuous passages in cell culture, and is administered as an oral vaccine. A pentavalent oral vaccine was approved in the USA in 2006: it is a mixture of five reassortants of human-attenuated bovine rotavirus WC3, whose genome segments 4 and 9 were replaced by the corresponding double-stranded RNA segments of human strains P1[8], G1, G2, G3 and GA. Both vaccines have a very good protective effect without an increased side effect rate. Ideally, vaccination should be done 6 and 24 weeks after birth.

Some Coltiviruses and Orbiviruses Occasionally Infect Humans and Cause Severe Diseases

Other reoviruses can occasionally infect humans by zoonotic transmissions. Colorado tick fever virus and Eyach virus of the genus *Coltivirus* and Kemerovo virus of the genus *Orbivirus* have pathogenic potential for humans. Colorado tick fever virus is transmitted by the tick *Dermacentor andersonii* in the Rocky Mountains in North America, especially to campers, hikers, hunters and forest workers. Rodents, such as squirrels, are considered as a reservoir for the virus. The incubation period of 3–6 days is followed by sudden fever accompanied by headache, retro-orbital pain and severe muscle and joint pain. Particularly adults recover only slowly. Severer progressive forms such as meningoencephalitis and haemorrhagic fever occur in about 5 % of patients, especially in children. The virus probably infects precursor cells of erythrocytes in the bone marrow. On the other hand, Eyach virus is found in European ticks. Antibodies against the virus have been found in patients with meningoencephalitis and polyneuritis. However, the causal relationship is unclear. Kemerovo virus is found in Siberian ticks and has been isolated from blood and cerebrospinal fluid of meningoencephalitis patients.

17.2.6 Animal Pathogenic Reoviruses

Important animal pathogenic reoviruses are found in the genera *Rotavirus* and *Orbivirus*. Rotaviruses cause a neonatal diarrhoea in several animal species, causing, to some extent, considerable economic losses. They are transmitted directly from animal to animal and by water or litter which is contaminated with the pathogens. In contrast, pathogens of the genus *Orbivirus* are typical arboviruses that are transmitted by arthropods and cause systemic infections in the host which are characterized by a viraemia. Their worldwide distribution is ultimately determined by the regional occurrence of the respective transmitting insects. Bluetongue virus of sheep and African horse sickness virus are the most important veterinary orbiviruses. Endemic orbivirus infections did not occur in central European countries until 2006. Subsequently, bluetongue virus serotype 8 was introduced, and spread almost throughout the whole of central Europe in just 2 years. Other orbiviruses, such as Ibaraki virus and epizootic haemorrhagic disease virus of deer, are less important, or are very restricted in their distribution. Human pathogenic orbiviruses have not been described so far.

17.2.6.1 Rotaviruses

Epidemiology and Transmission

Rotaviruses occur in many mammals and birds. Young animals are especially affected during the first few weeks of life. Types G6, G8 and G11 are predominantly

found in cattle, in association with types P[1], P[5] and P[11]. Types G3, G4, G5 and G11 in connection with P[6] and P[7] and rarely with P[13], P[19], P[23], P[26] and P[27] prevail in swine. Phylogenetic analyses increasingly reveal that porcine rotavirus isolates (G4) are closely related to human rotaviruses, which is a clear indication for zoonotic transmission.

Infected animals excrete the virus in large quantities in the faeces, which remains infectious in the environment for weeks. Contagious infections occur mainly through contaminated water and food. An indirect spread of the virus through equipment or litter is frequent. Animals with inapparent infections have also been described as a source of transmission. Infections in swine, cattle and – with certain restrictions – horses are economically important. Rotavirus infections do not play any role in dogs and cats.

Clinical Features

In the offspring of almost all domestic mammals, rotaviruses cause catarrhal enteritis, which is expressed as acute diarrhoea. The incubation period until the onset of disease symptoms is approximately 24 h.

Pathogenesis

The pathogenesis of animal rotavirus infections resembles largely that of humans (Sect 17.2.5). Even in this case, the virus infects enterocytes of the small intestine, affecting their function and destroying them. In young animals, this leads to a malabsorption of milk with the consequence that the remaining lactose in the gut exerts an osmotic effect on the intestinal mucosa, aggravating the diarrhoea. Neither a systemic infection nor viral replication in other tissues has been described so far.

Immune Response and Diagnosis

A local immune response arises in the intestinal mucosa during infection. In contrast, a detectable immune response in the blood is of minor importance. The diagnosis of rotavirus infection can be done easily by electron-microscopic imaging of virus particles from faeces samples. Alternatively, the viruses can also be detected in faeces using commercial antigen capture ELISA. However, the specificity of the antibodies used is crucial for the test. Therefore, only the detection of group A rotaviruses is possible, and this is not necessarily true for all G types. The different regional distribution of certain serotypes in various animal species can also be problematic for testing. The isolation of rotaviruses in cell culture is not always successful; hence, a negative result has only limited validity. The addition of trypsin to the culture medium is important for cultivation of these viruses, as trypsin facilitates attachment and penetration of the virus by cleavage of the VP4 protein.

Control and Prophylaxis

Antibodies present locally in the intestinal lumen protect animals from infection; thus, the administration of antibodies in the milk is the best prophylaxis.

Vaccination of mother animals before birth aimed at increasing the antibody concentration in the blood, and thus also in the colostrum, favourably influences the immune status of young animals. This type of vaccination is frequently practised especially in cattle. By contrast, active immunization of young animals is not successful because infections of animals – contrarily to those of humans – occur in the first few days and weeks of life, and a protective immunity develops only slowly.

17.2.6.2 Bluetongue Virus

Epidemiology and Transmission

Bluetongue virus infections are widespread, and cause some severe diseases in ruminants, particularly in sheep. The known isolates can be subdivided into 24 different serotypes. The virus is transmitted by midges of the genus *Culicoides*. These midges are very small (roughly 1 mm). The virus persists in midges, but a vertical transmission has not been demonstrated. Approximately 1 week after sucking on a viraemic animal, the midge excretes the virus in the saliva for the first time. Since midges suck blood on average every 3–4 days, a high density of insects leads very rapidly to the infection of an entire flock. Since midges swarm especially in summer and late summer, cases of bluetongue disease occur more frequently in July to October in the northern hemisphere. The virus can persist in infected ruminants for several weeks. However, the virus is ultimately eliminated, and the animals are immunologically protected against re-infections. A direct transmission from animal to animal has not yet been described. Nearly all 24 serotypes of bluetongue virus are present in Africa, whereas only a few serotypes are common in European countries. Whereas serotypes 1, 2, 4, 8, 9 and 16 have been detected in some Mediterranean countries, only serotype 8 has spread into central Europe, in 2006 3 years later, bluetongue virus serotype 1 and then serotypes 6 and 11 were detected sporadically. Today, the infection has probably become endemic in native populations of sheep, cattle and wild ruminants in several regions of southern and central Europe. The diversification of serotypes is also influenced by the distribution of certain *Culicoides* species: whereas the serotypes that prevail in Mediterranean countries are transmitted by *C. imicola*, serotype 8 is transmitted by *C. obsoletus*. The original geographical distributions of serotype 1 (southern France) and serotype 8 (including northern France, the Benelux countries and Germany) have constantly expanded and are converging. It remains to be seen whether the two serotypes develop preferences for different mosquito subspecies when they have a common regional occurrence.

Clinical Features

The clinical picture of ovine bluetongue disease resembles that of foot-and-mouth disease (▶ Sect. 14.1.6). The symptoms correspond to those of a febrile stomatitis and pneumonia. They are accompanied by coronitis (inflammation of the coronary band) with hyperaemia and formation of oedemas. The name of the disease originates from a commonly observed cyanosis (blue colour) of the tongue. The disease can be associated with a lethality of up to 80 %. The clinical picture of the disease

differs among different ruminant species, and the symptoms exhibit great variability even between several sheep breeds. Abortions and deformities have also been described in association with infections, which possibly result from attenuated vaccine virus strains. Infections with southern European bluetongue virus serotypes 2, 4, 9 and 16 are subclinical in cattle and goats; however, serotype 8 causes clinical cases in cattle. Similarly, bluetongue diseases have also been observed in cattle in France, and occurred during outbreaks of bluetongue virus serotype 1 infections. In cattle the disease entails considerable greater economic losses than in sheep.

The Biology of Midges Is Complex

The eggs of *Culicoides* midges (gnats) develop not in water, but – depending on the *Culicoides* species – in dry soil, decomposing plant material or in cattle or elephant dung. This partly explains the wide distribution of certain *Culicoides* species. Since it has not been possible to simulate these complex developmental conditions in the laboratory so far, the study of the biology of the virus-vector relationship is very difficult. For the establishment of the pathogen in a region, questions regarding a possible trans-stage transmission within the vector and the relevance of hibernation in the adult gnat are of particular interest.

Pathogenesis

The virus replicates in endothelial cells of blood vessels and in haematopoietic stem cells. Therefore, it can be isolated from lymphocytes and erythrocytes during the viraemic phase. In rare cases, it can also be isolated from the semen of viraemic rams. Nevertheless, a possible transmission of the virus without insects as intermediate hosts is epidemiologically irrelevant.

Immune Response and Diagnosis

During infection, the animals produce serotype-specific neutralizing antibodies which lead to the elimination of the virus and protect against re-infections. They can be detected by ELISA, whereby the selection of the antigen (whole virus or recombinant proteins) of the relevant serotypes is critical. The diagnosis of acute infections is performed by isolation of the virus from peripheral blood cells and cultivation in embryonated chicken eggs or in cell culture. Alternatively, detection of viral RNA is also possible by using the PCR technique.

Control and Prophylaxis

Bluetongue virus infection is a notifiable animal disease. Control is currently limited to observation of the outbreak and trade restrictions on infected animals. Vaccination against bluetongue disease is possible. Live vaccines are used in African and Mediterranean countries. This entails the inherent risk of the generation of reassortants between the vaccine virus and field viruses. In European countries,

the use of an inactivated vaccine with bluetongue virus serotype 8 has been favoured as a strategy for the control of infection. Since 2005, appropriate vaccines have been continuously introduced and approved in most European countries.

Ovine Bluetongue Disease: A Novel Virus Infection in Central Europe

The occurrence of bluetongue virus in central and northern Europe was not known until a few years ago. Since 2006, infections have spread from southern and western Europe into central European countries, including Germany, Austria and Switzerland. Midges (*Culicoides* spp.) have increasingly been identified as carriers of the infection in livestock populations of sheep, cattle and goats. It is believed that even other members of insect-borne orbiviruses are now able to spread in central and northern Europe under the influence of climatic changes. Bluetongue virus is very stable: owing to its insect-borne transmission, dissemination of the virus cannot be prevented by hygiene measures. Since bluetongue disease in sheep is very severe and associated with a high mortality rate, vaccination of all ruminants is mandatory in countries of southern and central Europe inactivated vaccines from three different manufacturers have been approved by the respective European authorities. According to statistical data, no side effects have been demonstrated by the use of those vaccines. However, the mandatory vaccination of animals led to violent protests in some regions, particularly in Germany. Some animal owners refused to vaccinate their sheep and cattle; this led to convictions and fines, which were converted into prison sentences by pressure from the anti-vaccination movement. These sentences were served, attracting considerable public attention. Even penitential pilgrimages were organized in some regions of Bavaria – in the hope of promoting understanding among the authorities responsible to achieve the cessation of compulsory vaccination. Unexpectedly, these actions were successful in the autumn of 2009: The mandatory vaccination was suspended for 2010. This approach was initiated against professional advice and beyond all reason. This measure will lead to a resurgence of bluetongue virus infection, which had almost disappeared. It will be of great economic importance and will cost many lives of ruminants.

17.2.6.3 African Horse Sickness Virus

Epidemiology and Transmission

African horse sickness virus is very similar to bluetongue virus and infects solidungulates. This virus is also transmitted by arthropods and is characterized by a pronounced antigenic diversity, which is manifested in at least nine serotypes. The virus is widespread in Africa. It is not endemic outside Africa, with the exception of Spain and Portugal. It is transmitted to horses and other equines such as asses and zebras mainly by arthropods of the genus *Culicoides*, but also by mosquitoes. African horse sickness also shows a seasonal occurrence in late summer owing to the preferred period during which these insects fly. Since these

insects fly especially at night, animals that are not housed during the night are primarily infected. It is essential that the pathogen of African horse sickness, like bluetongue virus, does not establish persistent infections in mammals. As zebras develop only a subclinical form of infection, they are thus considered to be both the main reservoir and the principal host.

Another orbivirus, equine encephalosis virus, is found in southern Africa. Probably, it also comprises different serotypes. It also infects solipeds, but it differs serologically from African horse sickness virus. It has been hardly studied, and it causes central nervous system symptoms in animals, which are probably consequences of cerebral oedema.

Clinical Features

The clinical signs and the severity of African horse sickness differ depending on the virulence of the serotype and the animal species affected. Horses are highly susceptible and show the highest morbidity and mortality, whereas the infection often develops subclinically in donkeys. Mules and hinnies exhibit intermediate susceptibility. The incubation period until the onset of symptoms is 1–2 weeks. Acute or hyperacute diseases are characterized by severe respiratory symptoms (pulmonary form), and are primarily manifested by high temperature and severe respiratory distress (caused by pulmonary oedemas). The fever phase lasts about 1 week. Afterwards, generalized oedema and bruising occur, and may result, in severe cases, in death of the animals. However, the more frequent form is a mild, protracted illness (cardiac form), which in addition to fever, displays signs of vasculitis and infections of superficial vessels (such as in the conjunctiva).

In Rare Cases, African Horse Sickness Virus Also Infects Other Mammals

There is evidence that besides solidungulates also other mammals can occasionally be infected with African horse sickness virus: sporadic infections of dogs which presumably had eaten infected meat of dead horses have been described. The dogs exhibited clinical symptoms that were similar to those of the pulmonary disease form of horses. Furthermore, illnesses of humans which were caused by infections with African horse sickness virus have also been reported. These were unexceptionally laboratory-borne infections within vaccine manufacturing companies. The symptoms observed in humans are multifarious, and include signs of encephalitis.

Pathogenesis

The pathogenesis of African horse sickness is poorly understood. The virus replicates in lymphatic organs and in the vascular endothelium. Similar to bluetongue disease, haematopoietic cells are infected in the course of infection. In severe cases, the animals finally die of the consequences of the resulting oedema and effusions (pericardial effusion, ascites).

Immune Response and Diagnosis

Animals develop an effective immune response during infection which eliminates the virus. It is serotype-specific, and thus it does not protect animals from infections by viruses of other serotypes. In animals that have survived African horse sickness, infections with other serotypes are usually considerably milder and are manifested mainly in the cardiac form.

Control and Prophylaxis

Attenuated live vaccines are available in Africa, and encompass all serotypes. They are not used outside Africa.

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Viruses with Single-Stranded RNA Genomes and Double-Stranded DNA as an Intermediate Product

18

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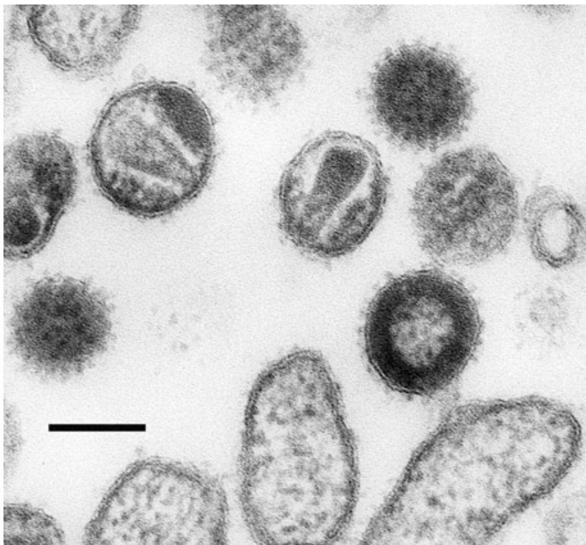
18.1 Retroviruses



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Retroviruses were described for the first time about 100 years ago: in 1908, Vilhelm Ellermann and Oluf Bang demonstrated the transmission of avian leucosis by cell-free filtrates; shortly afterwards, Peyton Rous discovered that avian sarcoma can be transmitted to healthy chickens by using filtered cell-free tumour extracts in 1911. Thanks to this discovery, he was awarded the Nobel Prize in Physiology or Medicine in 1966, and the retrovirus contained in the extracts has been named Rous sarcoma virus. Further evidence that retroviruses may cause tumours was provided by John J. Bittner through his research on the development of malignant mammary neoplasms in mice in 1936. He described mouse mammary tumour virus as the causative agent of the disease. This virus also displayed a previously unknown transmission mode: mouse mammary tumour virus is not only transmitted as an infectious, exogenous particle that is released by the cell (horizontal transmission), but it can also be passed on to the next generation as an endogenous constituent of the genome of germ-line cells (vertical transmission).

In 1970, Howard M. Temin and Satoshi Mizutani as well as David Baltimore (Temin and Baltimore were awarded the Nobel Prize in Physiology or Medicine in 1975) discovered that retroviruses possess the genetic information for a previously unknown enzyme that enables these viruses to reverse the general one-way flow of genetic information from DNA through RNA into protein: The reverse transcriptase is able to transcribe RNA into double-stranded DNA, an activity that is reflected in the term “retrovirus”. Viruses of this group had already been shown to be causative agents of tumours in animals.

Therefore, they are referred to as oncornaviruses. This neologism links the oncogenic potential with the RNA genome of the virus. The fact that the tumour-generating capability, which resides in the genetic information in form of viral “oncogenes”, has a cellular counterpart was described by Harold E. Varmus, J. Michael Bishop, Peter K. Vogt and Dominique Stehelin in 1976. Because of that discovery, Bishop and Varmus were awarded the Nobel Prize in Physiology or Medicine in 1989.

In 1980, Robert C. Gallo described human T-lymphotropic virus (HTLV) as the first retrovirus that can cause cancer in adults, namely T-cell leukaemia. Shortly afterwards, the groups of Luc Montagnier and Francoise Barre-Sinoussi at the Pasteur Institute in Paris identified human immunodeficiency virus (HIV) 1 and HIV-2 as retroviruses and causative agents of acquired immune deficiency syndrome (AIDS), for which they were awarded the Nobel Prize in Physiology or Medicine in 2008. The intensive research activity which followed these findings has resulted in many details of the molecular biology and pathogenesis of retroviral infections being well known today, and has also resulted in these pathogens belonging to the best studied viruses. In addition, a number of antiviral chemotherapeutic agents are available (▶ Chap. 9). It is impossible to discuss all retroviruses, the details of their replication cycles and their molecular biology within this chapter. For interested readers, there are numerous review articles on the biology of retroviruses. This chapter provides an overview of replication and infection mechanisms that are common to all retroviruses. Human pathogenic retroviruses, particularly the different types of HIV and HTLV, are described in detail.

Nonetheless, retroviruses also cause important diseases in domestic animals. Some infections are important for animal health purposes, e.g. enzootic bovine leucosis and equine infectious anaemia. Other virus types cause severe, lethal diseases in small animals, such as feline leukaemia and feline immunodeficiency. Particularly, the latter is of paramount importance, since infections by feline immunodeficiency virus (FIV) have become a valuable model for investigating AIDS and HIV.

18.1.1 Classification and Characteristic Prototypes

The family *Retroviridae* is divided into two subfamilies: *Orthoretrovirinae* and *Spumaretrovirinae*. Whereas the subfamily *Spumaretrovirinae* contains only the single genus *Spumavirus*, the subfamily *Orthroretrovirinae* comprises six genera, namely *Alpharetrovirus Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus* and *Lentivirus* (Table 18.1). The division was made on the basis of the characteristics of infections and the disease forms caused by them, as well on the basis of morphological and genetic differences among the viruses. Retroviral infections occur predominantly in vertebrates, where they cause many different symptoms (neoplastic diseases, immune deficiencies, neurological disorders, but also apparently completely harmless infections).

Furthermore, a distinction can be made between exogenous and endogenous retroviruses. The former possess all the genetic information which is required for the execution of an infection cycle, including the release of infectious virus particles. These viruses are able to spread from organism to organism. In addition, the genome of certain exogenous retroviruses contains oncogenes.

In contrast, endogenous retroviruses are integrated into the genome of all cells of an organism and are transmitted vertically through germ-line cells. Only under certain circumstances can they be activated for the production of exogenous, infectious particles, inasmuch as almost all of these viruses are defective, lacking some genetic information that is essential for a productive infection cycle, e.g. the gene encoding the surface protein. In some retroviruses isolated from mammals and birds, these missing functions are replaced by the sequences of viral oncogenes. To produce and spread infectious viruses, these defective animal retroviruses require the aid of another retrovirus (helper virus), which can complement the lacking functions. However, many of the endogenous retroviruses have been genetically reduced to such extent that even helper viruses cannot activate them any more, as only the long terminal repeat (LTR) regions, which once flanked the proviral genome, remain frequently preserved. Only the similarity of their DNA, which is integrated in the cellular genome, indicates that they constitute original retroviral sequences. These retrotransposons are widespread and compose, along with other retroelements, up to 8 % of the human genome. The fact that they can also be found in the genome of rodents (intracisternal A-type particles), yeast (Ty elements) and insects (copia elements) indicates that they are evolutionary highly conserved.

Table 18.1 Characteristic prototypes of retroviruses

Subfamily	Genus	Human virus	Animal virus	Type
Orthoretrovirinae	*Alpharetrovirus*		Avian leucosis virus	Exogenous/ infectious
			Rous sarcoma virus	Exogenous/ infectious
			Avian erythroblastosis virus	Exogenous/ infectious
			Avian myeloblastosis virus	Exogenous/ infectious
			Rous-associated viruses (1–50)	Exogenous/ infectious
			Rous-associated viruses	Exogenous/ infectious
	Betaretrovirus		Mouse mammary tumour virus	Endogenous and exogenous/ infectious
			Jaagsiekte sheep retrovirus (bovine pulmonary adenomatosis virus)	Exogenous/ infectious
			Mason–Pfizer monkey virus	Endogenous/ defective
		HervK family		Endogenous/ defective
	Gammaretrovirus		Feline leukaemia virus	Exogenous/ infectious
			Feline sarcoma viruses	Exogenous/ infectious
			Murine leukaemia virus	Exogenous/ infectious
			Simian leukaemia virus	Exogenous/ infectious
			Gibbon ape leukaemia virus	Exogenous/ infectious
			Murine sarcoma viruses	Exogenous/ infectious
			Moloney mouse sarcoma virus	Endogenous/ defective
			Harvey murine sarcoma virus	Endogenous/ defective
		Erv-3		Endogenous/ defective
		S71 family		Endogenous/ defective

(*continued*)

Table 18.1 (continued)

Subfamily	Genus	Human virus	Animal virus	Type
	Deltaretrovirus		Bovine leukaemia virus	Exogenous/ infectious
		Human T-lymphotropic viruses 1–3	Simian T-lymphotropic viruses 1–3	Exogenous/ infectious
		HRES-1		Endogenous/ defective
	Epsilionretrovirus		Various fish retroviruses	Exogenous/ infectious
			Walleye dermal sarcoma virus	Exogenous/ infectious
	Lentivirus	Human immunodeficiency viruses 1 and 2	Simian immunodeficiency virus	Exogenous/ infectious
			Feline immunodeficiency virus	Exogenous/ infectious
			Bovine immunodeficiency virus	Exogenous/ infectious
			Equine infectious anaemia virus	Exogenous/ infectious
			Maedi–visna virus	Exogenous/ infectious
			Caprine arthritis encephalitis virus	Exogenous/ infectious
Spumaretrovirinae	*Spumavirus*	Human spumaretroviruses[a]	Simian foamy virus[a]	Exogenous/ infectious
			Feline spumaviruses	Exogenous/ infectious
			Bovine spumaviruses	Exogenous/ infectious
			Equine spumaviruses	Exogenous/ infectious

HRES human T-lymphotropic virus related endogenous sequence
[a]Human spumaretrovirus has proven to be identical to simian foamy virus

They are responsible for a reverse transcriptase activity and particulate protein structures frequently being found in eukaryotic cells in connection with differentiation processes.

Spumaretroviruses: An Exceptional Class of Retroviruses
Spumaviruses are common in wild as well as in captive chimpanzees, in various Old Word and New World monkeys, cats, cattle and horses without

producing diseases in the host animals. Simian spumaretrovirus, also referred to as simian foamy virus (SFV), can infect exposed humans such as zoo-keepers. Even the zoonotic transmission and infection of humans do not lead to any disease. Human spumaretrovirus, which is also known as human foamy virus, is identical with SFV; it was isolated from a human nasopharyngeal carcinoma cell culture in 1971. At that time, this virus was considered to be the first retrovirus isolated from human cells, whereas it is now known to have been a contamination. Only the subsequent sequencing of the genome revealed the identity of the two viruses. Spumaviruses occupy an exceptional position within the family *Retroviridae*; they display a variety of specific features concerning their replication cycle which distinguish them from other retroviruses; therefore, they have been classified into a separate subfamily, *Spumaretrovirinae*. The Pol gene product is translated from a separate spliced messenger RNA (mRNA), independently of the Gag protein, i.e. a Gag–Pol precursor protein does not exist in spumaviruses. Another special feature of spumaviruses is an internal promoter in the 3′ region of the viral *env* gene, which is regulated by their own transactivator Tas (also called Bel1). Further open reading frames encode the accessory proteins Bel2 and Bet, whose functions remain to be explored. An RNA progenome is synthesized during replication, and is transcribed into DNA by the reverse transcriptase during morphogenesis, similar to hepatitis B virus (▶ Sect. 19.1.4). There is experimental evidence that infectious particles of spumaviruses already contain a DNA genome which can be directly integrated into the genome of the host.

18.1.2 Structure

18.1.2.1 Virus Particle

Infectious particles of the various retroviruses have a similar structure with a diameter of approximately 100 nm (Fig. 18.1). The capsid is surrounded by an envelope, which is derived from the cytoplasmic membrane. The viral glycoproteins are associated with the envelope, one of which is anchored as a transmembrane protein by a region of about 20 hydrophobic amino acids. On the other hand, the so-called exterior glycoprotein (EP) is non-covalently linked with the part of the transmembrane protein that is located outside the membrane. Both are synthesized as a common precursor protein. Cleavage and generation of the amino-terminal external moiety and the carboxy-terminal transmembrane domain is done by a cellular protease during viral morphogenesis which is associated with the endoplasmic reticulum (ER) and the Golgi apparatus. The external and transmembrane proteins of HIV-1, which are modified by sugar groups, have a molecular mass of 120 kDa (gp120) and 41 kDa (gp41), respectively. The functionally active complexes of gp120 and gp41 proteins are trimers. Cryo-electron-microscopic analyses revealed that about 14 and 73 such protein

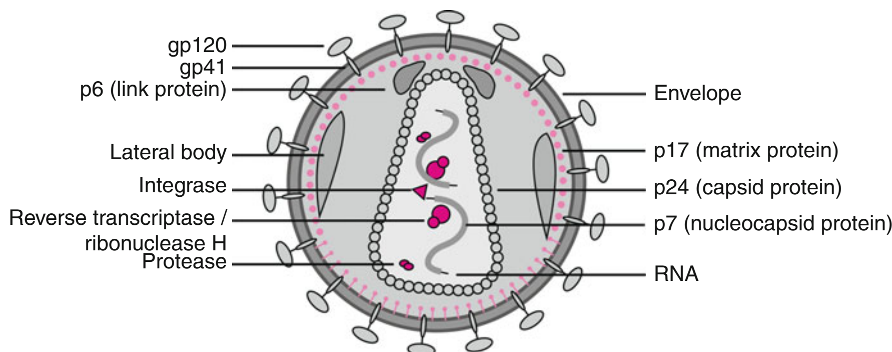


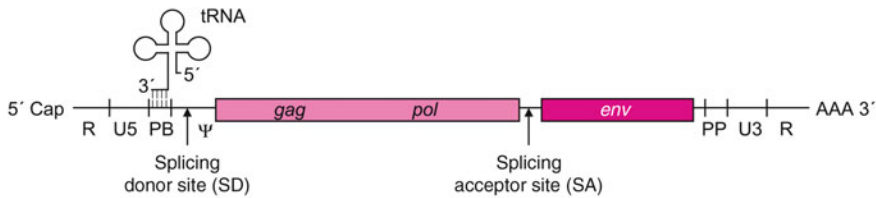
Fig. 18.1 Structure of a retrovirus particle represented by human immunodeficiency virus 1 (HIV-1). The conical capsid is situated inside the particle. It consists of the capsid proteins (p24) and contains two viral RNA genomes, which are complexed with nucleocapsid proteins (p7), exhibiting all the characteristics of a cellular messenger RNA (mRNA). The capsid is enclosed by an envelope containing the viral external and transmembrane glycoproteins gp120 and gp41. The membrane inside is coated by a matrix protein layer (p17). The link protein (p6) connects the capsid with the envelope. The lateral bodies consist mainly of matrix proteins, and probably represent electron-microscopic artefacts

complexes are associated with the envelope of HIV-1 and simian immunodeficiency virus (SIV) particles, respectively.

The matrix proteins (MA) are linked with the inside of the envelope by amino-terminally attached myristic acid residues. In lentiviruses, matrix proteins are trimeric and form a net-like protein layer, which confers an isometric structure on the virions. In the particle interior, the viral capsid or core is located mainly in the centre, which – depending on the virus type – has a spherical (in alpharetroviruses, betaretroviruses, gammaretroviruses, deltaretroviruses and spumaviruses) or a conical (in some betaretroviruses, such as Mason–Pfizer monkey virus, and lentiviruses) shape. Only in some betaretroviruses, such as mouse mammary tumour virus, is the capsid eccentrically arranged in the virus particle. It is composed of capsid proteins (CA), which are, like matrix proteins, components of the group-specific antigens (Gag proteins). The capsids harbour two identical single-stranded RNA genomes, which are neither covalently linked nor connected by base pairing with each other. The RNA molecules are complexed with nucleocapsid proteins (NC), which are also components of the Gag proteins. A further member of this protein group, the link protein (LI) forms the linkage between the capsid and the envelope. It has a molecular mass of 6 kDa, and was first described in HIV. Further viral components of the particle are the enzymes reverse transcriptase, integrase, and protease.

18.1.2.2 Genome Organization and Structure

The genome of retroviruses consists of single-stranded RNA, containing a 5′-cap structure and a 3′ poly(A) tail, i.e. all the features of eukaryotic mRNA (Fig. 18.2a). Depending on the virus type, the RNA can have a length of 7,000 nucleotides in



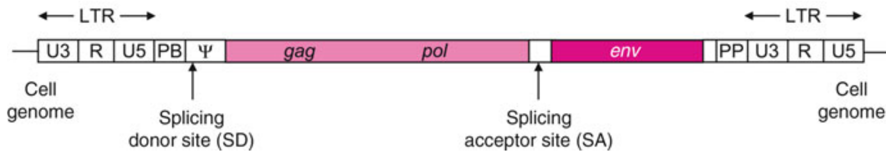


Fig. 18.2 Genome organization of retroviruses. The sequence elements and genes that are found in all retroviral genomes are shown. (**a**) Arrangement of the sequence elements and open reading frames that are found in the RNA genome of infectious retrovirus particles. The genome is modified at the 5′ end with a cap structure; the 3′ terminus is polyadenylated. A transfer RNA (*tRNA*) is bound at the primer binding site (*PB*), ψ indicates the sequence by which the RNA genome interacts with the nucleocapsid during morphogenesis. *R* redundant regions, *U3* and *U5* unique regions at the 3′ and 5′ termini, respectively, *PP* polypurine site. The coding regions for Gag, Pol and Env proteins are indicated by *coloured bars*. *SD* and *SA* indicate the location of the splice donor and acceptor sites for the synthesis of the mRNAs from which the Env proteins are translated. (**b**) Arrangement of sequence elements and open reading frames in the genome of the provirus after its integration into the genome of the host cell. *LTR* (long terminal repeat) indicates the arrangement of the sequence elements that are synthesized during reverse transcription

murine leukaemia virus, a member of the gammaretroviruses, about 9,000 or 9,200 nucleotides in HTLV and HIV, respectively, and up to 12,000 nucleotides in SFV. A cellular transfer RNA (tRNA) molecule is linked to the primer binding (PB) site, a sequence of 18 nucleotides located in the 5′ region of the genome. This interaction is mediated by complementary bases at the 3′ end of the tRNA. The amino acid specificity of the tRNA differs among the various virus types. It is $tRNA^{Lys}$ in HIV and SFV, and $tRNA^{Pro}$ in HTLV. The genomes of all infectious retroviruses encode Gag (group-specific antigen), Pol (polymerase, enzymatic activities such as reverse transcriptase, protease, integrase) and Env (envelope, glycoproteins) gene products. The complex retroviruses such as lentiviruses, spumaviruses and deltaretroviruses have additional genes that encode regulatory and accessory proteins. They are frequently encoded by several exons and are translated from multiply spliced mRNA species (Fig. 18.3).

The coding regions are flanked by important regulatory sequences at the 5′ and 3′ termini which are essential for reverse transcription and integration of the viral genetic information into the cellular DNA. The following regions can be distinguished (Fig. 18.2a):

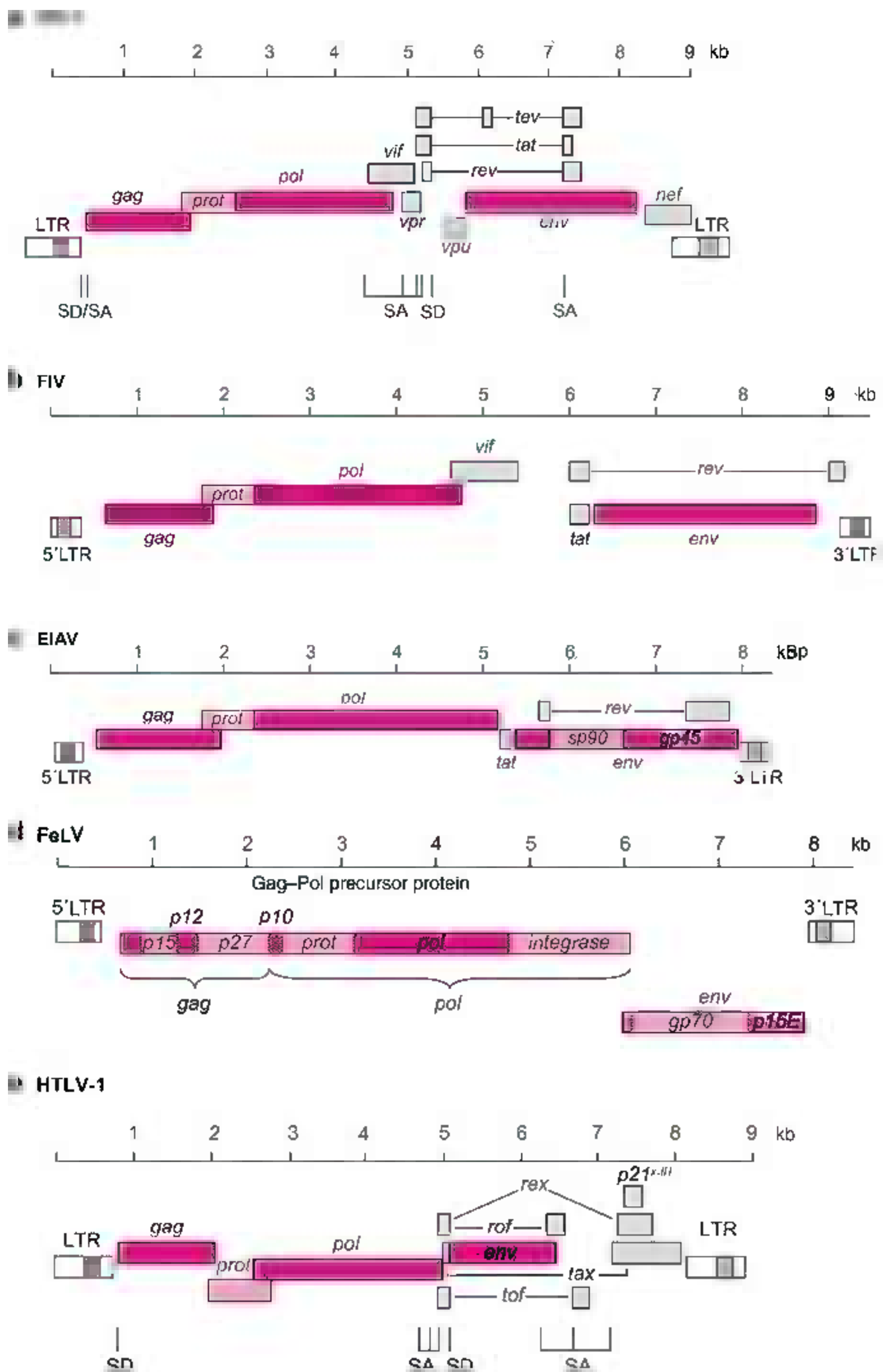


Fig. 18.3 (continued)

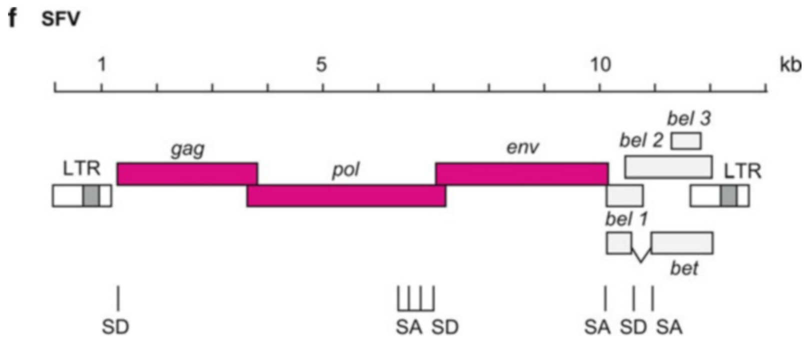


Fig. 18.3 Genome structure of different human and animal pathogenic retroviruses. The reading frames of the gene products are designated by abbreviations, which are explained in the text. Exons of the regulatory proteins that are translated from spliced mRNAs are interconnected by *lines*. The different shading of the open reading frames indicates the different reading frames that are used. *SD* and *SA* indicate the different splice donor and acceptor sites used during transcription. (**a**) Human immunodeficiency virus 1 (*HIV-1*). (**b**) Feline immunodeficiency virus (*FIV*). (**c**) Equine infectious anaemia virus (*EIAV*). (**d**) Feline leukaemia virus (*FeLV*). (**e**) Human T-lymphotropic virus 1 (*HTLV-1*). (**f**) Simian foamy virus (*SFV*)

1. The R region ("R" for "redundant") is between 15 (betaretroviruses) and 240 (HTLV and bovine leukaemia virus, BLV) nucleotides in length, and is located adjacent to the cap structure at the 5′ end of the genome; it is also present in identical sequence and orientation at the 3′ terminus.
2. The neighbouring U5 region ("U" for "unique") is situated downstream of the R region at the 5′ end of the genome. It encompasses 75 nucleotides in gammaretroviruses and up to 200 nucleotides in deltaretroviruses (HTLV and BLV); this region contains the nucleotide sequences that are essential for the integration of the provirus into the cellular genome.
3. The PB site has a length of 18 nucleotides and follows the U5 region; it is complexed with the 3′ end of a tRNA molecule via base-pairing.
4. The sequences located between the PB site and the start of the *gag* gene is known as the leader region; its length differs among the different retrovirus types and it can include up to 475 nucleotides (gammaretroviruses). A splice donor site is located in this region, and is used to produce all spliced mRNA molecules. Many retroviruses produce only one spliced mRNA form during the infection cycle, and this encodes the distinct glycoproteins. However, the same splice donor site is also used to generate many of the multiply spliced mRNA molecules, from which the regulatory and accessory proteins are translated in the complex retroviruses such as lentiviruses, HTLV, and spumaviruses. The short nucleotide sequence of the ψ site is located adjacent to the splice donor site. It is necessary to attach the RNA genomes to nucleocapsid proteins in the developing virus particle during morphogenesis.
5. Downstream of the leader region, there is the coding region, which, depending on the virus type, can have a different length, thus determining the respective

genome length (Figs. 18.2 and 18.3). A short non-coding region can follow afterwards. However, in deltaretroviruses, lentiviruses and spumaviruses, the transition from the coding region to the following sequence elements is direct, or overlaps with them. A polypurine tract resides at this site, a sequence of at least nine adenosine and guanosine residues that is characteristic of all retroviruses. It plays an important role in initiating double-stranded DNA synthesis during reverse transcription.

6. The polypurine tract is followed by the U3 region, which is denominated, analogous to the U5 region, according to its location at the corresponding genome terminus. Depending on the virus type, the U3 region has differing length: in betaretroviruses (mouse mammary tumour virus), it can encompass more than 1,200 nucleotides and contain coding sequences; it has about 450 and 800 nucleotides in lentiviruses and spumaviruses, respectively. Since, after transcription of the RNA genome into double-stranded DNA (Fig. 18.2b) these sequences constitute the 5′ end of the LTR, which is situated upstream of the genes, important regulatory sequences are also located in this region that mediate the integration process, similar to the U5 region. In addition, the U3 region is also essential for gene expression of the provirus that is integrated into the genome of the host cell, as it contains promoters and *cis*-active elements that are bound by transactivating cellular proteins, thus regulating viral transcription and gene expression.
7. The U3 region is followed – as mentioned above – by a further R region, which is also followed by a poly(A) tail of approximately 200 adenosine residues.

18.1.2.3 LTR Region and Promoter

After reverse transcription, the viral genetic information is integrated in the cellular genome as double-stranded DNA. This process is described in detail in Sect. 18.1.4. The integrated proviral genome has identical terminal sequences, which are generated during reverse transcription of single-stranded RNA into DNA. These sequences flank the viral genes, and are denominated "long terminal repeats" (LTR). These repeat units consist of the regions U3, R and U5, which are situated at the ends of the genome and have the same orientation. The LTR contains all *cis*-acting sequences, promoters and enhancers that control retroviral gene expression. An internal promoter occurs in the *env* gene region only in spumaviruses; it controls the transcription of the *bel* non-structural genes. Cellular, *trans*-acting proteins bind primarily to U3 sequences and induce transcription of the integrated viral genes. Besides the interaction of viral glycoproteins with specific cell surface components, this process ensures cell-type-dependent and differentiation-dependent infection. An example is mouse mammary tumour virus, in which DNA sequences have been characterized in the U3 region, to which activated glucocorticoid receptors bind. This virus is transmitted via maternal milk to the progeny; therefore, this ensures that gene expression and viral production is activated exclusively in the mammary glands of lactating mice.

Several different *cis*-acting control elements, which interact with specific cellular DNA-binding proteins, are located in the U3 region of human retroviruses. A very important regulatory sequence in HIV is the binding site for nuclear factor κB (NFκB),

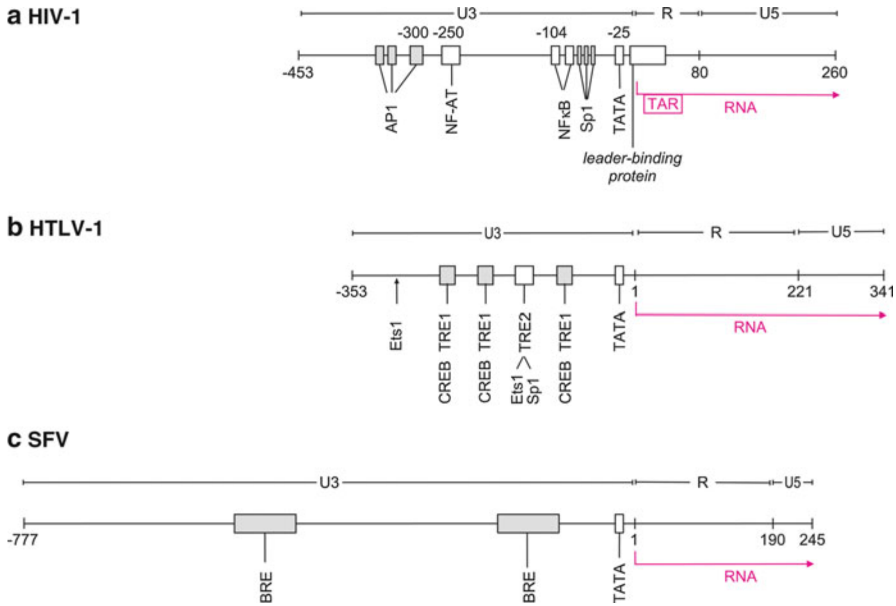


Fig. 18.4 The long terminal repeats (LTR) containing the regions U3, R and U5 in the proviral genome of human retroviruses. The start site of mRNA synthesis (*red*), the localization of the *cis*-acting control elements in the promoter region, its description and the cellular and viral factors that interact with them to influence the transcription activity are indicated. *BRE* Bel response element, *CREB* cyclic AMP response element binding protein, *NF-AT* nuclear factor of activated T cells, *NFκB* nuclear factor κB, *TAR* transactivation response, *TRE* Tax response element

a transcriptional activator protein. After stimulation by cytokines such as IL-1 and TNF-α, NFκB is present in its active form in T lymphocytes, inducing not only the expression of various immunologically active genes, but also that of viral genes. The NFκB-dependent gene expression is very important for the pathogenesis of HIV, as any stimulation of the immune system by other infections induces HIV gene expression. The particles formed infect, in turn, T lymphocytes and induce further damage to this cell population. Up to three NFκB-binding sites are found in the U3 region of the different HIV subtypes (clades). Three such *cis*-acting sequence elements have only the subtype C viruses, which are the most widespread today; compared with the viruses of other clades, they are present in much higher concentrations in infected individuals. This and the frequent transmission may possibly be associated with the threefold redundancy of NFκB-dependent control elements in the promoter region, which permit an efficient expression of the viral genome in T cells and macrophages. In addition to the NFκB-dependent, *cis*-acting sequences, several additional control elements are found in the U3 regions of HIV, including binding sites for the factors Sp1 and AP2 and for leader-binding protein 1 (Fig. 18.4).

The HTLV promoter also has a complex structure; it possesses several recognition sites for cellular transactivators as well. However, the HTLV gene expression

is additionally modulated by viral *trans*-acting proteins (Tax proteins), which interact, in association with cellular proteins, with multiple Tax response elements (TREs) in the U3 region. Similarly, the Tas or Bel1 protein of spumaviruses is a transactivator that enhances gene expression. It binds to Bel response element (BRE) sequences in the U3 region of these viruses. A similar *trans*-acting effect has long been postulated for the Tat protein of HIV; however, the expression-enhancing effect of this protein has a different molecular basis.

18.1.3 Viral Proteins

18.1.3.1 Group-Specific Antigens (Gag Proteins)

The matrix, capsid and nucleocapsid proteins of infectious virions belong to the complex of group-specific antigens. A link protein (p6) has been identified in HIV, which is also attributed to the Gag proteins, and links the vertex of the conical capsids with the envelope. During viral morphogenesis, Gag proteins are synthesized as a common precursor product that is cleaved by the viral protease into the individual components that can be found in the released infectious virus particles (Fig. 18.5). The molecular mass of the Gag precursor proteins is 55 kDa in HIV, 48 kDa in HTLV-1 and 74 kDa in SFV. The sequential arrangement of individual Gag components in the precursor protein coincides among the different retroviruses: the sequences encoding the matrix protein are located in the amino-terminal region; they are followed by the sequences encoding the capsid proteins as well as the nucleocapsid and link proteins at the carboxy-terminal region. Gag precursor proteins are synthesized on free ribosomes in the cytoplasm of the cell. The myristoylation reaction is performed cotranslationally at the α-amino group of a glycine at position 2. For this purpose, the amino-terminal methionine is removed. Cellular factors carry the modified Gag precursor proteins to the cytoplasmic membrane of the infected cell, with which they interact via fatty acids. Table 18.2 gives a comparative overview of the molecular characteristics and functions of proteins encoded by human retroviruses.

Gag proteins have various functions that contribute to viral infectivity:

1. Gag precursor proteins are necessary for the formation of particle structures; other viral proteins and RNA genomes are not required. If the Gag precursor proteins are produced per se in eukaryotic cells, they attach to the cytoplasmic membrane and form small, virus-like lipid–protein vesicles (virus-like particles) that bud from the cell surface. The pertinent active regions reside in the matrix and capsid protein moieties.
2. Nucleocapsid proteins contain amino acid sequences that specifically interact with the ψ sequences in the leader region of RNA genomes. In HIV and HTLV, this protein–nucleic acid interaction is mediated by domains that are similar to the zinc finger motifs of DNA-binding proteins. In spumaretroviruses, binding to the RNA is probably mediated by a basic, arginine-rich region in the nucleocapsid protein. After penetration of the virus into the cell, nucleocapsid proteins remain associated with the RNA genomes and are part of the preintegration

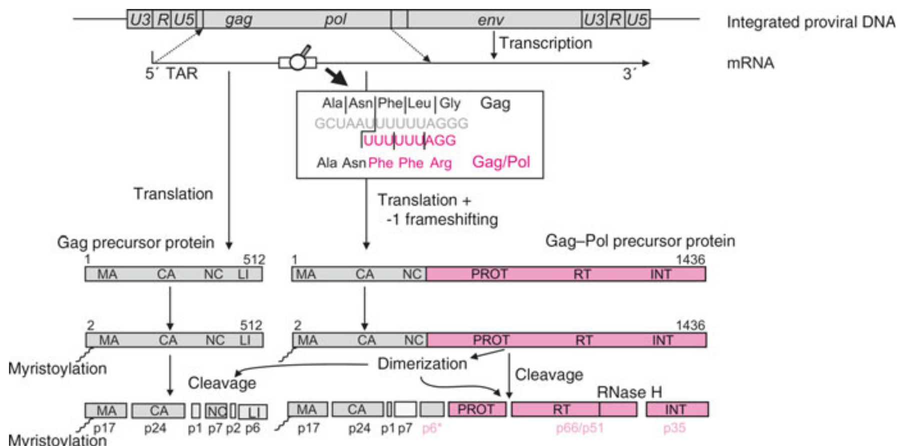


Fig. 18.5 Synthesis of Gag and Gag–Pol proteins in HIV-1 (strain BH102). An mRNA molecule is synthesized from the integrated proviral DNA, which starts at the R region in the 5′ LTR and includes the entire viral genome. The Gag and Gag–Pol proteins are translated from this transcript. A uridine-rich sequence is located in the region that encodes the nucleocapsid protein. A ribosomal −1 frameshift occurs in about 5 % of translation events, which has the consequence that the stop codon of the Gag polyprotein is skipped, resulting in the synthesis of a Gag–Pol fusion protein. Both the Gag protein and the Gag–Pol precursor protein are myristoylated at the amino terminus. The Gag–Pol product contains the viral protease in its sequence, which, after dimerization, autocatalytically cleaves itself from the polyprotein, and subsequently processes the precursor proteins into the individual components. *CA* capsid protein, *INT* integrase, *LI* link protein, *MA* matrix protein, *NC* nucleocapsid protein, *PROT* protease, *RT* reverse transcriptase

complex. They act as RNA chaperones and stimulate both reverse transcription and integration of the viral genome into the DNA of the cell.

3. In mature virus particles, the matrix proteins are associated with the inside of the envelope. In HIV, the phosphorylated matrix protein p17, which enters the cell as part of the virus particle, promotes the transport of the double-stranded DNA of the viral genome through the nuclear pores into the nucleus. Therefore, the matrix protein makes possible HIV infection of resting cells. This activity is based on nuclear transport signals in the amino acid sequence of p17; it distinguishes HIV from other retroviruses, which can only start the replication cycle in dividing cells in which the nuclear membrane is not present during mitosis.

Gag proteins induce specific humoral and cellular immune responses. The antibody specificity frequently overlaps, owing to the relatively high content of conserved amino acids among members of the same genus. Cytotoxic T-cell epitopes have been identified in matrix and capsid proteins of HIV. Possibly, they are crucial in determining the immunological control of infection, and thus the duration of asymptomatic HIV infection up to the outbreak of AIDS symptoms.

Table 18.2 Properties of human immunodeficiency virus 1 (*HIV-1*), human T-lymphotropic virus 1 (*HTLV-1*) and simian foamy virus (*SFV*) proteins

Proteins	HIV-1	HTLV-1	SFV	Features
Structural proteins				
Envelope proteins				
Precursor proteins	gp160	gp68	gp130	Glycosylated, trimer
Exterior glycoprotein (EP)	gp120	gp46	gp80	Glycosylated, trimer Attachment, induction of neutralizing antibodies, cell tropism
Transmembrane glycoprotein (TM)	gp41	gp21	gp45	Glycosylated, fusion, induces formation of neutralizing antibodies
Group-specific antigens (Gag)				
Gag precursor protein	pr55	pr48	pr74	Myristoylated, formation of virus-like particles
Matrix protein (MA)	p17	p19	p27	Myristoylated, phosphorylated, envelope-associated
Capsid protein (CA)	p24	p24	p33	Structural capsid protein
Nucleocapsid protein (NC)	p7	p15	p15	Zinc finger motif, association with the RNA genome
Link protein (LI)	p6	?	?	Link between the capsid and the envelope
Enzymes				
Gag–Pol precursor protein	pr160	pr160	pr190	
Gag–Prot precursor protein		pr60		
Reverse transcriptase	p51/66[a]	p95	p80	RNA- and DNA-dependent polymerase, RNase H
Protease	p9	p14	p10	Cleaves Gag and Gag–Pol precursor proteins into individual components
Integrase	p32	p60	p40	Nucleotidyltransferase, endonuclease, ligase, responsible for the integration of the viral genome into the host DNA
Regulatory proteins				
Transcriptional transactivator	p9/14 (Tat)	p40 (Tax)	p36 (Tas/Bel1)	Phosphorylated (Tax, Tas); Tat binds to the TAR element at the 5′ ends of the RNA and enables elongation; Tax and Tas bind to cellular factors that bind to the promoter and enhance initiation of transcription
Post-transcriptional transactivator	p19 (Rev)	p27 (Rex)	–	Phosphorylated; binds to the RRE and RxRE and promotes export of singly spliced and unspliced mRNAs from the nucleus
Accessory proteins (not essential)				
Virion infectivity factor (Vif)	p23	–	–	Interaction with APOBEC3G and APOBEC3F, inhibits the cytosine deaminase activity of these proteins, promotes the infection of peripheral blood lymphocytes

(*continued*)

Table 18.2 (continued)

Proteins	HIV-1	HTLV-1	SFV	Features
Virion-associated protein, rapid (Vpr)	p11/15	–	–	Virion-associated, transactivator
Viral protein U (Vpu; only in HIV-1)	p14/16	–	–	Phosphorylated, associated with the ER membrane, prevents intracellular complex formation between gp160 and CD4 receptors
Nef protein	p25/27	–	–	Myristoylated, similar to G proteins, associated with the cytoplasmic membrane, reduces the concentration of CD4 and MHC class I antigens on the cell surface, pathogenicity factor
Viral protein X (Vpx; only in HIV-2/SIV)	p13/16	–	–	Virion-associated
Bel2	–	–	p43	?
Bet	–	–	p56	Fusion protein between Bel1 und Bel2

The *numbers* refer to the molecular weight of the respective protein
ER endoplasmic reticulum, *mRNA* messenger RNA, *HIV-2* human immunodeficiency virus 2, *SIV* simian immunodeficiency virus, *RRE* Rev response element, *RxRE* Rex response element, *TAR* transactivation response
[a]Heterodimer

18.1.3.2 Enzymes (*pol* Gene Products)

Gag–Pol Precursor Protein

The genes that direct the synthesis of the viral protease, reverse transcriptase and integrase are located in the central region of the genome. The sequences encoding the protease are located in the 5′ region of the *pol* gene and overlap with the nucleocapsid protein coding sequences in most retroviruses; in feline leukaemia virus, these are directly adjacent (Fig. 18.3). The *pol* gene products are also synthesized as a precursor protein. It is a fusion protein between the Gag proteins in the amino-terminal region and the adjoining Pol domains (Fig. 18.5); these Gag–Pol precursor proteins have molecular masses of 160 and 190 kDa in HIV and spumavirus, respectively. Prerequisite for the synthesis of the Gag–Pol fusion protein is a ribosomal frameshifting event in a uridine-rich region of the mRNA, which is used for the translation of Gag and Gag–Pol proteins. In that region, the mRNA forms a hairpin structure, which retards protein synthesis. There is occasionally an erroneous codon recognition by the ribosomes owing to the homologous sequence of uridine residues. If the reading frame is shifted by −1 (in HIV) or +1 (in SFV), then the stop codon that terminates the synthesis of Gag proteins is skipped, translation continues, and the Pol domains are additionally synthesized. Such frameshifting events occur in approximately 5 % of all transcription processes, so the ratio of the Gag–Pol fusion products to the Gag proteins is approximately 5:95. The uridine-rich sequence is located in the region of the mRNA that encodes the nucleocapsid proteins of the precursor product. Therefore, the amino-terminal moiety of Gag–Pol precursor proteins consists of Gag polypeptides until the nucleocapsid protein region, to which the domains of the protease, reverse

transcriptase and integrase adjoin. Gag–Pol fusion proteins are also myristoylated at the amino terminus, and are associated with the cytoplasmic membrane by these modifications. The proteolytic cleavage reactions forming enzymatic active components occur mainly during viral maturation in the particles that have already been released from the cell surface.

Three different precursor fusion polypeptides are synthesized in HTLV-1. The first encompasses the sequences of Gag proteins. Another is synthesized by frameshifting in the nucleocapsid region, so the sequences of the protease are added, leading to a Gag–Prot fusion product, whose translation is terminated at a separate stop codon in HTLV-1. The continuous synthesis of the following Pol moiety is done only when a second frameshift of the reading frame occurs in the protease domain. This results in the synthesis of a Gag–Prot–Pol fusion protein.

Protease

The active HIV protease is a dimer of two identical protein subunits, which consist of 99 amino acids and have a molecular mass of about 9–10 kDa. The protein structure is known. Two functionally important aspartic acid residues are located in the active centre. Thus, the enzyme acts as an aspartate protease. It is responsible for the processing of Gag and Gag–Pol precursor proteins into the individual components, which are found in the infectious virus particles. Cleavage preferentially occurs between phenylalanine or thyrosine and proline residues. In addition, the three-dimensional folding of the Gag and Gag–Pol precursor proteins is decisive for recognition of cleavage sites. Synthetic peptides derived from the sequences of cleavage sites inhibit the protease. This knowledge has led to the development of various substances, such as ritonavir, indinavir and saquinavir, in recent years which inhibit the enzyme and prevent the production of infectious HIV particles (▶ Chap. 9).

Reverse Transcriptase

The Mg^{2+}-dependent reverse transcriptase has several functional activities: it can act as both RNA-dependent and DNA-dependent DNA polymerase, and has an additional RNase H activity, which degrades the RNA moiety of DNA/RNA heteroduplexes. The HIV reverse transcriptase is a heterodimer constituted of two protein subunits with molecular masses of 66 and 51 kDa, respectively. The crystal structure of this enzyme is also known. The smaller subunit arises by proteolytic cleavage and removal of the carboxy-terminal domain. Thus, the 51-kDa protein is identical with the amino-terminal region of the large subunit. The RNase H activity resides in the carboxy-terminal domain of the large subunit. The reverse transcriptase does not possess an exonucleolytic proofreading activity to control the accuracy of replication by recognizing and removing erroneously incorporated nucleotides. Therefore, wrong, mismatching nucleotides are incorporated into the newly synthesized strands with a relatively high probability of 10^{-3}–10^{-4}. Besides naturally occurring nucleotides, the reverse transcriptase can also use chemically altered derivatives. If nucleoside analogues such as azidothymidine (3′-azido-3′-deoxythymidine), dideoxyinosine and dideoxycytidine are available during

synthesis, they will also be incorporated into nascent DNA strands, leading to chain termination, and thus blocking of the synthesis of the double-stranded DNA intermediate, which is essential for the integration into the genome of the host cell.

Integrase

The integrase (32 kDa in HIV) is coded in the 3′ region of the *pol* reading frame. It acts as a nucleotidyltransferase, an endonuclease – it also cleaves double-stranded DNA – and also functions as a ligase. Following reverse transcription, the integrase binds to the ends of the linear, double-stranded DNA of the viral genome, and is responsible for its integration into the cellular genome.

18.1.3.3 Envelope Proteins

The *env* genes encode glycoproteins that are embedded in the cytoplasmic membrane of infected cells and in the envelope that surrounds the virus particle. These are complexes of an exterior glycoprotein (EP) and a transmembrane protein (TM), which are non-covalently linked to each other. Both proteins are translated as a common precursor protein from a singly spliced mRNA (gp160 in HIV, 68 kDa in HTLV, 130 kDa in spumavirus, 62–72 kDa in BLV, 80 kDa in feline leukaemia virus, 135 kDa in equine infectious anaemia virus), and the sequences of the exterior protein are located in the amino-terminal domain. An amino-terminal signal sequence is responsible for translation of the protein on the ER membrane, and for its translocation into the lumen. Membrane anchoring is mediated by a hydrophobic sequence of approximately 20 amino acids in the transmembrane domain (Fig. 18.6). The modification of proteins with carbohydrate groups occurs in the ER. The proteins aggregate into trimeric complexes and are cleaved into the exterior and the transmembrane glycoproteins after a sequence region containing basic amino acids. The cellular, Golgi-associated protease furin is responsible for this process in HIV. The exterior glycoprotein of HIV has a molecular mass of 120 kDa (gp120), whereas that of the transmembrane protein is 41 kDa (gp41). Particularly, gp120 exhibits a very high degree of modification with N-glycosidically linked carbohydrate groups. These represent more than half of the molecular mass of the protein.

Comparative analyses of the amino acid sequences of different isolates of HIV-1 revealed a high variability in regions V1–V5 of gp120 (Fig. 18.6). These are separated by relatively highly conserved regions (C1–C6). The variable regions differ not only in HIV isolates from different patients. Even succeeding isolates from the same patient display significant sequence differences in their V regions in the course of the disease. Apart from residues with different functional side groups, larger deletions and insertions, and also modified glycosylation patterns are found, which altogether change the structure and activity of variable regions. The variable regions possess all the characteristics that identify them as surface-exposed regions. They are responsible for the production of gp120-specific antibodies, which, in some cases, are able to neutralize the virus. Owing to the low fidelity of reverse transcription, the selection pressure exerted by these antibodies leads, in association with the high genetic variability of retroviruses, to the continuous formation of new

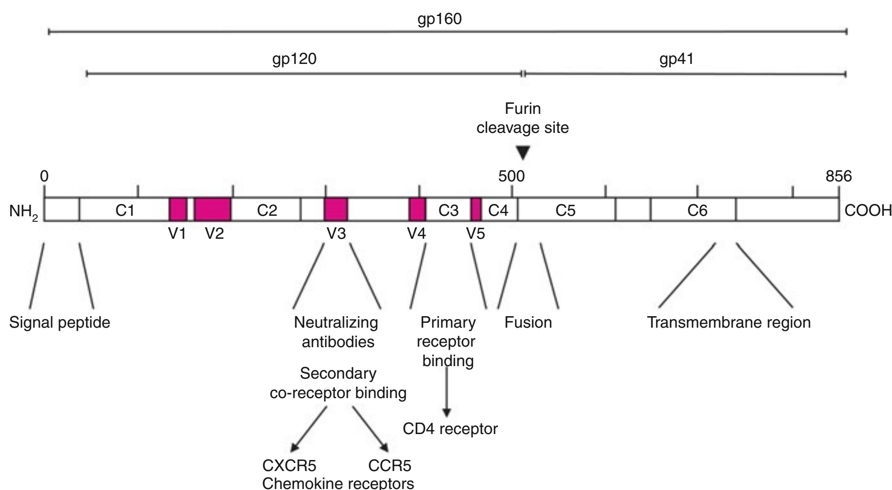


Fig. 18.6 The glycoproteins of HIV-1 (strain HIV/HTLV-IIIB). Glycoproteins are synthesized as a common precursor polypeptide (gp160). Its cleavage into gp120 (exterior glycoprotein) and gp41 (transmembrane subunit) occurs in the endoplasmic reticulum and Golgi apparatus, and is performed by the protease furin. Comparison of the amino acid sequences of different HIV isolates shows that the proteins are composed of highly variable domains (*V1*–V5, *red*) and conserved domains (*C1–C6*). The protein regions to which specific functions have been assigned are indicated

HIV variants (quasispecies), which are able to escape the neutralizing effect of immunoglobulins. The V3 domain has a special status among the variable regions: it is 30–35 amino acids long and is flanked by cysteine residues that form a disulphide bond, inducing the formation of a surface-exposed V3 protein loop. In almost all isolates, a conserved amino acid sequence (glycine–proline–glycine–arginine) is located at its centre, and forms a stable β-turn structure. However, the flanking V3 sequences exhibit a high variability in different isolates. The V3 domain is the only variable region of gp120 that is not glycosylated. V3-specific antibodies can neutralize the virus and prevent infection in vitro. The neutralizing effect of V3-specific immunoglobulins is restricted to the HIV isolate which has induced the immune response. V3 regions of other isolates with slight variations in the amino acid sequence are not bound with the affinity that is necessary for a neutralization effect. However, the C regions are not exposed to the selective pressure exerted by the antibody response, and have predominantly conserved amino acid sequences, which shape the protein scaffold of the exterior glycoprotein. The common functions of envelope proteins of all HIV isolates are associated with them, e.g. attachment to the CD4 receptor.

During infection, envelope proteins have different functions, which can be assigned to specific domains:

1. The exterior glycoproteins are responsible for attachment to cell surface components. Whereas little is known about the nature of receptor binding and the

cellular structures involved in the vast majority of retroviruses, the viral and cellular interaction partners of HIV are well known: HIV-1 interacts with the first immunoglobulin-like domain of CD4 – a member of the immunoglobulin superfamily – on the surface of T-helper cells by sequences in the third conserved C3 domain of gp120. Further sequences in other regions of gp120, which are exposed by the three-dimensional folding of the protein, are involved in this interaction. The V3 domain is also involved in binding of HIV-1 particles to target cells – this is suggested by the neutralizing effect of V3-specific antibodies. The V3 regions are responsible, together with the V1/V2 domains, for the different tropisms of individual HIV isolates for macrophages or monocytes (R5 virus isolates) as well as T-helper cells (X4 virus isolates). They influence interaction of p120 with different chemokine receptors, which are used as co-receptors by the viruses. Virus variants with tropism for macrophages bind to the chemokine receptor CCR5, by contrast, viruses with tropism for T cells bind to the CXCR4 receptor.

2. Viral envelope proteins contain epitopes, against which neutralizing antibodies are directed. In the case of HIV-1, the V3 domain is the epitope – as already mentioned – which primarily induces the production of isolate-specific, neutralizing antibodies. In addition, other amino acid regions of p120 have been described which also elicit the generation of neutralizing immunoglobulins. These appear to be based on the three-dimensional structure of the protein. An epitope of gp41 is known to elicit neutralizing antibodies against different virus isolates.
3. The proteolytic cleavage into exterior and transmembrane protein components and the interaction of gp120 with the CD4 receptor cause a rearrangement of a hydrophobic sequence at the amino-terminal region of gp41, which mediates the fusion of the viral envelope with the cytoplasmic membrane of the cell which is being infected, but also that of infected cells with other $CD4^+$ T lymphocytes. The HIV fusion domain is approximately 25 amino acids long. In terms of its sequence and localization at the amino terminus of the transmembrane protein domain, it shows a striking similarity to the fusion active regions of both the paramyxovirus F protein and the orthomyxovirus haemagglutinin (▶ Sects. 15.3 and ▶ 16.3).

Structural Analysis of gp120 Has Proven Difficult

The amino acid sequence of gp120 is characterized by its high degree of glycosylation and the presence of highly variable domains. Both attributes complicate the structural analysis of the protein. Therefore, the structure of gp120 has been only partially resolved by X-ray structure analysis so far. Joseph Sodroski, Wayne Hendrickson and colleagues deleted the V1, V2 and V3 domains, replacing them with tripeptides composed of glycine–alanine–glycine. The carbohydrate groups were then enzymatically removed, producing a protein with a molecular mass of 35 kDa that constituted only 67 % of the original gp120, which, however, was still able to bind to the CD4 receptor,

and to interact with some gp120-specific monoclonal antibodies. The X-ray crystallographic structure analysis of this core fragment of gp120 has been solved, revealing a heart-shaped conformation. It is formed of an outer and a highly conserved inner domain; both domains are connected by a bridge of four β-sheets. CD4 receptor binding occurs at a transition site between the outer and the inner domain as well as the bridging region, which is located at the tip of the heart-shaped gp120 core fragment. It contains two cavities, one of which is filled with H_2O molecules, allowing little direct contact of gp120 with CD4; therefore, variations in the amino acid sequence are tolerated. The second, very deep cavity is lined by hydrophobic amino acids. The interaction with the phenylalanine residue at position 43 of the CD4 receptor, which is crucial for binding, occurs at that site. Binding to the co-receptors CCR5 and CXCR4 occurs by neighbouring protein structures, whose conformations are rearranged by the gp120–CD4 interaction. Mutation analyses have revealed that amino acids of the V3 domain and the V1/V2 regions are also directly involved in this interaction. However, since these variable regions were deleted in the gp120 core fragment, precise asseverations are not possible. Recently, other domains of the surface proteins have been structurally analysed; however, the structure of the entire protein complex is still not yet available. Nevertheless, cryo-electron-microscopic analyses allow inferences regarding the arrangement of the proteins and their structure.

18.1.3.4 Transactivators

Tat Proteins

Tat (for "transactivator of transcription") proteins have been identified only in lentiviruses. They are encoded by two exons, and are synthesized in the early phase of the infection cycle; their functions are developed in the nucleus. Depending on the HIV-1 isolate, they are 86–104 amino acids long and have a molecular mass of 9–14 kDa. Similar molecular masses are also found in animal pathogenic lentiviruses. In FIV, the Tat protein is mainly encoded by the second exon, which is transcribed from ORF2. In addition to the Tat proteins that are encoded by two exons, truncated versions lacking the sequences of the second exon and Tev proteins are also found in HIV-1, in which the first exon is combined with segments of the *env* and *rev* genes by alternative splicing ("Tev" from "*tat*", "*env*", "*rev*"). All Tat protein variants have similar transactivating functions, so it is assumed that these reside within the first exon of the amino-terminal region, which, in turn, can be divided into five domains. The amino-terminal region, which has differing length in different virus isolates, contains conserved acidic amino acid motifs, which possibly form an amphipathic α-helix structure. It is followed by a cysteine-rich, conserved domain, which binds Zn^{2+} and Cd^{2+} ions and has a length of about 15 amino acids. It is followed by a core region that comprises ten amino acid residues. Amino acid alterations or deletions in this region influence the transactivating effect of Tat proteins. Further downstream, there is a region with

a high content of basic amino acids, which mediate both transport of Tat proteins into the nucleus and RNA binding (Fig. 18.7a). The carboxy-terminal sequences of exon 1 enhance the activity of the protein. The function of the domain encoded by exon 2 is not clear. However, a sequence motif (arginine–glycine–aspartic acid) which is usually involved in protein–protein interactions is located in this region.

Tat proteins exert some of their activities in the nucleus of infected cells, into which they are translocated through nuclear pores by a mechanism mediated by transport signals located in the basic domain. The transactivating effect of Tat proteins, which enhance transcription from the LTR promoter by several-hundred-fold, is mediated by their binding to the transactivation response (TAR) element. It is localized at the 5′ end of all viral mRNA species. In that region, RNA molecules have a pronounced secondary structure, forming a partially double-stranded RNA hairpin structure that includes the first 59 nucleotides in HIV-1 (Fig. 18.7b), and 123 nucleotides in HIV-2. The HIV-1 Tat protein binds to a bulge within the TAR element, which is formed by three non-base-pairing nucleotides. A cellular TAR–RNA-binding protein (TRP) 1 complex, which consist of two subunits (p185 and p90), binds to the apical loop of the TAR element. The following model explains the effect of the Tat protein. The cellular RNA polymerase II complex and pertinent transcription factors bind to the LTR promoter of the integrated proviral genome and initiate RNA synthesis. The synthesized products are short, but they contain the 5′-terminal sequences of TAR elements, which interact with TRP1. In this situation, elongation of mRNA synthesis is blocked; the short transcripts are unstable, and are degraded with the exception of the TAR regions, which are protected against degradation, and accumulate in the cytoplasm. If Tat proteins are present in the nucleus, they will bind to the bulge within the hairpin structure of the TAR element, preventing termination of transcription. The initiation complex is stabilized and translatable transcripts are synthesized. Simultaneously, an increased initiation rate leads to enhanced transcription as well. The situation is more complicated because of the fact that a cellular TRP2 complex composed of four proteins competes with Tat proteins for binding to the TAR element. It is not clear how this affects Tat binding. Furthermore, Tat-binding cellular proteins (TBP) have been identified which presumably interact with the carboxy-terminal domain of the protein. Both Tat-enhancing (TBP-1, p50) and Tat-inhibiting (p36) effects have been described.

In addition to these properties that are predominantly manifested in the nucleus of infected cells, additional functions have been reported for Tat proteins. Some of these proteins are secreted after synthesis and released into the environment. The secreted Tat proteins can bind to integrins on the surface of other cells by their arginine–glycine–aspartic acid motif at the carboxy-terminal domain, whereby they are incorporated into the cells, in which they exert their transactivation effects on cellular genes. Alternatively, binding to cell surface proteins can trigger a signalling cascade, which in turn induces an altered gene expression pattern. Depending on the type of cell to which Tat proteins bind, they change the expression of cytokines and enzymes which are involved in increased cell proliferation, as well as of adhesion factors, such as integrins and growth receptors. Secreted Tat proteins are possibly involved in several processes during pathogenesis of HIV infection.

Fig. 18.7 The HIV-1 Tat protein and its binding to the TAR element at the 5′ terminus of viral mRNA molecules. (**a**) Functional domains of the Tat protein and its localization in the amino acid sequence. The Tat protein is encoded by two exons, whose position in the amino acid sequence is depicted schematically. Various protein domains are indicated by different *shades of red*, and *numbers* refer to amino acid positions (counting from the amino terminus). The respective functions the protein domains are indicated *below the bars*. (**b**) Structure of the TAR element at the 5′ end of all viral mRNA species, which start at the beginning of the R region within the LTR. The first 59 nucleotides form a largely double-stranded region. The Tat protein binds to the single-stranded bulge that interrupts the double-stranded stem of the hairpin structure. By contrast, cellular proteins (Tat-binding protein 1, *TBP-1*; see the text) interact with the apical loop of the hairpin-shaped TAR element

Tax Proteins

The denomination of the transactivating Tax proteins is derived from the X region of the HTLV genome, which is situated between the *env* gene and the LTR region, and whose function was initially unknown. Proteins with similar molecular masses and functions are also found in BLV. Their mode of action differs from that of Tat proteins. The nuclear TAX_1 and TAX_2 proteins of HTLV-1 and HTLV-2 influence *cis*-acting TRE sequences in both viral LTR promoter regions flanking the integrated proviral genome and several cellular promoters. Tax_1 protein is phosphorylated, has a length of 353 amino acids and has a molecular mass of approximately 40 kDa. Tax_2 protein (37 kDa) lacks 22 amino acids at the carboxy terminus. The sequences of both proteins are conserved, and have about 80 % homology. A zinc-finger-like motif that binds Zn^{2+} ions is found in the amino-terminal region of Tax_1 and Tax_2 proteins. Possibly, Tax proteins are active as homodimers. They do not exert their transactivating effects by direct interaction with the TRE_1 and TRE_2 elements (21 nucleotides) located in the U3 region of the LTR (Fig. 18.4). TRE_1 sequences are present in triplicate and interact with several cellular factors of the cyclic AMP response element binding protein (CREB protein) family and with NFκB. Cellular factors such as Sp1 and Ets, inter alia, bind to TRE_2. Tax proteins interact with several of these cellular factors that bind to the different TREs, thus exerting an indirect transcriptional activation effect on viral mRNA synthesis. This also explains why Tax proteins are able to activate the expression of a plethora of different cellular genes: they induce the expression of all cellular genes that are regulated by NFκB and serum response factor (SRF). These include, on the one hand, granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-2 and the α chain of IL-2 receptor, as well as the chemokine receptors CCR4, CCR7 and CXCR4 and, on the other hand, the cellular oncogenes c-*fos*, *fra-1*, *egr1* and *egr2*, as well as cell cycle regulators such as cyclin D2, CDK4, CDK6 and p21/Waf1. There is evidence that this activation of cellular cytokines and cytokine receptors is closely related to the pathogenesis of HTLV-mediated T-cell leukaemia.

Tas Protein

The Tas (for "transactivator of spumaviruses") protein, occasionally also referred to as Bel1 protein, is a nuclear phosphoprotein with a molecular mass of 36 kDa. It binds to BRE sequences in the U3 region of the LTR of spumaviruses (Fig. 18.4). Whether this involves the direct interaction with the DNA sequence or whether the transactivation is exerted by interacting with cellular factors remains to be elucidated.

18.1.3.5 Post-Transcriptional Transactivators

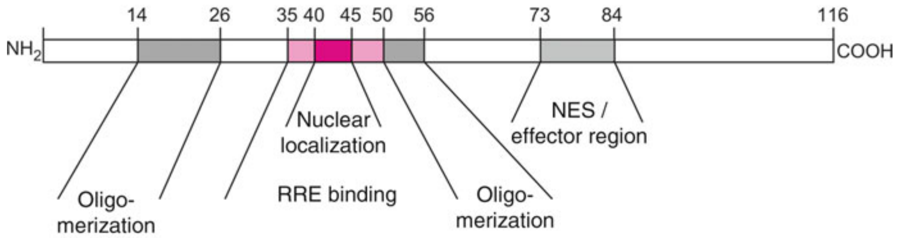
Rev Protein

The extremely compact genome organization of complex retroviruses containing a single promoter in the LTR requires that these viruses rely on mechanisms that enable them to regulate gene expression even after transcription. Besides ribosomal frameshifting during translation of Gag–Pol proteins and alternative splicing to

generate the mRNA species for regulator and envelope proteins, the Rev proteins (for "regulator of expression of virion proteins") also play an important role in this context. This class of post-transcriptional transactivators was initially discovered in HIV. They are essential for the temporal regulation of gene expression during the replication cycle. With the exception of the synthesis of Rev, Tat, Tev and Nef proteins, the translation of all other viral polypeptides is dependent on the activity of this regulator.

The Rev protein (13 kDa) is encoded by two exons that overlap with those of the Tat protein. Its translation occurs, however, in another reading frame (Fig. 18.3a). In HIV-1, it is 116 amino acids long, is phosphorylated and is present in tetrameric form or higher molecular aggregates. The Rev proteins of animal pathogenic lentiviruses have molecular masses in the range from 16 to 19 kDa. The Rev protein is synthesized early during infection, and is accumulated in the nucleus and nucleoli, where it binds to a Rev response element (RRE) of roughly 240 nucleotides in length, which is present in all singly spliced and unspliced mRNA molecules. These encode the proteins Env, Vif, Vpr and Vpu, as well as the Gag and Gag–Pol precursor polypeptides. The interaction with RRE leads to a preferential translocation of these mRNA species to the cytoplasm, where they are translated into the corresponding proteins. In the case of multiply spliced mRNA species that serve for translation of Tat, Tev, Rev and Nef proteins, the RRE is removed by splicing as part of an intron, so their export and translation are independent of the binding of the Rev regulator.

The Rev protein has two functionally important domains: the amino acids of the amino-terminal half are responsible for binding to RRE motifs, nuclear localization, and oligomerization. Rev binding to RRE is mediated by the arginine-rich domain of amino acids 35–50. The nuclear localization signal has been mapped to residues 40–45 (Fig. 18.8a). The sequences responsible for oligomerization flank the RRE binding site. According to computer calculations, the nucleotide sequence of the RRE is folded into a sophisticated secondary structure, which includes several double-stranded regions (Fig. 18.8b): the predicted structure displays a double-stranded stem containing four stem-loops. The Rev protein binds to a bulge of unpaired nucleotides in the branched stem-loop II. This causes the accumulation of additional Rev proteins, so the RRE structures of transcripts are finally complexed with Rev oligomers. A nuclear export signal has been identified at the carboxy-terminal half between amino acid residues 73 and 84, which are essential for the transport of RRE-containing mRNAs from the nucleus to the cytoplasm. The nuclear export signal is also referred to as an effector region. Important interaction sites for cellular proteins are located in this domain, and are critical for the activity of Rev. These include exportin-1, also known as CRM1 (chromosome maintenance gene), which is known to bind to nuclear export signal sites. Exportin-1 interacts, in turn, with the GTP-modified form of Ran protein. This leads to the transport of mRNA–Rev–exportin-1–Ran-GTP complexes through nuclear pores. After translocation, exportin-1 and Ran dissociate from the complex under GTP hydrolysis, leading to the release of the Rev proteins from mRNA, which thereby becomes available



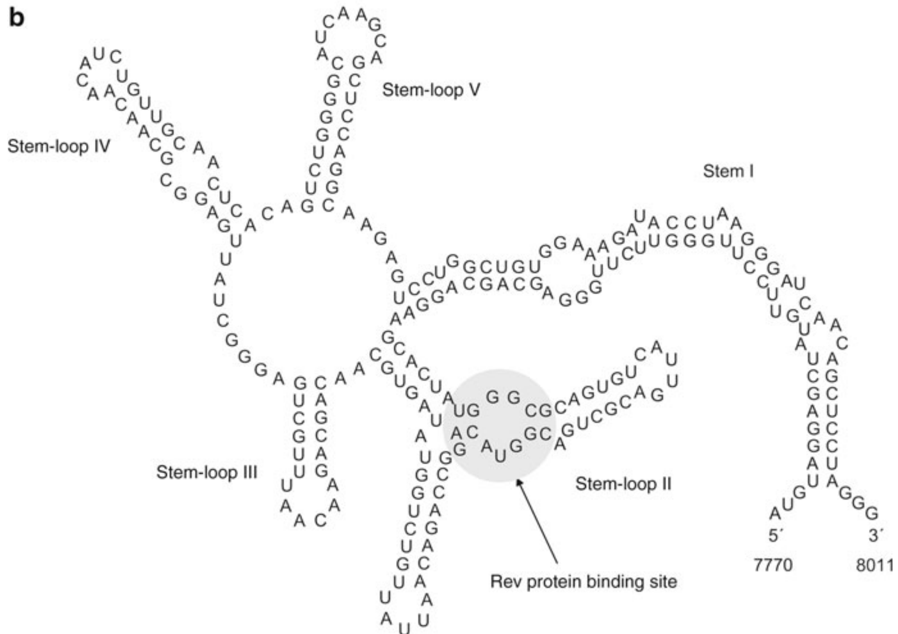


Fig. 18.8 The HIV-1 Rev protein and its binding to the Rev response element (*RRE*), which is located within the Env protein coding region. (**a**) Functional domains of the HIV-1 Rev protein and their location in the amino acid sequence. The various protein domains are indicated by different *shades*, and *numbers* refer to amino acid positions (starting from the amino terminus). The respective functions of the protein domains are indicated *beneath the bars NES*: nuclear export signal. (**b**) Predicted secondary structure of the RRE region in mRNA. An approximately 240-nucleotide-long mRNA region, situated within the Env protein coding sequences, is able to adopt a highly structured conformation that is characterized by the formation of partial double-stranded regions, which are folded into four well-defined stem-loops (*II–V*) with a long stem region (*stem I*). The binding site of the Rev protein is indicated by a *shaded circle*. The *numbers at the 5′ and 3′ ends* refer to the location of the RRE in the unspliced mRNA form (counted from the transcription start)

for translation. This leads to the clearance of the arginine-rich domain of the Rev protein, which thereby becomes accessible for interacting with the cellular protein importin-β, which in turn interacts with Ran-GDP, triggering its reimport into the nucleus.

Rex Proteins

The function of Rex proteins, whose second exon resides in the X gene region of HTLV-1 and HTLV-2, is equivalent to that of the Rev protein of HIV (Fig. 18.3b). They are phosphorylated and have molecular masses of 27 kDa (Rex1) and 26 kDa (Rex2). The nuclear localization and RNA-binding regions are located in the amino-terminal domains, whereas the regions essential for transactivation reside in the centre. The 255-nucleotide-long Rex response element (RxRE) sequences of HTLV-1 are located in the 3′ LTR at the transition site between the R and U3 regions. They overlap with the polyadenylation signals, and form a highly ordered stem-loop secondary structure. An additional RxRE region has been identified at the R–U5 transition site in the 5′ LTR. If RxRE is present in the transcripts, then the singly spliced and unspliced mRNA molecules accumulate in the cytoplasm. Rex1 and Rex2 are also able to bind to RRE regions of HIV, and to complement the function of the Rev protein; however, the converse complementation is not possible.

18.1.3.6 Accessory Proteins

Vif Protein

The gene encoding the Vif (for "virion infectivity factor") protein of lentiviruses is located between the *pol* and *env* genes (Fig. 18.3a). It is translated from a singly spliced mRNA, contains 192 amino acids and has a molecular mass of 23 kDa in HIV-1. Vif proteins of animal pathogenic lentiviruses have molecular masses of 22 kDa (caprine arthritis encephalitis virus) and 30 kDa (FIV). The Vif protein is a component of the nucleoprotein complex of infectious virus particles; it is associated with RNA and influences the infectivity of progeny viruses. Vif-defective viruses exhibit an altered particle morphology, including an inhomogeneous incorporation of capsid proteins. If such virus mutants are used for new infection experiments, an incomplete double-stranded DNA synthesis can be observed. This relies on the lack of interaction of Vif protein with the cellular proteins APOBEC3G and APOBEC3F: these proteins are cytosine deaminases, which alter and influence the sequences of single-stranded DNA regions during reverse transcription. As a result, defective nucleic acid strands are synthesized, which are degraded by nucleases. Hence, APOBEC3G and APOBEC3F have been characterized as intracellular antiviral factors. Vif proteins bind to the cellular cytosine deaminases and inhibit their activity. Inasmuch as APOBEC3G and APOBEC3F are not synthesized in comparable quantities in all cell types, the function of Vif proteins is not always necessary. For the production of infectious HIV during the infection of peripheral blood lymphocytes the Vif protein is essential, however.

Vpr Protein

The *vpr* reading frame overlaps with that of the *vif* gene and with the first exon of the *tat* gene. Vpr (for "virion-associated protein, rapid") is also translated from a singly spliced mRNA. The 78–96 amino acid Vpr has been identified in almost all lentiviruses and has a molecular mass between 11 and 15 kDa. It interacts with

the domain of the link protein in the carboxy-terminal p6 region of Gag precursor proteins, and thus is included in the mature virus particles. Each virus particle contains about 100 Vpr molecules. Its main function is the participation in transporting the preintegration complex to the nucleus. This complex includes the double-stranded DNA viral genome, the integrase, the reverse transcriptase and some other structural proteins; it is transported into the nucleus by the interaction of Vpr with nuclear pore proteins, whereby Vpr develops its effect along with the matrix protein p17. In addition, Vpr blocks the cell cycle in the G_2 phase by inhibiting activation of cyclin B. Thereby, it is able to induce apoptosis.

Vpu Protein

Vpu (for "viral protein U") is encoded in the genome of HIV-1 and the closely related SIV of chimpanzees (SIVcpz). Apart from these viruses, it has been found that the caprine arthritis encephalitis virus X protein has a function similar to that of Vpu. However, it is considerably smaller (8 kDa). The 80–82 amino acid long Vpu of HIV-1 (16 kDa), which is phosphorylated by casein kinase 2, is translated from a bicistronic mRNA that also contains the sequences encoding the Env proteins, promoting, especially in resting cells, the release of progeny viruses. It possesses hydrophobic amino acid sequences in the amino-terminal region, through which it inserts in the ER membrane and in the cytoplasmic membrane, where it interacts with tetherin, a ligand of cyclophilin. If the Vpu protein is absent, tetherin inhibits the chaperone function of cyclophilin, which constitutes an essential activity during HIV morphogenesis. In comparison with the wild-type virus, Vpu-defective variants exhibit a delayed or impaired release of progeny viruses. Furthermore, Vpu binds to the cytoplasmic domain of the CD4 receptor, inducing ubiquitination and degradation of the receptor. Possibly, HIV-2 and other lentiviruses do not necessarily need this function of Vpu, because the affinity of their surface proteins for the CD4 receptor is lower than that of gp120 of HIV-1. In addition, there is evidence that Vpu also acts as a proton channel. However, it is not known how this influences the infection cycle.

Vpx Protein

The *vpx* (for "viral protein X") gene exists solely in HIV-2 and SIV, and is located between the *pol* and *env* genes. It has a pronounced homology with the neighbouring *vpr* gene, and has probably evolved by gene duplication. The protein Vpx (14–16 kDa) comprises approximately 112 amino acids, is translated from a singly spliced mRNA and can be detected in the virions between the capsid and the envelope. It enhances the ability of viral replication in macrophages and peripheral blood lymphocytes; however, it does not have any significance in other systems. Presumably, it enters the cell during infection and stabilizes the viral RNA–protein complex or facilitates its transport into the nucleus. There is evidence for the existence of further accessory protein variants which arise by alternative splicing. However, their functions have hardly been investigated.

Nef Protein

The gene encoding the Nef (for "negative factor") protein is located between the *env* gene and the 3′ LTR, and overlaps with the U3 region. The expression of this gene is independent of the effect of the Rev protein, and occurs by translation of a multiply spliced mRNA. The Nef protein is abundantly expressed at early stages of the infection cycle. It has a length of 206 amino acids on average, and has a molecular mass of 25–27 kDa in HIV-1 and about 10 kDa in HIV-2. Nef proteins exhibit a high variability of 17 % in their amino acid sequences. Moreover, differently sized deletions or insertions have been found in the *nef* gene in diverse HIV isolates, whereas in others – such as HIV-1 strain HXB2 – even a truncated product has been observed, which lacks up to 124 amino acids. Functionally active Nef proteins can be divided into four domains: an amino-terminal myristoylated region, which anchors the protein in the cytoplasmic membrane of infected cells, a loop region containing a proline-rich segment, a conserved, well-structured globular domain, and a flexible carboxy-terminal moiety. Approximately, ten to 100 Nef proteins have been found even in purified virus particles; their presence in the virions increases their infectivity. Nef proteins in the particles are cleaved by the viral protease into a soluble carboxy-terminal fragment and a 57 amino acid long amino-terminal moiety, which remains associated with the viral membrane. The Bel3 gene product of spumaviruses probably resembles the Nef protein.

The functions of the Nef protein are varied during replication. They have mainly been studied using the model system of SIVmac239. This virus causes no disease in its natural host, the African sooty mangabey (*Cercocebus ays*), whereas Asian rhesus monkeys develop an AIDS-like immunodeficiency. Nef-defective SIV variants are attenuated, and establish an asymptomatic infection. Nef protein activities have been unravelled by mutation of individual amino acids and by deleting *nef* gene sequences coding for specific domains. The Nef protein also plays a pivotal role in the pathogenesis of HIV-1 infections: in infected individuals, Nef-defective HIV-1 mutants induce a virus carrier state without illness, or a very late developing disease. The analysis of HIV isolates from infected individuals with different infection courses – such as those with an asymptomatic carrier stage or those with rapidly progressing symptoms – revealed that the Nef functions of the SIVmac239 model are very comparable with those of human HIV infections.

The following functions of this versatile protein have been characterized so far:

- Nef protein induces the synthesis of specific antibodies and cytotoxic T cells.
- Nef protein is phosphorylated by protein kinase C, and possibly by other cellular kinases, at a threonine residue (position 15 in the HIV-1 Nef protein). Some publications have shown that Nef protein possesses autophosphorylating activity. Phosphorylation of Nef protein induces a signal transduction pathway, and enhances the expression of NFκB, AP1 and nuclear factor of activated T-cells (NF-AT). This leads to activation of HIV-infected T cells, whereby expression of the proviral genome is increased. In addition, the synthesis of the chemotactic factors macrophage inflammatory protein (MIP) 1α and MIP-1β as well as T-cell-activating factor is induced. As a result, more T lymphocytes are attracted and

activated, which serve as target cells for the released virus particles. Furthermore, the Nef-induced signalling cascade triggers the synthesis of Fas ligand, whereby an apoptosis signal is transmitted to HIV-specific cytotoxic T cells.

- Nef proteins reduce the amount of CD4 proteins on the surface of infected cells at an early stage during the replication cycle. If amino acids 64–67 of the Nef protein of SIVmac239 are deleted, this property is lost, and the mutant virus is significantly less virulent. Thereby, the Nef proteins bind to the sorting signal of two leucine residues in the cytoplasmic moiety of the CD4 receptor, leading to the interaction with the adapter protein AP2; as a consequence, CD4 receptors are transported from the cell surface and the ER to the lysosomes, where they undergo proteolytic degradation. This process increases the incorporation of viral Env proteins in the cytoplasmic membrane, and facilitates release of HIV particles. This prevents binding of newly synthesized virus to the surface of the infected mother cell, and thus also prevents its overinfection. In addition, the same mechanism leads to a reduction in CD28 – an important cofactor for T-cell activation and induction of the cellular immune response – protein levels on the cell surface. Infected cells are then no longer able to exert antigen-specific cellular immune responses.
- Nef proteins reduce the concentration of MHC class I antigens on the cell surface, especially those of the HLA-A and HLA-B alleles; however, the amounts of HLA-C and HLA-E proteins remains largely unaltered. This has two consequences: on the one hand, infected cells are not attacked by antigen-specific cytotoxic T lymphocytes, and on the other, as these cells are not completely depleted of HLA, they cannot be destroyed by natural killer cells. The activity responsible for the reduction of the concentration of MHC class I antigens resides in the carboxy-terminal region of Nef, whose amino acid sequences mediate the association of AP1 proteins with the cytoplasmic carboxy-terminal regions of MHC class I antigens, which are anchored in the membrane of the ER and the Golgi network; in MHC class I proteins that are embedded in the cytoplasmic membrane, the interaction with AP1 induces endocytosis. In both cases, AP1 binding results in the transport of the complexes to the lysosomes and their subsequent degradation.
- Depending on the particular virus isolate with the respective Nef variant, it has also been found that the amount of other cell surface proteins can be altered. In some cases, the concentrations of CD3 as well as MHC class II proteins and chemokine receptors, especially CXCR4, are decreased. In contrast, an increased synthesis of the invariant chain (Ii, CD74), which interacts with MHC class II antigens and prevents their association with specific binding foreign peptides (▶ Chap. 7), has also been found. Possibly, these effects also result in HIV-specific peptides not being bound and presented on the cell surface; as a result, the induction of an appropriate T-helper response does not occur.

Bet and Bel2 Proteins

The Bet protein of spumaviruses arises by an alternative splicing mechanism, which fuses the first 88 codons of the *bel1* reading frame to the *bel2* gene The Bet protein has a molecular mass of approximately 60 kDa and is phosphorylated. The Bel2

protein (44 kDa) is identical to the carboxy-terminal region of the Bet protein. The function of both proteins has not been elucidated so far.

Besides CD4, HIV Uses Additional Components for Attachment to Cells
CD4 and the chemokine receptors CCR5 and CXCR4 are considered as the main receptors of HIV. Nonetheless, there is evidence that infection of certain cell types occurs independently of them. Some HIV strains can infect cells which do not have CD4 receptors on their surface. These include neuronal cells such as oligodendrocytes and Schwann cells. The infection of such cells can be inhibited by antibodies against galactosylceramide, a glycolipid, which is probably the interaction partner of gp120 in brain cells; additionally, galactosylceramide seems to mediate penetration of the virus into intestinal and vaginal mucosal cells. An interaction of viral surface proteins with C-type lectins such as dendritic-cell-specific intercellular adhesion molecule 3 grabbing non-integrin (DC-SIGN, CD209) has also been described; in addition, these also interact with the cellular membrane proteins leucocyte-function-associated antigen (LFA) and intercellular adhesion molecule (ICAM). Recently, binding of HIV to integrin $\alpha_4\beta_7$ has been discovered; this subtype of integrin is present preferentially on the surface of $CD4^+$ memory T cells. This may explain the early loss of such cells in the gastro-intestinal tract. Antibodies directed against epitopes of gp120/gp41 that do not exert a neutralizing effect can also mediate CD4-independent attachment. On the one hand, they interact with virus particles, and on the other, they bind to Fc receptors on the surface of T lymphocytes, macrophages and other cell types, which subsequently become infected. This antibody-mediated attachment – such immunoglobulins are referred to as infection-enhancing antibodies (▶ Sect. 14.5) – is possibly of great importance for the pathogenesis of HIV infection, as it allows entry of HIV into cells that otherwise cannot be infected.

18.1.4 Replication

Attachment of retroviruses to target cells is mediated by the exterior component of the envelope protein complex. The cellular interaction partner of HIV has been identified. It is CD4 (55 kDa), a member of the immunoglobulin protein superfamily, which consists of four extracellular immunoglobulin-like domains. The amino-terminal domain displays three regions with resemblance to the complementarity-determining regions (CDR). HIV binds with high affinity (dissociation constant 10^{-9} M) to the CDR2 and CDR3 domains of CD4 by the conserved C3 region of gp120 near the carboxy terminus (Fig. 18.6). A prerequisite for binding is that gp120 be present in its native conformation as a trimeric, glycosylated complex. Therefore, in addition to the amino acids of the C3 region, further folding-dependent determinants are also involved in the interaction. The CD4 receptor is present on the

surface of T-helper cells as well as dendritic cells, macrophages and monocytes. During induction of the cellular immune response, it binds in complex with T-cell receptors to a constant region of MHC class II proteins. Low concentrations of CD4 proteins have also been detected in other cells such as fibroblasts.

CD4 receptors are not solely responsible for binding of HIV particles. After binding, a conformational change occurs in gp120, through which further domains of the viral glycoprotein are able to interact with additional factors on the cell surface. The V3 region of gp120 is involved, along with the V1/V2 domains, in the interaction with chemokine receptors, which function as accessory interaction partners, i.e. as co-receptors. Since the V3 region has, except for the characteristic β-turn region (Sect. 18.1.3), a high sequence variability, the preference of certain virus isolates for particular cells and tissues is based, at least in part, on the properties of V3. The pertinent chemokine receptors are members of the rhodopsin receptor family, which are anchored in the cytoplasmic membrane by seven transmembrane regions. By interaction of chemokine receptors with chemokines, signal transduction pathways are triggered, which are further routed into the cell by interacting with G proteins (▶ Chap. 8). The chemokine receptor CXCR4, which was originally called fusin by Yanru Feng and colleagues, and interacts with the chemokine stromal cell derived factor 1 (CXCL12) as a natural ligand, is used as a co-receptor by lymphotropic HIV variants (also referred to as X4 viruses) for entry into T lymphocytes. Some moieties of the viral glycoproteins, particularly regions of the V3 and V1/V2 domains, bind to the CXCR4 receptor. This increases the binding affinity between the virus and the cell surface, and facilitates the fusion between the viral envelope and cell membrane, thus promoting entry of the virus into the cell. On the other hand, the chemokine receptor CCR5 and related receptors, which interact with the CC chemokines RANTES, MIP-1α and MIP-1β, are responsible for the preferred binding of virus variants with tropism for macrophages (R5 viruses) to monocytes and macrophages. Binding of the natural chemokine ligand blocks attachment of the virus, thus exerting an inhibitory effect on HIV infection. Interaction of the external surface protein (gp51) with the cellular protein X4 has been demonstrated in FIV. This protein is the CXCR4 chemokine receptor homologue occurring primarily on haematopoietic cells of cats.

Most neutralizing antibodies are directed against the V3 region. However, they do not inhibit binding of gp120 to the CD4 receptor, but affect the rearrangements of the protein complex, which are important for the subsequent steps, including interaction with chemokine receptors, cell entry and membrane fusion. The following process is conceivable. The virus binds by its exterior glycoprotein gp120 to the CD4 receptor and the corresponding chemokine receptors, which act as co-receptors. This induces further rearrangements in the protein structure of the glycoprotein complex. The conformational change activates the fusogenic activity of the hydrophobic domain at the amino terminus of the transmembrane gp41 protein, which is similar to the fusogenic regions of F_1 proteins of paramyxoviruses or HA_2 proteins of orthomyxoviruses (▶ Sects. 15.3 and ▶ 16.3). It embeds in the cytoplasmic

membrane, and mediates the fusion with the viral envelope, leading to the penetration of the capsid, which harbours the single-stranded RNA genome as well as the enzymes reverse transcriptase, protease and integrase (Fig. 18.11).

The structure of the penetrating capsid remains intact, even if its conformation is altered, possibly by the action of the protease. By this procedure, some capsid proteins are released and the reverse transcription complex becomes permeable to nucleotides. The reverse transcriptase, which is associated with the ribonucleoprotein complex, transcribes the RNA genome into double-stranded DNA in the cytoplasm. This process begins at the free 3′-OH end of the tRNA molecule, which is bound to the PB site in the 5′ region of the RNA genome, and facilitates initiation of the polymerase reaction. In the first step, starting from the tRNA, the reverse transcriptase synthesizes a DNA strand, which is complementary to the U5 and R sequences. The inherent RNase H activity of reverse transcriptase degrades the RNA moiety of the short hybrid molecule (Fig. 18.9). This leads to a single-stranded DNA, which contains sequences complementary to U5 and R, and is covalently linked with the 3′ end of the tRNA. This molecule is transferred to the 3′ end of the RNA genome, where it hybridizes with the respective sequences of the redundant R region, and serves as a primer for the synthesis of a continuous DNA strand, which is complementary to the entire RNA genome. Whether the primer transfer occurs intramolecularly to the 3′ end of the same RNA strand or intermolecularly to that of the second RNA genome in the capsid is not clear. The RNA moiety of the RNA/DNA hybrid formed is in turn degraded by the RNase H function, but not completely: the short RNA sequence of the polypurine tract remains intact. The structure of the RNA/DNA hybrid in this region is apparently very stable, and its digestion by the RNase H activity is retarded. The free 3′-OH end of the RNA moiety is used in the following reaction as a primer for the synthesis of the first double-stranded DNA segment, which includes the sequences of the PB region containing the still-bound tRNA. The tRNA is now degraded. There remains a partially double-stranded DNA molecule with a 3′ overhanging sequence, which is complementary to the PB region present at the 3′ end of the continuous DNA strand synthesized first. Both sequences can hybridize with each other, thus providing the primer structure required for the subsequent synthesis of double-stranded DNA. The ends are filled up. Finally, the viral genome is present as a double-stranded DNA molecule in which the coding sequences are flanked by the U3, R and U5 regions of the LTR regions (Fig. 18.9). Since the reverse transcriptase does not possess an exonucleolytic proofreading activity to enhance the fidelity of DNA synthesis, misincorporations occur with a probability of 10^{-3}–10^{-4} during synthesis of double-stranded DNA – a process that contributes to the high mutation rate of retroviruses.

The double-stranded viral DNA genome remains associated with the protein components, and it is transported with them as a preintegration complex into the nucleus. In HIV, the proteins involved are Vpr and the matrix protein p17. In the virus particle p17 is associated with the inside of the envelope. A phosphorylated

form of p17 remains associated with the preintegration complex and facilitates, along with Vpr and cellular nuclear import factors, the transport of the double-stranded DNA genome through nuclear pores into the nucleus, thus facilitating the infection of quiescent cells. By this property, lentiviruses differ from all other

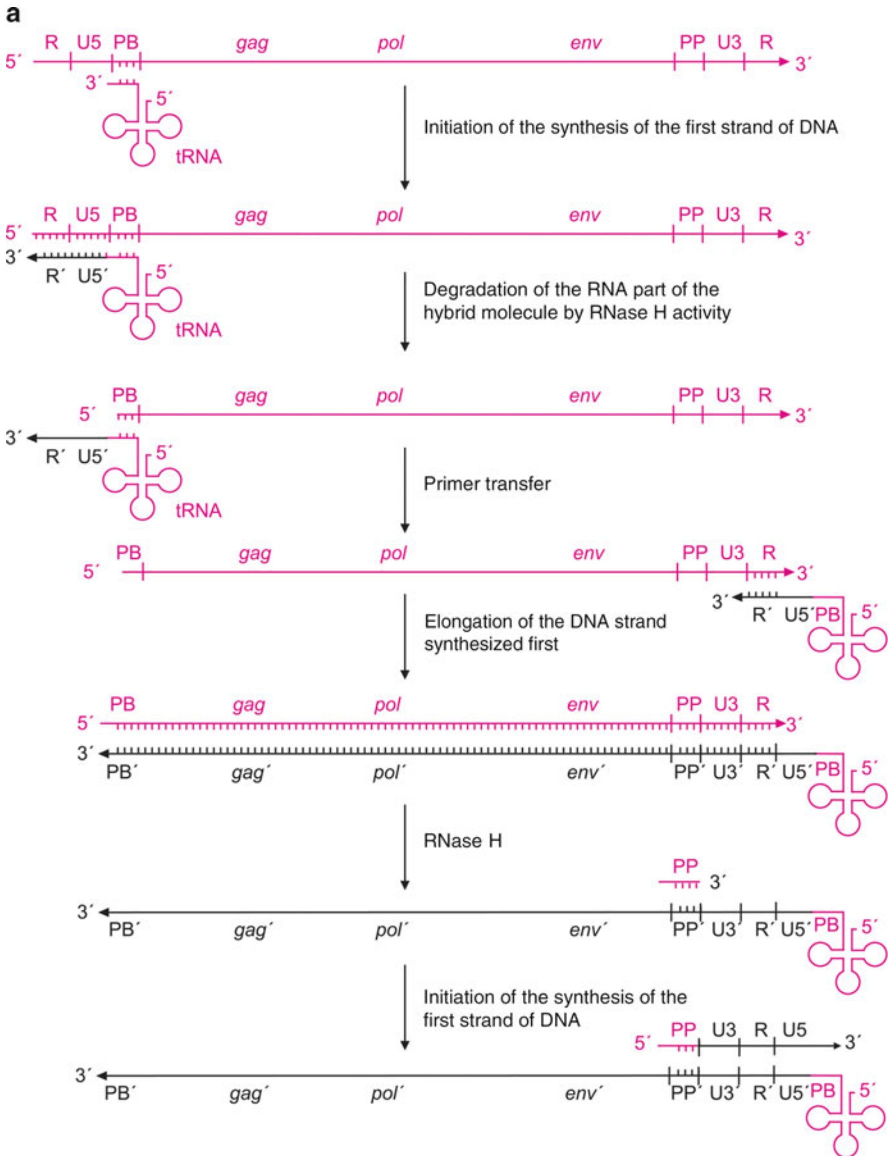


Fig. 18.9 (continued)

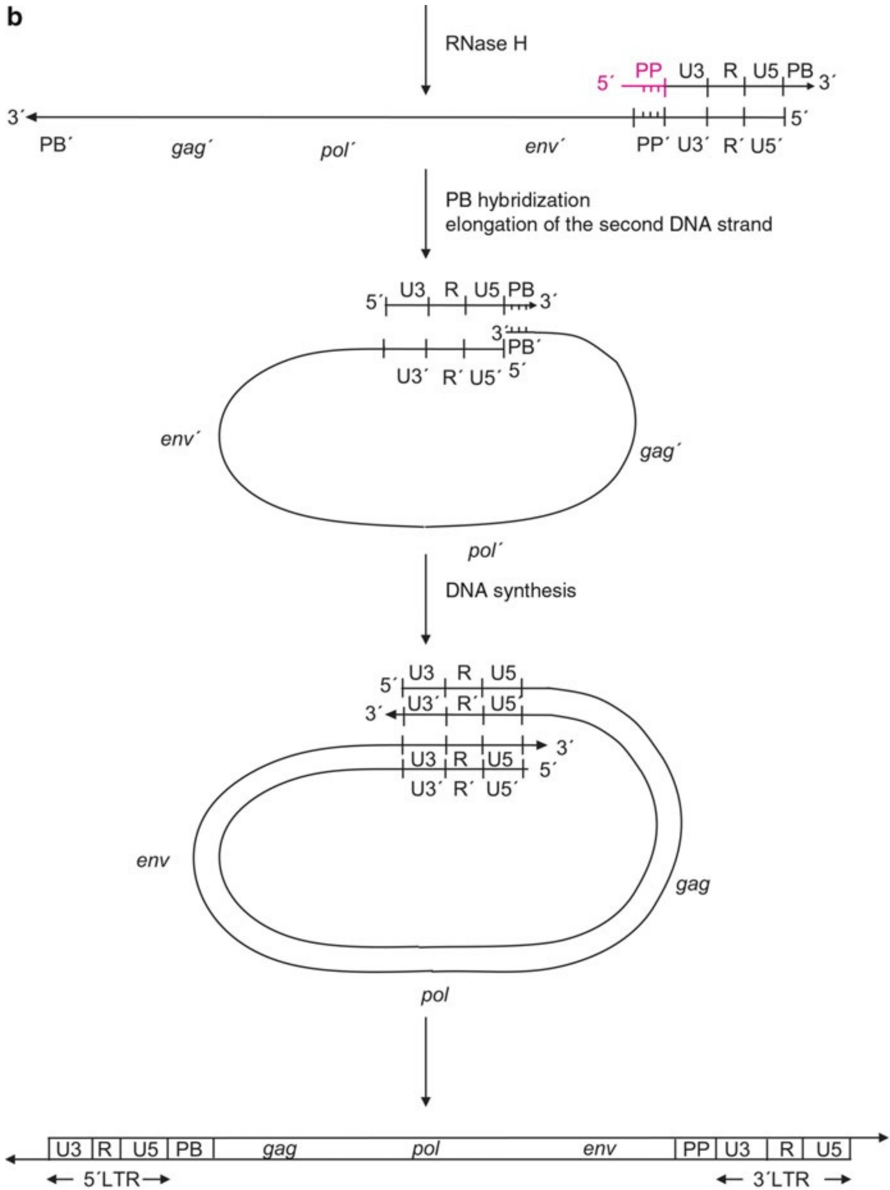


Fig. 18.9 Processes involved in transcription of the retroviral single-stranded RNA genome into double-stranded DNA by reverse transcriptase. The mRNA viral genome and its associated tRNA are depicted in *red*; the sequences that are reverse-transcribed into DNA are represented in *black*. Sequence elements and open reading frames are represented by the usual abbreviations. Sequences that are complementary to the RNA sequences of the genome are marked by the addition of *primes*

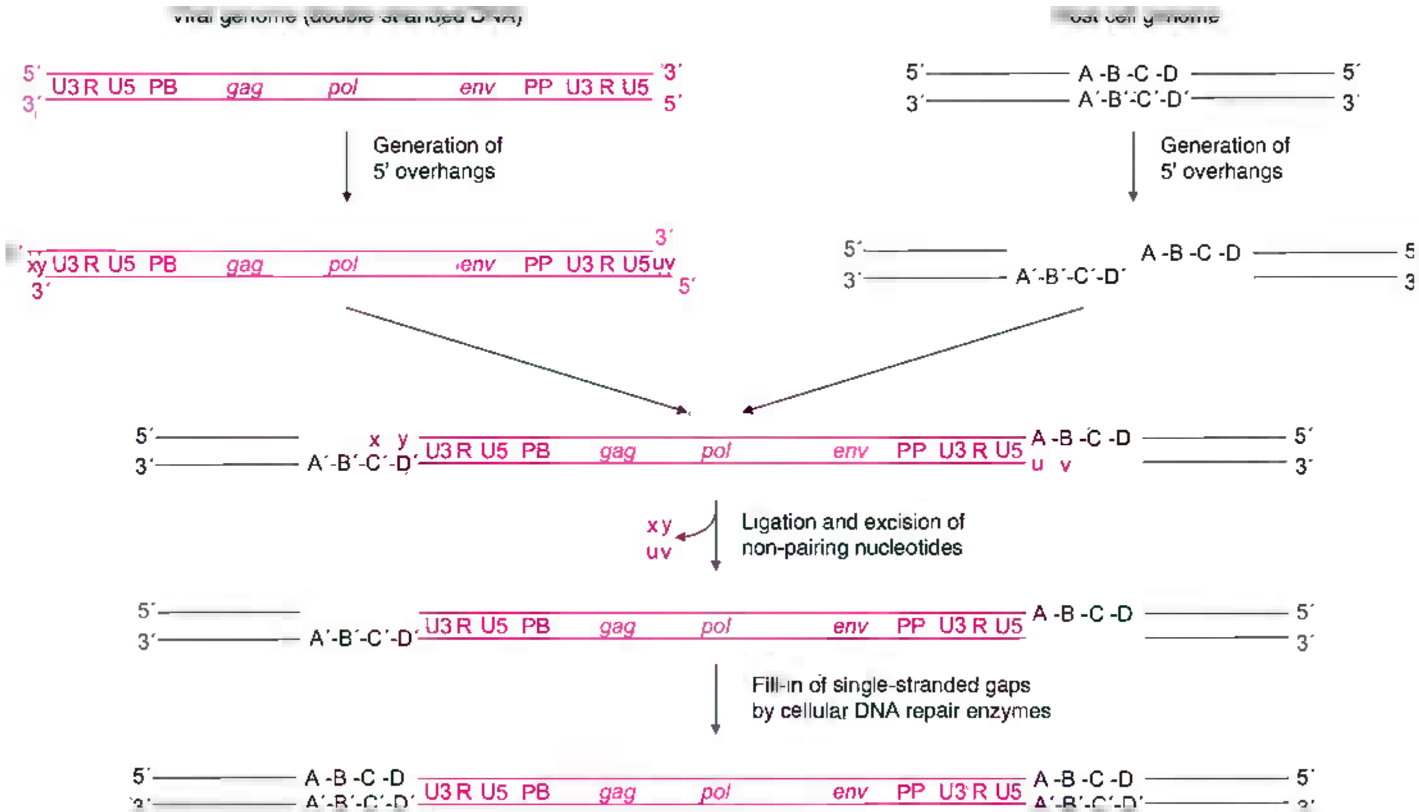


Fig. 18.10 (continued)

retroviruses, which can infect only dividing cells. The preintegration complex has a size of approximately 20–30 nm. Therefore, it cannot penetrate through the nuclear pores without the assistance of additional, nuclear-transport-promoting factors. Hence, most retroviruses can only transport and integrate their double-stranded DNA genome into the chromosomal DNA of host cells when the nuclear membrane disintegrates during mitosis. For this reason, the integration of the viral genome, and hence the infection, is usually possible only in mitotic (dividing) cells. In the case of lentiviruses, a segment of the polypurine tract region spanning about 99-nucleotides has been observed to remain as a triple helix containing three nucleic acid strands. The gap that arises during initiation and elongation of the second DNA strand in the overlapping region (also called flap) is necessary for transport and remains intact during this process. Proteins of the preintegration complex interact with the triple helix and mediate the interaction with nuclear pore components. Lentivirus mutants carrying a deletion of the polypurine tract are not able, like other retroviruses, to transport the preintegration complex into the nucleus; it is retained in the cytoplasm.

In the nucleus, the double-stranded DNA molecule is integrated into the genome of the host cell. This process is performed by the third enzymatic component of the Pol protein complex: the integrase removes two nucleotides from the 3′ end of each LTR, resulting in a short 5′ overhang of two single-stranded nucleotides. The endonucleolytic activity of the integrase also cleaves the cellular DNA at an arbitrary site, which, however, must be accessible in the euchromatin. For example, this is the case in actively transcribed regions. The cleavage generates overhanging 5′ termini, which – depending on the virus type – may include four to six nucleotides. The 5′ ends of the cellular DNA are covalently linked with the recessed 3′ termini of the viral genome by phosphodiester bonds, the 5′ overhanging dinucleotides of the viral genome are removed and the remaining single-stranded gaps are filled by cellular repair systems and covalently linked to double-stranded DNA by ligases (Fig. 18.10). During this process, the viral genome loses its two terminal nucleotides, and four to six bases of the cellular DNA flanking the integrated provirus are duplicated. Integration of proviral DNA alters the genome of the cell and has consequences: depending on the position of the insertion site, cellular genes can be destroyed (insertional mutagenesis), or their expression can come under the control of the 3′ LTR promoter, which can activate them. On the other hand, the integration site can also influence the activity of the 5′ LTR promoter: if the

Fig. 18.10 Integration of the double-stranded DNA viral genome into the genome of the host cell after completed reverse transcription. *Red* sequences of the double-stranded DNA provirus. *Black* genomic DNA sequences of the host cell. The letters *u* and *v* and *x* and *y* represent the single-stranded nucleotides at both termini of the non-integrated provirus, which are later removed during integration. The letters *A*, *B*, *C* and *D* represent an arbitrary genomic DNA sequence of the host cell that is cleaved by the integrase, which produces 5′ protruding ends. After integration, this nucleotide sequence is present as identical directed repeats flanking both ends of the proviral DNA

integration occurs within a highly methylated or genetically inactive chromosomal DNA region, then only marginal or no transcription of the integrated proviral genome occurs; as a result, no progeny viruses can be generated, and a latent form of infection is established.

Fundamentally, integration of the viral genome is a prerequisite for gene expression. Depending on the virus type, various cellular factors bind to *cis*-acting sequences in the U3 region of the LTR, and induce transcription by the cellular RNA polymerase II. In the case of HIV, NFκB is such an important protein, whereas CREB and Ets factors, among others, bind to *cis*-acting sequence elements in the U3 region of HTLV-1. In HIV-1, sufficient levels of the cellular factors NFκB (which is activated after stimulation of infected T lymphocytes by the immune system) and Sp1 are necessary to initiate low level transcription. The transcripts are usually terminated after the sequences of the TAR element. However, an elongated mRNA is occasionally synthesized, which is multiply spliced and translated into Tat protein in the cytoplasm. It increases the transcription rate by about 100-fold by transport into the nucleus, binding to TAR elements at the 5′ end of viral mRNAs, stabilizing them and facilitating their elongation.

In this early infection phase, there are three size classes of viral mRNA in the nucleus:

1. Multiply spliced mRNA molecules with a length of approximately 2,000 nucleotides, which encode the proteins Tat, Rev and Nef, and lack the RRE, which was removed together with an intron.
2. Singly spliced mRNA species with a length of about 4,000 nucleotides, encoding the proteins Env, Vif, Vpr and Vpu.
3. Unspliced mRNA forms, which are equivalent to the RNA genomes, and from which the Gag and the Gag–Pol proteins are translated (Fig. 18.11).

The multiply spliced mRNA species predominate. They are transported to the cytoplasm and translated into the respective proteins.

The activity of the Rev protein is a precondition for the transition from the early to the late phase of the replication cycle. Inasmuch as it is translated from a multiply spliced mRNA molecule, it is already present at an early stage. After transport into the nucleus, it binds to the RRE of singly spliced and unspliced mRNA species, allowing their export into the cytoplasm, where they are either translated or packaged as RNA genomes into new virus particles. This form of Rev- or Rex-dependent mRNA export is found only in lentiviruses and deltaretroviruses. The other retroviruses, including spumaviruses, have a conserved RNA sequence in their unspliced transcripts, which is called a constitutive transport element (CTE) and is located adjacent to the polyadenylation site. Cellular proteins bind to this element, facilitating the transport of CTE-containing mRNAs from the nucleus to the cytoplasm.

Synthesis of Env proteins occurs on the ER membrane. The amino-terminal domain acts as signal peptide and is responsible for binding of the signal recognition particle. The nascent amino acid chain is translocated through the ER membrane, to which it is anchored by the hydrophobic domain of the carboxy-terminal

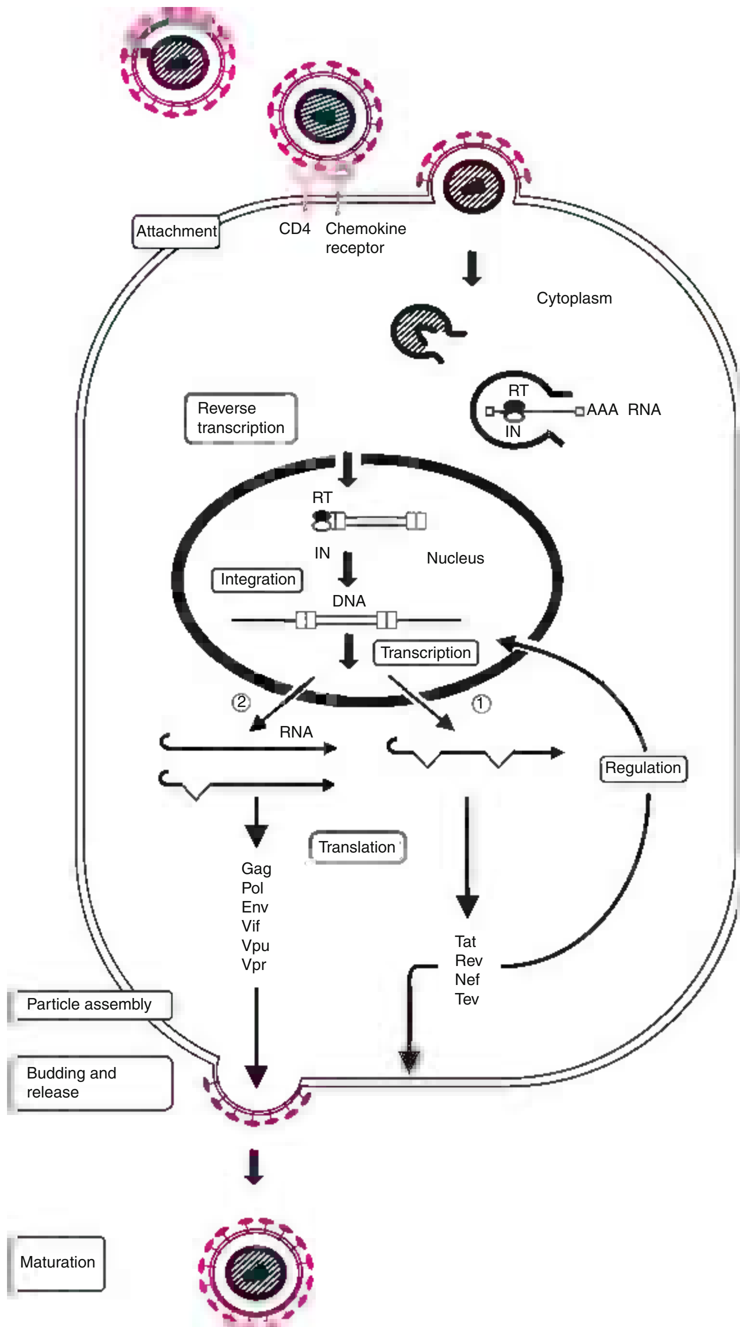


Fig. 18.11 Events that occur during HIV infection of CD4$^+$ T lymphocytes or macrophages/monocytes. In the *upper part*, the virus attaches with its exterior glycoprotein gp120 to the CD4

region (Fig. 18.6). The Env protein regions that are localized in the lumen of the ER are glycosylated at asparagine residues. The polypeptides interact with each other forming trimeric complexes. The activity of Vpu molecules, which are also associated with the ER membrane, prevents viral glycoproteins from interacting with the CD4 receptors in the ER lumen. Maturation cleavage into the exterior (gp120) and membrane-anchored (gp41) protein components occurs during trafficking through the Golgi apparatus to the cell surface. The glycoprotein complexes can be detected on the surface of infected cells. There, they exert their fusogenic activity by binding to CD4 proteins of uninfected cells – chemokine receptors serve as cofactors – and by inducing the fusion of the membranes. In this way, the HIV genome can be transmitted from cell to cell, enabling the virus to spread in the organism in a particle-independent fashion.

Translation of all other virus proteins occurs on free ribosomes in the cytoplasm. The amino termini of Gag and Gag–Pol precursor proteins are myristoylated during their synthesis, and cellular factors mediate their transport to the cytoplasmic membrane and the ER membrane, in which they embed. In HIV-infected T-helper cells, these steps of infectious virion morphogenesis occur almost exclusively on the cytoplasmic membrane, and the particles of progeny viruses are released from the cell surface. In contrast, assembly of viral proteins and the viral genome in HIV-infected monocytes and macrophages occurs on intracellular membranes, mainly on the ER membrane. This has the consequence that the resulting virus particles are released into the lumen of the ER or Golgi vesicles. They are present as intracellular infectious particles, and cannot be detected by neutralizing antibodies during that period. Infected macrophages are a reservoir for the viruses, which can transport the pathogens to other tissues, e.g. to the brain.

However, the molecular assembly processes are similar in both cases: by intermolecular interactions of matrix and capsid protein moieties, Gag and

Fig. 18.11 (continued) receptor and the chemokine receptor CXCR4 on T lymphocytes or CCR5 on macrophages. Structural rearrangements within the viral gp120/gp41 surface protein complex activate the fusogenic activity of the transmembrane protein gp41, whereby the viral envelope fuses with the plasma membrane of the cell. The viral RNA genome is transcribed into double-stranded DNA by the reverse transcriptase. In the nucleus, the viral integrase mediates the integration of the viral DNA into the genome of the cell. The proviral DNA is transcribed by the cellular RNA polymerase II, whereby multiply spliced mRNAs are initially synthesized. The regulatory proteins Tat, Rev, Nev and Tev are translated in the cytoplasm and are subsequently transported to the nucleus, where they enhance transcription and synthesis of singly spliced and unspliced RNA molecules. After transport into the cytoplasm, these serve not only as mRNAs for translation of the viral structural and accessory proteins, but also as viral genomes, which assemble with the protein components on the cytoplasmic membrane. As a result, immature virus particles are generated by budding, and are released from the cell surface (*lower part*). Maturation to infectious viruses is performed through the viral protease by cleaving the Gag and Gag–Pol precursor proteins within the virus particle. *IN* integrase, *RT* reverse transcriptase

Gag–Pol precursor proteins accumulate in the respective membrane compartments that can be visualized by electron microscopy. Additional interactions occur between Gag precursors and membrane-anchored glycoproteins. Amino acids of the matrix proteins and the intracellular domains of transmembrane proteins are involved in this interaction. Cellular chaperones such as cyclophilin, a prolyl isomerase, play an important role in this process. The genomic RNA molecules – i.e. the unspliced mRNA species from which the Gag and Gag–Pol precursor proteins are translated – contain the ψ element in the leader region between the U5 sequences and *gag* genes. It is lacking in all other mRNA species, as it is removed by splicing. This ψ element enables the RNA genomes to bind to the zinc finger motifs of nucleocapsid protein moieties in the Gag and Gag–Pol precursors. This mechanism ensures that only complete RNA molecules interact with viral components on the membrane. It is unclear how it is ensured that always just two RNA genomes are attached to the corresponding regions and are packaged into the resulting particles. Upon contact with RNA molecules, the pertinent regions fold outwards and form membrane vesicles, which then bud from the cell surface or into the lumen of the ER. Gag–Pol precursor proteins are present in high local concentration in these still immature virus particles, which have a slightly acidic pH of about 6.0–6.2 owing to the amino acid composition of viral proteins. Under such conditions, the protease domains, which are located at the transition between the Gag and the Pol region, can dimerize, and exert their activity effectively in the moderately acidic environment. The mature protease is cleaved from the precursor polyprotein by an autocatalytic process. Subsequently, the Gag and Gag–Pol precursors are processed into matrix, capsid, nucleocapsid and link proteins, as well as reverse transcriptase and integrase. At the same time, the virus particles undergo structural rearrangements to form the conical capsid and become infectious. Since the protease is activated just in the released virus particle, it is ensured that the precursor proteins are not cleaved in the cytoplasm. In such a case, the association with the membrane would be lost, and the proper morphogenesis and assembly with the RNA genome would be impossible. The untimely proteolytic activity would also affect cellular proteins, and the cell would be prematurely destroyed. The cells die by viral replication; apoptosis is induced.

The replication cycle of HTLV is very similar to that of HIV. Also in this case, translation of the multiply spliced mRNA species and synthesis of the Tax and Rex proteins occur in the early phase of the cycle. Tax proteins bind to cellular factors which are associated with the U3 region, thus increasing transcription of viral genes. The Rex proteins interact with RxRE regions located close to the 5′ terminus of unspliced mRNA species, which serve as genomes and also encode the Gag, Gag–Prot and Gag–Prot–Pol precursor proteins, and mediate their nuclear export and translation. The morphogenesis processes are largely similar to those described for HIV.

Simple retroviruses do not encode accessory or regulatory proteins or transactivators. Gene expression of the integrated provirus is induced only by cellular factors that specifically interact with distinct *cis*-active elements of the

U3 regions. The transition from the early to the late infection phase is not regulated, as these viruses do not possess gene products that are equivalent to the Rev proteins. However, the processes of reverse transcription and integration of viral sequences and the formation of infectious particles are comparable to those described above. Unlike HIV, these viruses are not cytolytic, but they are capable of transformation if they possess viral oncogene gene products. They induce a persistent (productive) infection, during which progeny viruses are continuously generated without destroying the cell.

The Diploid Genome is Responsible for the Emergence of Novel Retrovirus Types

The fact that all infectious retrovirus particles contain two RNA genomes suggests that intermolecular processes can occur during reverse transcription. The diploid RNA genome is considered as the basis for the emergence of new virus types and viruses containing oncogenes. Reverse transcription occurs under preservation of the particulate structures in which the two RNA genomes are present close to each other. During DNA synthesis, the reverse transcriptase switches back and forth between the two identical template molecules and synthesizes pseudo "chimeric DNA strands", which consist of successive alternating sections of the original genomes (copy choice recombination). In the case of double or multiple infection of a cell with different virus subtypes, this feature promotes the formation of recombinants (Sect. 18.1.5). Cellular genes are assumed to have become an integral part of the viral genetic information by similar processes: The viral genomes correspond to unspliced mRNA species. Their packaging is mediated by the ψ sequences, which are located in front of the *gag* genes at the 5′ ends. Occasionally, an erroneous transcription occurs during the replication cycle, which can lead to reading and synthesis of genome sequences of the host cell that flank the proviral genome, which thus become part of the RNA genomes, and are incorporated into the virions. These particles are infectious, the capsids are able to attach to and penetrate target cells and chimeric DNA strands can arise during reverse transcription, whereby the cellular sequences become an integral part of the retroviral genome. In subsequent infection cycles, these "foreign" sequences change so much owing to the high error rate of transcription that they largely lose their original function, and their origin can only be inferred by the sequence similarity to the cellular genes from which they have been derived. In this way, genes encoding cellular membrane proteins could have been integrated in the mRNA of the evolutionarily very old retroelements, which eventually mutated into the *env* genes of exogenous retroviruses. Analogously, other sequences encoding cellular regulatory proteins could similarly have evolved into the oncogenes of the alpharetroviruses, betaretroviruses and gammaretroviruses as part of the viral genetic information.

The Variability of Retroviruses Is Primarily Based on the Low Fidelity of the Cellular RNA Polymerase II Activity

Viral genomes are synthesized by the cellular RNA polymerase II, which incorporates nucleotides with a relatively high error rate during transcription because it does not possess exonucleolytic proofreading activity. High-fidelity synthesis is not necessary for the processes that are performed by this cellular enzyme. It is usually responsible for the synthesis of relatively ephemeral products such as mRNA molecules, which are subsequently translated into proteins. Hence, RNA synthesis errors are not inherited. However, errors that arise during synthesis of the mRNA-like genomes of retroviruses are manifested as mutations in the genetic information of progeny viruses, which are eventually released from infected cells. The reverse transcriptase also contributes to viral variability, but to a lesser extent than the cellular RNA polymerase II. The reverse transcriptase is required only once during the infection cycle, namely for transcribing the RNA genome into DNA before integration into the genome of the host cell. However, the many thousand RNA genomes that are packaged into progeny viruses are synthesized by the cellular RNA polymerase II. Therefore, the quasispecies that have been particularly observed in HIV infections can likely be attributed to the error-prone RNA synthesis by the cellular enzyme.

Retroviral Vectors Resemble the Genome of Defective Retroviruses

Retroviral vectors are increasingly applied in gene therapy approaches. These vectors are generally derived from murine leukaemia viruses; they contain all the sequences necessary for integration of the LTR regions, including the ψ element, which is responsible for packaging. Recent developments are based on lentiviral vector systems, which facilitate treatment of resting, not actively dividing cells. In such vectors, the regions coding for viral proteins are replaced by specific heterologous genes and their regulatory sequences which are desired to be transduced into human cells The vectors are produced in so-called helper or packaging cell lines that contain a copy of a complete retroviral genome, which, for safety reasons, are usually partitioned into discrete DNA sections on several plasmids. It synthesizes all the proteins necessary for replication and infection; however, it is not able to package its genomic viral RNA into particles since it has a designed defect in the ψ sequences. If retroviral vectors are transduced into helper cells, they are transcribed, and the resulting transgenic mRNA interacts through its own ψ element with the structural proteins of the helper virus, leading to its packaging into virus particles. Recombinant virions, which do not have the genetic information for viral components, attach to respective target cells by their surface proteins, the capsids can penetrate into the cytoplasm, and the

transgenic RNA is transcribed into double-stranded DNA, which is subsequently integrated into the genome of the host cell. The advantage of this system resides in the stable integration of recombinant genes, which can be inherited by daughter cells. However, an important drawback is the retroviral property of unspecific, indiscriminate integration into random sites in the genome of the cell. Furthermore, an intracellular recombination of LTR regions of the integrated vector with similar sequences of retrotransposons and endogenous retroviruses of the human genome cannot be excluded with the vector systems that are currently used. This process could lead to translocation of recombinant genes.

18.1.5 Human Pathogenic Retroviruses

18.1.5.1 Human Immunodeficiency Virus

Epidemiology and Transmission

AIDS was first described in 1981 among a group of homosexual men who had severe opportunistic infections of the lung. The way of transmission strongly suggested the involvement of viral contamination of blood or blood products. In 1983, this assumption was confirmed when the groups of Luc Montagnier at the Pasteur Institute in Paris and Robert Gallo at the National Cancer Institute in Bethesda isolated retroviruses from lymphocytes of AIDS patients. The name “human immunodeficiency virus” (HIV) has been in use since 1986. Different simian species are naturally infected with similar immunodeficiency viruses, and more than 40 different SIV types have been identified. However, monkeys usually do not develop diseases. HIV-1 has its origin in Africa, and is the result of zoonotic transmissions of SIV to humans. For example, an immunodeficiency virus was isolated from chimpanzees, SIVcpz, which is very closely related to HIV-1 and is considered to be its direct ancestor. However, recent data suggest that in contrast to other SIV types, SIVcpz can cause AIDS-like symptoms in chimpanzees: by observing wild chimpanzees over a period of 9 years, Beatrice Hahn and colleagues found that SIVcpz-infected chimpanzees died earlier than uninfected chimpanzees; AIDS-like symptoms could be diagnosed in the carcasses of infected chimpanzees. This chimpanzee virus was possibly repeatedly transmitted to humans some decades ago, first in the 1930s, and developed subsequently to the human pathogenic HIV-1. A variant of HIV-1, which is very similar to SIVcpz, was found in the serum of a member of the Bantu tribe originating from 1959. Retrospective serological studies have also revealed that antibodies against HIV were sporadically present in the population before 1980. In the early 1980s, the HIV-1 epidemic began nearly simultaneously in large cities of Central Africa and the USA. Since then, this infection has spread throughout the world, and has become a pandemic, especially in the Third World countries of Africa, India, Southeast Asia and South America. According to the World Health Organization, 33 million people were infected in 2007. Because of sequence differences of at least 30 %, HIV-1 is divided into three subgroups: M (major), O (outlier) and N (new or

non-major). Infections with viruses of subgroup M are found in the vast majority of HIV cases, whereas only about 100,000 cases or very few infection cases are known for viruses of subgroups O and N, respectively. The viruses of subgroup M can be subdivided into eight subtypes (clades) – A, B, C, D, F, H, J and K – whose sequences differ by up to 15–20 %. Worldwide, infections with HIV-1 subtype C are most frequent, whereas clade B is the commonest variant in western Europe and the USA. The HIV isolates that were originally assigned to subtypes E and I are, on closer examination, variants that have arisen by recombination; the same is assumed for subtype G viruses.

The genome sequences of HIV-2 differ by up to 40 % from those of HIV-1. HIV-2 was originally isolated from a West African AIDS patient in 1986. The infection with HIV-2 is attributed to sooty mangabeys, which are frequently kept as pets in West Africa, and are asymptomatically infected with a similar virus (SIVsmm). This virus has also been repeatedly transmitted from simians to humans. Eight independently originating subgroups of HIV-2 are known today (A–H); the most frequent subtypes are A and B. HIV-2 infection was originally confined particularly to West Africa. However, meanwhile, cases have been detected in India and occasionally also in many other countries. Thus, this virus has also transcended continental borders. However, a characteristic feature of HIV-2 infections is that they are frequently asymptomatic.

Besides humans, HIV-1 can infect only chimpanzees. They also develop symptoms of immune deficiency after 10–15 years. SIVmac causes an AIDS-like disease in rhesus monkeys. By contrast, vervet monkeys, the natural hosts, survive infections without signs of disease. In addition, HIV-related immunodeficiency viruses have also been found in cattle (bovine immunodeficiency virus) and cats (FIV; Sect. 18.1.6). These pathogens can be cultivated in T-cell lines or macrophage cell lines.

In humans, HIV is transmitted through semen or vaginal fluids in homosexual and heterosexual sexual intercourse and by contaminated blood (e.g. during transfusions by using contaminated needles or other medical instruments, and by common use of syringes by drug addicts) and blood products (not completely inactivated coagulation factors or immunoglobulin preparations). Furthermore, the virus is mainly transmitted from infected women to children at delivery or during lactation. Sexual contact is now responsible for most new HIV infections. Very large amounts of virus can be found in the semen of some HIV-infected men. Apart from such "super producers", there are also "super transmitters": Jan Münch and colleagues detected specific proteins (semen-derived enhancer of viral infection, SEVI) in the semen of such individuals which form amyloid-like fibrils and promote HIV transmission. The fibrillar SEVI proteins facilitate attachment of the virus to its target cells. However, HIV primarily enters the organism not as a free particle, but through infected donor cells, which contain not only the integrated proviral genome in the chromosomal DNA, but also newly produced viruses. In the next step, the HIV-infected cell can further transmit the virus to other cells of the immune system – such as macrophages, dendritic cells and T lymphocytes – or to cells of the vaginal or intestinal mucosa. In 2008,

investigations by Brandon F. Keele and colleagues demonstrated that in most cases only one virus is transmitted, and causes the infection.

Emergence of New Variants of HIV-1

The finding that recombinants can be generated between different virus subtypes in HIV-1-infected patients is relatively new. These recombinant HIV variants additionally contribute to mutation events, and to the generation of new genotypes and subtypes. HIV recombinants can be generated when a cell is concomitantly infected by two or more HIV subtypes, or when an infected cell is infected with another HIV subtype at a very early stage of infection, i.e. before establishing the persistence and latency stage. During viral replication, two RNA genomes are packaged into a particle. If the RNA molecules are from different subtypes, then this "diploid" status of an HIV particle will promote recombination between the two nucleic acid strands during reverse transcription after entry into the cytoplasm of a cell. Meanwhile, virus isolates have been detected whose genomes represent mosaics of up to seven different HIV subtypes.

Clinical Features

The course of HIV infection can be divided into three phases:

1. The first phase or primary infection is frequently inapparent. Only in 20–30 % of cases is it associated with flu-like or infectious mononucleosis-like symptoms, an uncharacteristic rash and swollen lymph nodes. These symptoms are developed after an incubation period of up to several weeks, and are usually recognized retrospectively after HIV seroconversion. The viral load, i.e. the concentration of genome equivalents, can be determined by quantitative PCR. The patients are viraemic at this early stage, and large quantities of virus of up to 10^6–10^8 genome equivalents per millilitre of blood can frequently be detected. In this phase, the virus population multiplies with a doubling time of approximately 6–10 h. Every infected cell leads to 20 new infected cells in the following replication round. Temporarily, the titre of $CD4^+$ lymphocytes falls below 500 cells per microlitre and the CD4/CD8 cell ratio changes to less than 0.5. This phase of primary infection usually lasts about 6 months.
2. The primary infection is followed by a prolonged, symptom-free stage of latency or chronic infection. In this period, HIV-specific antibodies and T lymphocytes can be detected in patients. In addition, infected cells, usually depleted of MHC class I proteins, are recognized and lysed by natural killer cells (Fig. 18.12). As a result of the antiviral immune response, viral load decreases to levels below 2×10^4 genome equivalents per millilitre in the peripheral blood. If values less than 10^3 genome equivalents per millilitre of blood are measured, then a very long asymptomatic phase is established in these patients. Nowadays, so-called elite controllers are known among HIV-infected patients who do not exhibit detectable HIV production in peripheral blood, and in which the asymptomatic

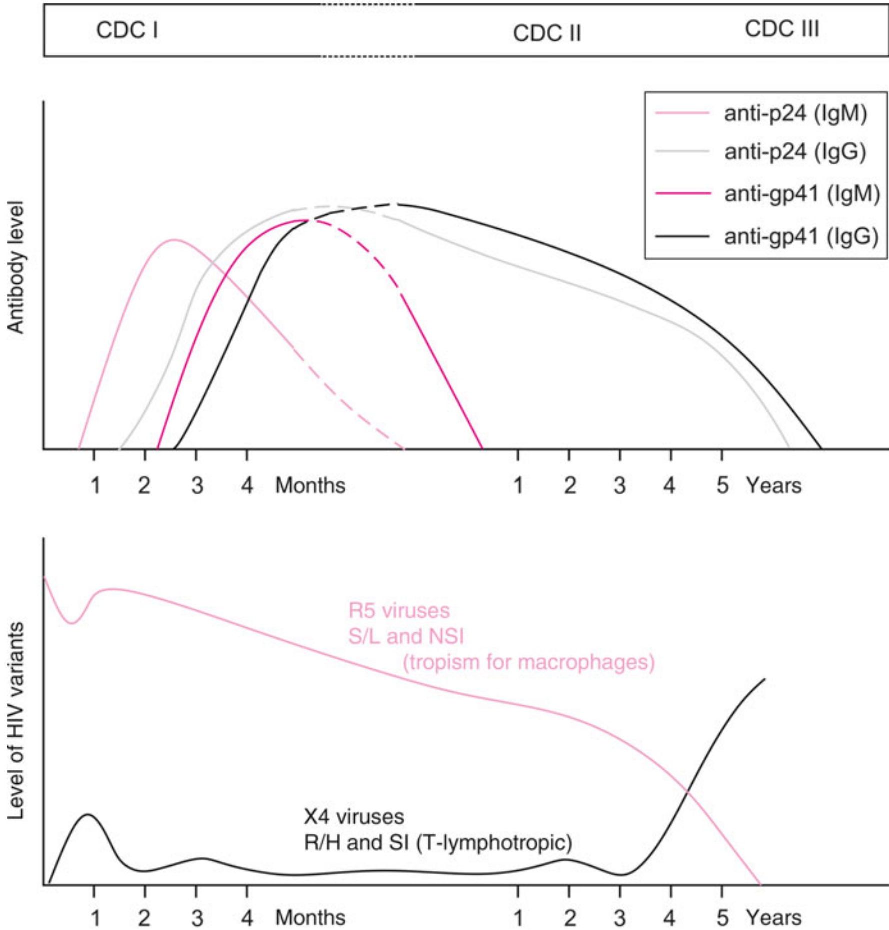


Fig. 18.12 Clinical stages of the disease according to the Centers for Disease Control and Prevention (*CDC*) classification (*top*) in connection with the serological course of HIV infection (*middle*) and the titre of HIV in the peripheral blood (*bottom*). The production of IgM antibodies against the capsid proteins (*light red*) and the successive synthesis of IgM antibodies against the glycoproteins (*red*) can be detected early after infection. IgM antibodies are followed by IgG antibodies against the capsid proteins (*light grey*) and the glycoproteins (*black*) Whereas IgM is no longer detectable a few months after infection, the concentration of IgG remains relatively constant for prolonged periods. It diminishes during the transition to CDC stage III. HIV variants (R5 viruses) which correspond to the slow/low growth type (*S/L*, *red line*) in cell culture and do not induce syncytia formation (*NSI*) are predominantly found in infected cells at the beginning of infection. Their levels decrease during the progression of infection, and they are continuously replaced by HIV variants (X4 viruses) which are prone to induce syncytia (*SI*, *black line*) and rapidly multiply to high concentrations (*R/H* rapid/high) in culture

phase lasts for over 20 years. However, if the viral load does not drop below 10^4 genome equivalents per millilitre of blood, then the asymptomatic latent period has an average or short duration, and lasts only a few years. As a consequence of viral replication, the concentration of $CD4^+$ cells decreases gradually. At this stage, a balance predominates between viral replication, cell death and production of new $CD4^+$ cells through differentiation of progenitor cells in the bone marrow. Although the viral infection destroys cells faster than they can be reproduced, the organism is able to compensate for this condition.

3. In approximately half of HIV-infected patients who are not treated with antiviral drugs, the asymptomatic phase lasts for more than 10 years. This is followed by the third infection phase, which is characterized by the appearance of the clinical symptoms of HIV infection and AIDS. One or 2 years before the onset of disease symptoms, the number of $CD4^+$ cells decreases rapidly and the concentration of the virus increases in the peripheral blood. A lymphadenopathy syndrome can be established in AIDS patients, and can last for several weeks to years. It is characterized by an enlargement of at least two peripheral lymph nodes, which persists for more than 3 months. The lymphadenopathy syndrome can develop into the AIDS-related complex. In this phase, fever, nocturnal sweating and weight loss occur, and are occasionally accompanied by opportunistic infections such as oesophageal candidiasis, herpes zoster, cytomegalovirus-associated retinitis, and Kaposi's sarcoma, which is caused by human herpes virus 8. However, the symptoms can disappear spontaneously. HIV-specific antibodies are present in the serum of patients during this time, and the number of $CD4^+$ cells can decrease below 400 per microlitre. The HIV infection can remain stable in the lymphadenopathy syndrome/AIDS-related complex phase for years, or can develop into the complete clinical picture of AIDS within a few weeks and months. A sign of further deterioration of the clinical situation is usually a persistent reduction in the level of $CD4^+$ cells. The critical limit is 200 $CD4^+$ cells per microlitre of blood. Increasing defects in cell-mediated immune response, which primarily lead to repeated infections with opportunistic pathogens and/or the occurrence of malignant tumours (especially Kaposi's sarcoma in homosexual men and lymphomas), characterize full-blown AIDS. Neurological symptoms (subacute encephalitis) are common in the late phase of the disease. Some patients develop dementia syndromes with brain atrophy. Viruses are detectable in the cerebrospinal fluid and brain of such patients, but also in HIV-positive individuals without neurological manifestations. The levels of HIV-specific antibodies and $CD4^+$ cells decline in the late phase of the disease, and infectious viruses are detectable in very large quantities in the peripheral blood. HIV patients commonly die of life-threatening opportunistic infections.

According to the criteria of the Centers for Disease Control and Prevention, HIV disease can be divided into phases which reflect the state of infection and have prognostic significance. They are composed of the three clinical categories of the disease (Table 18.3) and the number of $CD4^+$ cells, which are also assigned to three groups (1–3): category 1 for patients who have more than 500 $CD4^+$ cells

Table 18.3 Clinical categories of human immunodeficiency virus (*HIV*) infection from the Centers for Disease Control and Prevention

Category A	Acute HIV infection: mononucleosis-like clinical picture Acute (primary) HIV infection with symptoms persistent, generalized lymphadenopathy (LAS)
Category B	Diseases and symptoms that are neither in category A nor in the AIDS-defined category C, which are causally related to HIV infection, or infer a perturbation of the cellular immune system: Bacillary angiomatosis *Candida* infections of the oropharynx Chronic or severe vulvovaginal *Candida* infections Cervical dysplasia or carcinoma in situ Persistent temperature above 38.5 °C Diarrhoea lasting longer than 4 weeks Oral hairy leucoplakia Idiopathic thrombocytopenic purpura Relapsing herpes zoster (shingles) Peripheral neuropathy
Category C	AIDS-characteristic diseases: *Pneumocystis carinii* pneumonia Toxoplasma encephalitis *Candida* infections of the oesophagus, bronchi, trachea and lungs Chronic herpes simplex infections with ulcerations, herpes bronchitis, herpes pneumonia, herpes oesophagitis Generalized cytomegalovirus infection, cytomegalovirus retinitis Extrapulmonary cryptococci infections Disseminating or extrapulmonary tuberculosis (typical and atypical mycobacteria, *Mycobacterium avium* or *M. kansasii*) Kaposi's sarcoma (HHV-8) Malignant lymphomas (Burkitt's lymphoma, immunoblastic or primary cerebral lymphoma, EBV-associated lymphomas) Invasive cervical cancer (human papillomavirus) Progressive multifocal leucoencephalopathy associated with JC virus HIV encephalopathy Wasting syndrome

AIDS acquired immunodeficiency syndrome, *EBV* Epstein–Bar virus, *HHV-8* human herpes virus 8, *LAS* lymphadenopathy syndrome

per microlitre of blood (the standard value is 800–1,000 $CD4^+$ cells per microlitre of blood), category 2 for patients with 200–499 $CD4^+$ cells per microlitre of blood and category 3 for those with values below 200 $CD4^+$ cells per microlitre of blood. Hence, this results in nine categories (A1–A3, B1–B3 and C1–C3) in which patients can be classified by their $CD4^+$ cell counts and clinical symptoms: e.g. patients without symptoms and with $CD4^+$ cell counts above 500 are assigned to category A1; symptomatic patients with $CD4^+$ cell counts between 200 and 499 belong to category B2 and individuals with AIDS-defining diseases (clinical category C) and with fewer than 200 $CD4^+$ cells per microlitre of blood are classified into category C3. Patients classified in categories A3, B3 and C1–C3 are considered to have AIDS.

Pathogenesis

Virus particles and virus-infected cells reach the vaginal or intestinal mucosa through sexual contact, or enter the bloodstream directly by injuries. The first target cells of the virus are presumably Langerhans cells of the skin, dendritic cells and macrophages, which migrate into the mucous membranes. Macrophages constitute one of the long-term reservoirs for persistent viruses. They are of particular importance owing to their presence at the sites of entry of HIV into the organism. The R5 viruses (with tropism for macrophages) bind to the surface of macrophages by interacting with the CD4 and CCR5 receptors. It has been shown that people who do not produce the chemokine receptor CCR5 owing to a 32-base-pair homozygous deletion in the CCR5 gene cannot be infected with HIV by sexual contact, and thus are resistant to HIV infection. Heterozygous individuals become ill much later, and have a slower progression of the disease. This also means that HIV variants with tropism for T lymphocytes (X4 viruses) do not play an essential role in the very early phase of infection when they are inoculated through mucous membrane regions, since they do not encounter T lymphocytes at these entry sites. HIV variants with tropism for T lymphocytes that use the CXCR4 receptor as a cofactor for attachment only develop from viruses that originally have tropism for macrophages by continuous mutations during the progression of infection in the individual patients. Binding of viral surface proteins to the different cellular chemokine receptors also activates a signalling cascade, which is similar to that triggered by the natural ligands. Thereby, infected macrophages produce a series of chemotactic chemokines that attract other macrophages and dendritic cells, which in turn are used by the virus as new target cells.

Infected cells are transported from the mucosal regions to the nearest lymph nodes through the afferent lymphatic vessels. The lymph nodes, which then contain HIV in their network of follicular dendritic cells, constitute the reservoir for the virus. In lymph nodes, the virus infects monocytes, macrophages and primary T lymphocytes, in which it proliferates after binding to CD4 and CCR5 receptors on the cell surface, thus stimulating both the cell-mediated and the humoral immune response. Macrophages transport the virus to the brain and other organs. Macrophages release minute, CCR5-containing membrane vesicles from their surfaces. These microparticles are able to merge with the membranes of other cell types, which thereby gain the CCR5 receptor, even if only transiently. This might be a way by which HIV infects other cell types such as astrocytes and endothelial cells which do not express the chemokine receptor. In this manner, these cells may then be infected, constituting another reservoir for persistent viruses.

During the latent phase, active viral replication occurs, although free infectious HIV particles are rarely detected in the blood. Only one of 10^4–10^5 $CD4^+$ cells is infected with HIV in the peripheral blood, and the architecture of the lymph nodes is initially intact. Nevertheless, high concentrations of HIV-infected cells and viruses complexed with antibodies are found in their germinal centres. During this early infection phase, the virus replicates not only in the lymph nodes, but also in other lymphoid tissues, such as the spleen, tonsils and Peyer patches. In these organs, viral replication causes an initial hyperplasia with resultant swelling of

lymph nodes (lymphadenopathy) and the final destruction of the tissue (lymphatrophy). However, approximately 10^{10} new infectious virus particles are produced and released daily also in the peripheral blood during the early, still asymptomatic phase of infection, and they destroy almost as many $CD4^+$ cells. This corresponds to about 5 % of the total lymphocytes of an individual. Physiologically, $CD4^+$ lymphocytes have a very short half-life of only a few days and are subject to high turnover. Initially, they can be almost completely regenerated by continuous differentiation of progenitor cells from the bone marrow. Viruses are captured and eliminated by the immune system, which is still intact at this early infection stage. Thus, extensive viral proliferation occurs, but, it is controlled by the immune defence. In the further course of the disease, this balance is broken during transition to the symptomatic phase. The immune system is no longer capable of controlling the released viruses and virus-producing cells. The progressive damage to the populations of $CD4^+$ lymphocytes, macrophages and dendritic cells leads to the failure of the associated immunological functions. Inasmuch as cytokine production and secretion are also affected, $CD8^+$ lymphocytes are not indirectly activated, and thus are no longer available for immune reactions. This ultimately leads to opportunistic infections with pathogens that can normally be controlled by the immune system (Table 18.3, Fig. 18.13).

The HIV genome is subject to high mutation rates; owing to the low fidelity of transcription, each virus produced contains, on average, at least one erroneously incorporated nucleotide. Because of the selection pressure exerted by the immune system, viruses constantly change their epitopes for both humoral and cellular immune response, thereby evading the defence mechanisms. At the same time, along with the emergence of novel variants that can no longer be controlled by the immune system, viruses change their replication properties, and exhibit an altered cell tropism (Fig. 18.12). Whereas R5 viruses are mainly isolated in the early infection phase, infect macrophages and monocytes, proliferate slowly generating low levels of progeny viruses in vitro, and induce only a few syncytia by gp120/CD4-mediated fusion of cell membranes (no syncytium induction) – whence they are called S/L (for “slowly/low”) or NSI (for “no syncytium induction”) strains – X4 viruses of late infection stages have other features: they exhibit a pronounced tropism for infecting T lymphocytes, replicate rapidly, yield high virus titres in cell culture and mediate the formation of a large number of giant cells (R/H, for “rapidly/high”, or SI strains) In summary, it can be concluded that highly virulent virus variants are steadily generated by continuous mutations during infection. These evade the immune response, multiply explosively in the lymphoid tissues and finally destroy them. Consequently, the architecture of lymph nodes is destroyed in the AIDS phase, the network of follicular and interdigitating dendritic cells and germinal centres is disintegrated and all remaining $CD4^+$ T lymphocytes produce viruses.

Several factors are responsible for the progressive reduction of the number of $CD4^+$ cells:

1. The cytolytic effect of HIV. This mainly affects $CD4^+$ T lymphocytes, which are destroyed as a result of viral replication.

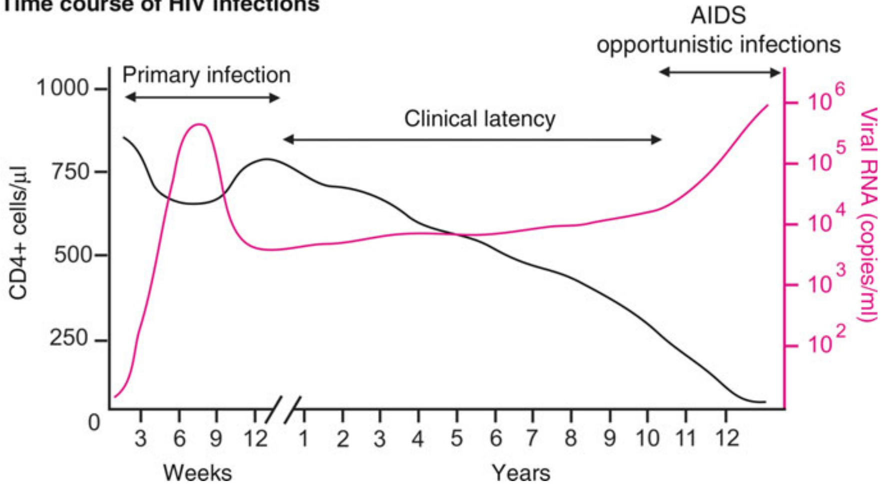


Fig. 18.13 Clinical progression of HIV infection. The *black curve* represents the time course of the number of $CD4^+$ cells per microlitre of blood; the *red curve* shows the number of viral genome copies per millilitre of blood. The time axis includes the first few weeks of infection up to a period of more than 10 years. The symptoms of category A (here, primary infection), the clinical latency phase and the AIDS-defining opportunistic infections (category C) are shown. The steady increase of viral load can be calculated mathematically (about 0.1 log units per year of infection). In the final stage, the number of $CD4^+$ cells decreases rapidly, whereas both the concentration of the virus and the number of opportunistic infections increase. Finally, the patient dies of a complete failure of the immune response because of opportunistic pathogens

2. Apoptotic processes. Monocytes and macrophages infected with R5 viruses secrete cytokines and chemokines (including TNF-α, IL-8, RANTES and MIP-1) in the early phase of infection, and these bind to their receptors on the surface of T lymphocytes, inducing apoptotic processes, i.e. programmed cell death. Apoptosis is also triggered by binding of gp120–antibody complexes to CD4 receptors of uninfected cells. The exterior glycoprotein gp120 is – as described in Sect. 18.1.3 – non-covalently linked with the transmembrane protein gp41 and other components of the virus particle. Therefore, it can be shed as a soluble gp120, which reacts with circulating antibodies and triggers the cell death pathway upon binding to the CD4 receptor. Tat proteins, which are secreted by infected cells, are also able to induce apoptosis.
3. Cellular immune responses against $CD4^+$ cells. The binding of gp120 to the cell surface of uninfected T lymphocytes and its subsequent incorporation is responsible for this. The protein is cleaved by cellular proteases, and the resulting peptides can be presented by MHC molecules. These lymphocytes simulate an infection state and can be eliminated by cytotoxic T cells.

All these mechanisms contribute to the fact that steadily more $CD4^+$ cells are destroyed during the progression of infection than are regenerated. As a consequence, the immune system is less and less capable of controlling HIV proliferation as well as opportunistic infections.

Particularly, $CD8^+$ cytotoxic T lymphocytes are responsible for the immunological control of infection. They recognize and lyse HIV-infected cells, which present viral peptide fragments by MHC class I antigens. The activity of the cell-mediated immune response is regulated by type 1 T-helper cells (T_H1 cells), a subpopulation of $CD4^+$ lymphocytes. They secrete IL-2 and interferon-γ, which stimulate the activity of cytotoxic T cells. By contrast, type 2 T-helper cells support the humoral, antibody-mediated immune response by releasing IL-4, IL-6, IL-8 and IL-10. At the same time, the respective cytokines facilitate mutual control of the two subpopulations in healthy people, which prevents an uncontrolled reaction of the one at the expense of the other. The secretion of cytokines by HIV-infection-damaged T_H1 cells continuously decreases during HIV latency. This results in the loss of the ability of $CD8^+$ lymphocytes to eliminate infected cells. Likewise, the concentration of chemokines that are secreted by immunologically activated T lymphocytes, macrophages and dendritic cells diminishes. These natural ligands of CCR5 and CXCR4, which are used as co-receptors by HIV, usually inhibit attachment of the virus to target cells, and thus prevent infection. In addition to the high genetic variability of the virus, the impaired cytokine production is possibly also one of the fundamental reasons for the inability of the cellular immune system to control the viral infection and to maintain a long-term symptom-free state.

Cytotoxic T Cells Are Very Important for the Duration of the Asymptomatic Carrier Stage

The significance of cytotoxic T cells in controlling HIV infection can be demonstrated by studies with long-term survivors. These are patients who exhibit an asymptomatic latent phase that lasts, in part, for more than 15 years. They have significantly higher levels of cytotoxic $CD8^+$ lymphocytes than patients with normal disease progression. Long-term survivors also have normal levels of cytokines and an intact T_H1 cell population, and their $CD8^+$ cells secrete large amounts of a cytokine that suppresses viral replication. Probably, this group of patients has MHC class-I subtypes which present T-cell epitopes of viral protein regions that are functionally important for viral replication. The virus can not tolerate mutations in such epitopes, since this would lead to a loss or impairment of protein function. There are also indications that HIV infections cannot be established in certain people: some prostitutes who demonstrably had contact with HIV in Gambia's major cities have shown no seroconversion, and the virus has not been detected in their lymphocytes. HIV-2 was initially much commoner than HIV-1 in this region of Central Africa. However, this situation has been reversed in recent years. After the protein sequences of both virus types had been compared, peptides were sought in the conserved domains which can be presented by HLA-B35 and HLA-B5. Both HLA types are very common in the population of Gambia. It has been demonstrated that these prostitutes possess cytotoxic T lymphocytes that recognize and lyse HIV-infected cells in vitro. Therefore, contact with the virus has presumably occurred without an infection

being established. Obviously, these women have cytotoxic memory T cells that probably protect them from infection after subsequent contact with the virus.

Immune Response and Diagnosis

The time course of antibody response is depicted in Fig. 18.12. A few weeks may elapse between infection and detection of the first immunoglobulins. The reasons for the delayed antibody production in some patients are unclear. It is believed that both the quantity of the virus that infects the organism and the immune status of the patient are important for this effect. This "serological window" must be taken into account in the diagnosis, since this could lead to false-negative findings, although patients are highly infectious at that stage. IgM antibodies against Gag and Env proteins are detectable approximately 3–4 weeks after infection for several months by ELISA and Western blotting. These are followed by IgG antibodies against structural proteins. Antibodies against the Nef protein are also detectable early during infection. Antibodies against structural proteins remain at constant concentrations during latency. However, their concentration decreases when the clinical situation is aggravated. They are present in lower concentrations in the serum during the full clinical picture of AIDS. Viruses can be detected by means of HIV p24 antigen capture ELISA as well as HIV p24 combo ELISA (detection of p24 and antibodies against p24) soon after infection, or by culturing of infectious viruses; viral nucleic acids can be verified by PCR. The number of viral RNA genomes which are detectable in the blood plasma after primary infection during the latent phase is reliably well correlated with the duration of the latency period. However, as only about 20 % of white blood cells are present in the peripheral blood (the rest reside in the lymphoid tissues), cell numbers and virus amounts provide only an imperfect picture of the overall situation in the organism.

In daily diagnostic routine, a staged diagnosis is used as a screening test; it is based on an extremely sensitive p24 combo ELISA for detection of p24 and antibodies against p24. Every positive reaction has to be confirmed by other serological methods, e.g. by an immunoblot assay. To exclude ambiguity errors, a second independent serum sample from the patient must additionally be examined. The result is considered "positive" only when these conditions are fulfilled. The viral load is determined by quantitative reverse transcription PCR (RT-PCR) from plasma; this method ascertains the amount of free, unbound virus particles. The quantitative RT-PCR technique is also used for continuous monitoring of therapeutic success. If the viral load increases again in spite of antiviral treatment, then the emergence of resistant viruses has to be considered. The underlying mutations in the viral genome are usually determined by RT-PCR and subsequent sequencing of amplification products. Resistant virus variants can also be phenotypically analysed by propagation of the virus and direct resistance testing in cell culture, as well as by amplification of viral genome segments encoding the reverse transcriptase or protease and their cloning into appropriate recombinant HIV test viruses.

The cellular immune response was described in detail in the previous section. Virus-specific cytotoxic T cells and T-helper cells are detectable in the early and latent infection stages. T-cell epitopes have been found in almost all viral proteins. Their recognition depends to a large extent on the prevailing virus variant and on the MHC class I and class II types of the patient.

Therapy and Prophylaxis

Several nucleoside and non-nucleoside inhibitors of essential viral enzymes are available for the treatment of HIV infection (▶ Chap. 9), more precisely, inhibitors of reverse transcriptase, integrase and protease. The indication for therapy depends on the viral load, the $CD4^+$ cell counts and the clinical symptoms. Patients belonging to clinical categories B and C always require antiviral treatment, whereas the antiviral therapy in infected individuals of category A depends on the viral load and $CD4^+$ cell counts. The primary infection is not treated. The antiviral drugs used to treat HIV patients are always applied in combination in order to inhibit as many of the independent functions which are essential for viral replication as possible. This highly active antiretroviral therapy (HAART) includes combinations of at least two different inhibitors of reverse transcriptase and a protease inhibitor. The most commonly used nucleoside analogues are azidothymidine, dideoxycytosine, dideoxyinosine, lamivudine and abacavir. These compounds compete with the respective natural nucleotides for the active site of the reverse transcriptase; they are incorporated into nascent DNA strands, leading to chain termination. Non-nucleoside inhibitors such as nevirapine, delavirdine, loviride and efavirenz bind to other functionally important domains of the reverse transcriptase, such as the pyrophosphate binding site. Saquinavir, indinavir, nelfinavir, and ritonavir are available protease inhibitors. Similar to nucleoside analogues, they also compete with the natural cleavage sites in the precursor proteins Gag and Gag–Pol for the active centre of the viral protease (peptidomimetics). There are also integrase inhibitors (raltegravir). The HIV drug enfuvirtide (also called T20) is a novel antiretroviral inhibitor; it was approved in the European Union in 2003. It comprises the 38 amino acid residues of the heptad repeat unit from the central domain of g41, which is located in front of the transmembrane region of this protein. Upon attachment of the virus to the receptor and co-receptor, these amino acids form a coiled-coil structure, which is a prerequisite for the subsequent fusion of the viral envelope with the cell membrane. If enfuvirtide, modified with a membrane anchoring region is present in the membrane of T lymphocytes and macrophages, it prevents the structural rearrangements in the gp41 protein which are necessary for the fusion step and entry of the virus; hence, the cells cannot be infected. It is being attempted to combine enfuvirtide with other inhibitors. Other antiviral drugs that are already being tested in clinical trials are aimed at preventing entry of the virus (attachment inhibitors, co-receptor antagonists) or budding of the virus. For example, maraviroc, a co-receptor antagonist, was approved in 2007. It blocks the CCR5 receptor and is accordingly only suitable as a therapeutic agent to prevent R5 virus infections. Another application of such HIV drugs is postexposure situations, such as needlestick injuries in an HIV-positive patient, in which either immediately

or at least during the first few hours after injury HAART is initiated and performed for 4 weeks to prevent infection if possible.

A difficult problem is the rapid emergence of resistant HIV variants, whose replication can no longer be blocked by the inhibitors. Apparently, each inhibitor induces certain types of mutation. Therefore, the emergence of resistant viruses is monitored during therapy by detecting specific mutations in the viral genome (e.g. in the genes encoding the reverse transcriptase or protease) by amplification of the relevant genome sequences using PCR and subsequent sequencing of the PCR products. The mutations are then assigned to specific inhibitors. If mutations are identified during HAART which indicate the development of resistant variants, then – if possible – the composition of the drug cocktail is changed. Because of the selection pressure exerted by the antiviral drugs, the infection rate with already resistant HIV variants has also increased, but without exceeding a limit of approximately 15 %.

Owing to antiretroviral therapy, the concentration of virus in the peripheral blood can be decreased below the detection limit, thus extending the asymptomatic phase of infection for several years. However, HAART is only able to retard the progression of HIV disease to the full clinical picture of AIDS, and thus to extend the life span of patients; a cure, i.e. the complete elimination of the viral genetic information from the cells of the organism, is not possible to date. This means that the virus possesses reservoirs in the organism in which it is not affected by the inhibitors. Probably, these are principally HIV proviruses which were integrated into the genome of resting T cells during the early infection phase. Only when these T cells are activated are they able to produce infectious viruses. Therefore, attempts are being made to activate resting cells by the use of cytokine inhibitors and the simultaneous application of antiviral drugs, thereby making the virus accessible to the inhibitor effect before the emergence of resistant virus variants. A very promising new approach was a bone marrow transplant of an HIV-infected patient with cells from a donor who carried a homozygous mutation in the CCR5 locus (CCR5del32) and whose cells were resistant to infection by R5 viruses.

In addition to antiretroviral therapy, opportunistic infections are directly controlled by administration of antibiotics, antiviral drugs and fungicides. Obviously, the use of HAART also implies that the medium- and long-term side effects of antiviral chemotherapy should be considered and adapted to the individual responses of the patients. These particularly include changes in adipose tissue (hypertrophy and atrophy) and serious cardiovascular incidents. These side effects have largely led to the fact that non-HIV-associated effects exceed HIV-associated deaths in AIDS patients today.

So far, all classic approaches to develop a preventive vaccine have failed, and this also applies to therapeutic vaccines. The administration of inactivated HIV, recombinant vaccinia viruses that synthesize HIV-specific glycoproteins, surface proteins produced by genetic engineering, virus-like Gag particles or synthetic peptides from various viral proteins has not induced protection from infection with the wild-type virus in animal experiments. The reason for this probably resides in the high variability, which allows the pathogen to evade the induced antibodies and cytotoxic T cells, and the failure of inactivated vaccines to induce a sufficient cellular immune response.

18.1.5.2 Human T-Cell Leukaemia Viruses

Epidemiology and Transmission

HTLV-1 was initially isolated from a patient with adult T-cell leukaemia (ATL) by Robert Gallo and colleagues in 1980. This form of T-cell leukaemia occurs only in adults, and is especially common in Japan, the Caribbean region, South America and Africa. In these regions, approximately 5–15 % of the population has antibodies against HTLV-1. Studies in Japan have revealed that 3–5 % of HTLV-1-positive individuals aged between 20 and 70 years develop ATL. All ATL patients are seropositive, and HTLV-1-infected T cells are detectable in them. This and the fact that the genome of all leukaemia cells harbours integrated HTLV-1 gene sequences strongly suggests a causal relationship between viral infection and disease. Furthermore, HTLV-1 infection causes chronic progressive myelopathy in tropical countries, namely tropical spastic paraparesis (TSP) or HTLV-1-associated myelopathy (HAM). The latter occurs in some regions of Japan where HTLV-1 is endemic.

The virus is transmitted by blood transfusions, sexual contact and mainly through milk of infected mothers during breastfeeding. The virus is transmitted not as free particles, but by infected cells. Thus, cell-free blood plasma of seropositive donors is not infectious. Myelopathic diseases are especially observed after HTLV-1-positive blood transfusions, and their occurrence has been considerably reduced since the introduction of serological testing of all blood products in countries with high prevalence of HTLV.

HTLV-2 and HTLV-3 is closely related to HTLV-1. Their genomes have about 60 % sequence homology. HTLV-2 was first isolated from an immortalized $CD8^+$ T-cell line originating from a hairy cell leukaemia by the group of Robert Gallo in 1982. Further isolations of the virus have been successful only in some sporadic cases. It is not clear whether HTLV-2 induces leukaemia or other diseases in humans. The distribution of the virus is also not well known. Seropositive individuals have frequently been observed in the indigenous people of North America and South America, and also among intravenous drug users in North America and Europe. In contrast to HTLV-1 and HTLV-2 a third human HTLV-type (HTLV-3) has been isolated from individuals in Cameroon.

Clinical Features

The primary infection with HTLV-1 is asymptomatic, and most infected individuals remain asymptomatic carriers of the virus for life. Acute ATL is characterized by enlarged lymph nodes, liver and spleen, skin lesions and infiltration of leukaemic cells into various organs. Most patients exhibit an increased calcium concentration in the blood. Besides acute ATL, there is also a chronic form of ATL, in which patients have only a few morphologically altered T cells in the blood, and do not display symptoms of the disorder over long periods. Chronic ATL can spontaneously progress to the acute form. The development of acute ATL usually has a fatal outcome within 1 year.

TSP with bilateral paralysis of the limbs and loss of control over bladder and bowel functions emerges about 2 years after transmission of the virus by

contaminated blood bags. In both TSP and HAM, histopathologically detectable perivascular inflammations develop in the spinal cord and spinal cord membranes (meninges). A large number of infected, morphologically transformed T lymphocytes can be found in patients, and they infiltrate the spinal cord, especially in the thoracic spine region. It is assumed that both disorders are caused by immunopathogenetic mechanisms. This is additionally supported by the observation that certain HLA types (HLA-B5401) frequently occur in patients, whereas the types HLA-A2 and HLA-Cw8 are only rarely found in HAM patients.

Pathogenesis

In vivo, HTLV-1 is found exclusively in $CD4^+$ cells, whereas HTLV-2 preferentially infects $CD8^+$ T lymphocytes. The cellular receptors of both viruses are unknown; because both viruses are capable of infecting many different cell types in vivo, it has been assumed that the cellular receptor is a ubiquitous cell surface molecule. All ATL cells of a patient carry the HTLV-1 provirus integrated at the same site in the genome of the host cell. This indicates that all leukaemia cells originated from a common precursor cell. However, the integration site is unlike that in a different patient. Therefore, the mutations that result as a consequence of the integration process do not seem to be associated with the pathogenesis of the disease. Even the tendency to develop ATL seems to be connected with certain HLA types. It has been found that carriers of HLA-A26, HLA-B4002, HLA-B4006 and HLA-B4801 are not able to or are only partially able to bind to epitopes of the Tax protein and to present them, and thus do not possess cytotoxic T cells for detection of Tax-producing cells.

Large amounts of the IL-2α receptor are present on the surface of leukaemia cells. Furthermore, these cells also produce increased amounts of IL-2, IL-1β and GM-CSF. Probably, this can be ascribed to the transactivating effect of the Tax protein, which induces NFκB-, CREB- and SRF-dependent cellular promoters, enhancing the expression of genes that are controlled by these regulatory elements, which also govern the expression of IL-2, IL-1ß, GM-CSF and IL-2α receptor. The effect of NFκB is additionally increased, as Tax protein binds to the inhibitor IκB and induces its degradation via the proteasome. Thereby the transactivator NFκB is released from the complex and transported into the nucleus, where it activates NFκB-dependent promoters (▶ Chap. 8). The cytokines are released, bind to their respective receptors that are present in abundant quantities on the surface of infected cells and induce proliferation of these lymphocytes; hence, proliferation of lymphocytes is activated by an autocrine mechanism. The Tax protein exerts a repressive effect on other cellular promoters. These include p53-dependent promoters. Regardless of these functions that regulate cellular gene expression, Tax proteins also directly inhibit some tumour suppressor proteins. Tax interacts with inhibitors of cyclin-dependent kinases and inhibits their function. Thereby, cyclin-dependent kinases are activated, and phosphorylate, among others, the tumour suppressor protein RB105. This induces the release of E2F transactivators, which initiate the expression of genes encoding proteins that promote the transition from the G_1 phase to the S phase of the cell cycle.

The tumour-inducing properties of Tax protein have also been demonstrated in Tax transgenic mice, which develop mesenchymal tumours. However, like other viral products, the Tax protein is not synthesized in immortalized cells. It is probably mainly involved in the initial stages of immortalization and is no longer required for the maintenance of the transformed state. Moreover, it is expected that other still unknown cellular factors may also be involved.

Immune Response and Diagnosis

IgG antibodies against Gag, Env and Tax proteins can be detected by ELISA and Western blotting; most antibodies are directed against Gag proteins. However, because of the pronounced sequence homology, a distinction between HTLV-1-specific and HTLV-2-specific immunoglobulins is impossible by these test systems. Distinction is performed, like determination of viral load, by detection of integrated viral genomes using PCR. In the patients there are $CD8^{+}$ cytotoxic T lymphocytes that are specifically directed against Tax protein epitopes, and are presumably involved in the immune pathogenesis of TSP/HAM. Their amount correlates with the viral load. The number of proviral genomes per 100 lymphocytes in peripheral blood is determined by quantitative PCR. The value is between 0.1 and 0.5 in asymptomatic HTLV carriers, whereas it is between 5.0 and 10.0 in TPS or HAM patients.

Therapy and Prophylaxis

Presently, there is neither a vaccine that prevents infection with HTLV nor are there effective antiviral agents for the treatment of ATL or TPS. Although azidothymidine, cidofovir and other antiviral drugs inhibit viral replication in vitro, their effects have proven to be rather disappointing in vivo. There are no clinical studies evaluating the use of virostatic drugs. Bone marrow transplants have been performed as a therapeutic measure in ATL patients. Highly dosed glucocorticoids are applied in HAM/TPS patients. Interferon has been approved by the Food and Drug Administration for the treatment of hairy cell leukaemia in the USA.

18.1.6 Animal Pathogenic Retroviruses

18.1.6.1 Avian Leucosis Viruses

Epidemiology and Transmission

Avian leucosis virus and Rous sarcoma virus, members of the genus *Alpharetrovirus*, are found worldwide in poultry populations, and virtually every herd is infected. However, diseases are very rarely observed. The virus can be transmitted to the following generations both horizontally from animal to animal and vertically from hens to eggs. Infected chicks excrete the virus lifelong. Depending on the virus type, the virus can cause tumours with different frequencies in poultry flocks, which can range from less than 3 % up to 20 % of the animals of a herd. In addition, there are a number of endogenous alpharetroviruses in chickens which do not have oncogenic potential, but that affect the progression of infection with exogenous retroviruses.

Clinical Features

Infected flocks show a multilayered clinical picture and a high loss rate. Symptoms of osteopetrosis are frequently observed, which is manifested as a malalignment of chicken legs (stand) as a result of infection-related bone degeneration. The commonest form of cancer is lymphatic leucosis.

Pathogenesis

Lymphatic leucosis is characterized by proliferation of B lymphocytes in several organs, including the liver, spleen and bursa of Fabricius. Transformed B lymphocytes do not differentiate into plasma cells, but remain, if they produce antibodies, in the stage of IgM-producing lymphoblasts. The bursa of Fabricius is the central organ in the pathogenesis of this disease. Its removal leads to tumour regression.

The osteopetrosis is characterized by infection of osteoblasts, including subsequent bone abnormalities or osteosarcomas. These symptoms appear when chickens are infected by a retrovirus that does not carry an oncogene. Therefore, there is slow tumour development. By contrast, if a viral oncogene is present in the genome of the retrovirus, it can induce fast tumour development, such as with Rous sarcoma virus, which bears the v-*src* oncogene. In such cases, fibrosarcomas develop rapidly. Other oncogenes such as v-*erb*, v-*myb* and v-*myc* also lead to the development of sarcomas or haemangiomas. Affected chickens then develop the symptoms of lymphoid leucosis.

Some chicken breeds are genetically resistant to infection with avian leucosis virus; consequently, they develop no tumours. The resistance is restricted to certain virus subtypes. In such cases, all cells of a chicken cannot be infected, including cell cultures (chicken embryo fibroblasts). This permits the breeding of resistant chicken cell lines. The genes that are responsible for resistance reside within the MHC locus. Presumably, the resistance is based on the lack of subtype-specific receptors for attachment of the virus which are not synthesized in all chicken breeds.

Immune Response and Diagnosis

In the course of infection, the animals develop immune reactions that can retard or prevent tumour formation, but that do not lead to the elimination of the virus. The fetal infection of embryos in the egg by vertical transmission of the virus (congenital infection) can lead to the development of a virus-specific immune tolerance, in which chickens excrete the virus lifelong. A clinical disease is rare in such animals.

Control and Prophylaxis

Vaccines are not available. The control of avian leucosis viruses is only possible by elimination of carriers of the viruses; therefore, the aim is to establish chicken populations that are free from infection. Horizontal transmission can be prevented by hygiene measures. This includes, in particular, all in–all out management (culling of infected flock, and establishment of a new herd) accompanied by the implementation of thorough disinfection measures in the period between livestock changes.

18.1.6.2 Feline Leukaemia Virus

Epidemiology and Transmission

Feline leukaemia virus is significant pathogen of cats; which is widespread worldwide. It belongs to the gammaretroviruses, and is striking in several respects; it is the only retrovirus whose infection can be controlled with vaccines; furthermore, it coexists along with feline sarcoma virus in the cat population. The latter is a defective, replication-incompetent retrovirus carrying a deletion of the *env* gene, which instead possesses a viral oncogene (v-*myc* or v-*fms*). Its replication is dependent on the simultaneous infection of cells with feline leukaemia virus, which provides helper functions, such as Env proteins for mediating morphogenesis and release of feline sarcoma virus progeny.

There are three subtypes of feline leukaemia virus (A, B and C), and they can be defined by variations in the envelope protein. Subtype A is isolated in more than 80 % of infected cats. Subtypes B and C are found in about 20 % and in less than 2 % of infected cats, respectively. Subtype B is a recombinant of subtype A with endogenous retroviruses, and subtype C is a variant of subtype A that has been altered by mutation of the *env* gene.

Feline leukaemia virus is transmitted from animal to animal, particularly by droplet infection with virus-containing saliva, but also through other metabolic products of infected cats. However, a successful transmission requires close contact between animals for long periods, or a parenteral infection. Therefore, kittens are mainly infected by the dam during the lactation period, or adult cats, especially male cats, can be infected during territorial disputes. The cats establish an infection that induces an immune response, which frequently leads to elimination of the virus from the peripheral blood. Whether the virus is completely eliminated from the organism during these abortive infections is not clear. However, the cats do not excrete viruses, and are without epidemiological importance for the spread of infection; they do not develop tumours. These occur only in cats that do not completely eliminate the virus from the blood, and that establish a persistent feline leukaemia virus infection. Besides horizontal transmission, vertical transmission plays an epidemiologically important role. The intrauterine infection of kittens is common and leads, depending on the stage of pregnancy at the time of infection, to fetal death and abortion, or to the birth of kittens with persistent production and excretion of pathogens.

Clinical Features

Because of the relatively short incubation period of retroviruses lasting only a few weeks, and the rapid development of tumours, infections with feline leukaemia virus and its associated disease are especially found in young cats aged between 1 and 2 years. Cats older than 6 months seem to be less susceptible to infection (age-related resistance). Infections with feline leukaemia virus have a broad spectrum of different clinical manifestations. Apart from lymphosarcomas, bone marrow tumours and immunosuppression are also frequently found. If pregnant cats are infected, this can lead to infertility and abortions. Furthermore, pure red cell

aplasia (erythroblastopenia), a particular disease, is associated with subtype C of feline leukaemia virus. In such cases, the production of erythrocytes is impaired and the cats become anaemic.

Pathogenesis

Cats are especially infected by bite wounds or through oral ingestion of the virus. Initially, it multiplies in the lymph nodes near the infection site, and migrates from there into the other lymphoid tissues and the bone marrow during a primary viraemia. The infection of the bone marrow is of particular pathogenetic interest as it determines whether the virus is eliminated, or whether a persistent infection is established. If the virus is already immunologically controlled and eliminated in this phase of infection, no other symptoms will appear. If this fails, a second viraemia develops, which disseminates the virus in almost all tissues. These cats will most probably develop tumours or an immunosuppression with fatal consequences within 2 years, and this had already been named "cat AIDS" prior to the discovery of FIV.

Immune Response and Diagnosis

The diagnosis of feline leukaemia virus infections is easily possible by detecting viral antigens in the blood (antigenaemia). A variety of test systems (immunochromatography, ELISA) are available, and tests can be performed in veterinary practice. These tests rely on the detection of p27. On the one hand, this capsid protein is present in infectious virus particles, and on the other, it is secreted by virus-infected cells as a glycoprotein. Consequently, an infection occurs when the antigen test is positive. A second examination within an interval of 4–6 weeks is necessary to distinguish a transient antigenaemia from a persistent infection. If this sample also tests positive, it can be assumed that a persistent infection has been established.

Control and Prophylaxis

Natural infections typically confer a reliable immunity, which can also be similarly achieved by a vaccine. Feline leukaemia virus is currently the only retrovirus whose infection can be successfully controlled by vaccination. Vaccination prevents the outbreak of the disease in infected cats; however, the infection remains. Proviral sequences integrated in the genome of the host can be detected.

A genetically engineered vaccine which is based on the envelope protein of a subtype A virus that is produced in *Escherichia coli* has proven to be effective. Inactivated vaccines which are based on killed viruses that were grown in cell culture are also available. A new approach is a recombinant vaccine in which the *env* gene is inserted into the genome of a canarypox virus. Vaccination with these genetically engineered poxviruses leads to expression of the early gene products of poxviruses, including the Env protein of feline leukaemia virus. However, infectious recombinant poxviruses are not produced, as canarypox virus is not able to grow lytically in mammalian cells (▶ Sect. 19.6); it is solely capable of

infecting mammalian cells. Even though the early genes are transcribed and translated into the corresponding proteins, the late genes are not transcribed, and thus no structural proteins are available to assemble recombinant progeny poxviruses.

18.1.6.3 Bovine Leukaemia Virus

Epidemiology and Transmission

The causative agent of enzootic bovine leucosis is bovine leukaemia virus (BLV), a deltaretrovirus. Its natural host range includes only cattle; however, sheep and goats can be infected experimentally. The virus is present in the blood of infected animals and is excreted in the milk. Transmission occurs primarily through blood contact, by injuries or iatrogenic causes, e.g. through the use of non-disinfected needles, surgical instruments or other devices. Intrauterine transmissions have been described, but are not the rule. The infection is not contagious; therefore, a spatial separation of seropositive from seronegative animals is sufficient to avoid infections. There is no evidence for a possible human pathogenecity of this virus. In many respects, it has similarities with HTLV-1 and HTLV-2, which have also been assigned to the genus *Deltaretrovirus*.

Clinical Features

Most infections with BLV are subclinical. Only about 30 % of infected cattle develop a persistent lymphocytosis, and less than 1 % shows the malignant form, whose denomination is derived from the name of the virus: lymphatic leukaemia. It is associated with the development of fibrosarcomas and malignant lymphomas in various organs, such as heart, liver, spleen, ovary, lymph nodes and brain. In contrast to cattle, almost all sheep which are infected experimentally with BLV develop the tumorous form of the disease.

Pathogenesis

The target cells of BLV are B lymphocytes. BLV differs from HTLV in this step of the infection cycle, as the latter replicates in T cells (Sect. 18.1.5). The mechanisms that lead to activation and proliferation of B cells are unknown. Similarly, the processes that contribute to tumorigenesis in some animals remain unknown as well. Some Tax protein variants of BLV increase their transcriptional activator functions, giving rise to an increased production of the virus. They also enhance the activity of some cellular promoters, such as that of the proto-oncogene c-*fos*. This induces c-*fos* gene expression. It is unclear whether this process contributes to the pathogenesis of the disease.

Immune Response and Diagnosis

The diagnosis of enzootic bovine leucosis is usually performed by detection of virus-specific antibodies in ELISA or immunodiffusion tests (Ouchterlony test). The antibodies are directed against the gp51 envelope protein of BLV and have virus-neutralizing properties. The detection of antibodies is technically simple, and

is performed routinely. Isolation of the virus is also possible, and the PCR technique can also be used for detection of proviral DNA and viral RNA. However, both methods are technically complex.

Control and Prophylaxis

A vaccine that prevents BLV infections is not yet available. Despite their in vitro neutralizing effect, antibodies against the envelope protein gp51 are not able to eliminate the virus from the organism. However, it appears possible in principle that a vaccine that induces the formation of gp51-specific antibodies can confer protection from infection with BLV. Enzootic bovine leucosis has been successfully controlled, and is considered to be eradicated from most countries. The disease is notifiable: monitoring of the disease is implemented by serological testing of the animals or by examination of bulk milk samples for the presence of virus-specific antibodies. Animals that test positive are slaughtered. These measures ensure that infections with BLV do not spread.

18.1.6.4 Maedi–Visna Virus and Caprine Arthritis Encephalitis Virus

Epidemiology and Transmission

Both maedi–visna virus and caprine arthritis encephalitis virus are members of the genus *Lentivirus*. Both viruses are very similar and exhibit a pronounced serological cross-reactivity among their proteins. The infection of sheep with maedi–visna virus is the prototype of a slow virus infection. The term was coined by the Icelander Bjorn Sigurdsson and points to the particular biology and epidemiology of this infection: a long incubation period, a persistent infection with a slowly developing clinical picture and an inevitably lethal outcome.

Maedi–visna was first described in Iceland in 1930; it is ascribed to the introduction of the virus by Karakul sheep from Germany. Transmission occurs through direct contact with secretions of the respiratory tract of infected sheep. Clinically normal sheep excrete only small amounts of the virus in the saliva; oral transmission to other animals is rare. However, the infection can be very efficiently transmitted from these animals by virus-containing milk. Therefore, they are very important for the dissemination of the virus in a herd, as the lambs are mainly infected by virus-containing cells present in the colostrum. A high livestock density facilitates intensive contact between animals, and thus the transmission of the virus, e.g. when sheep are kept in stables during the winter months. In addition, iatrogenic transmissions through unclean surgical instruments are possible. Moreover, stinging arthropods are also responsible for the passive dissemination of the virus.

Caprine arthritis encephalitis virus infects goats, and its infection is very similar, in terms of epidemiology and transmission, to maedi–visna virus infection. Again, the virus is transmitted to lambs principally through the colostrum of infected ewes. Further transmission of the virus within goat herds is possible, but requires intensive contact between the animals. Commonly used and not disinfected milking equipment can contribute to the dissemination of the virus. Maedi–visna virus and caprine arthritis encephalitis virus can be transmitted experimentally to the respective other host animals.

Jaagsiekte Sheep Retrovirus and Maedi–Visna Virus Cause a Similar Disease
Ovine pulmonary adenocarcinoma is an economically important disease of sheep. It was initially described as jaagsiekte in South Africa in the late nineteenth century, and is found virtually worldwide today. This disease is caused by jaagsiekte sheep retrovirus (a betaretrovirus), and is similar to maedi–visna virus infections in terms of the clinical picture. However, in contrast to maedi, it is a progressive malignant disease of the respiratory tract with a neoplastic transformation of lung tissue. Adenocarcinoma cells, which develop in the course of the disease, produce a surfactant factor, a protein that is necessary for the surface tension and functionality of alveoli. However, in the case of the disease, the surfactant factor promotes the occlusion of small airways. The virus is transmitted horizontally by droplet infection. After an incubation period of up to 3 years, the always fatal ovine pulmonary adenocarcinoma develops slowly. Currently, there is no feasible way to diagnose the infection. Control of the disease seems to be possible only by separation of normal (possibly virus-free animals) from suspicious, sick animals. The latter are killed. Interestingly, the Env protein of jaagsiekte sheep retrovirus is the transforming protein. Owing to the existence of numerous endogenous retroviruses in sheep, no protective antiviral immune response is induced. This disease-promoting effect of endogenous retrovirus sequences has clearly been demonstrated experimentally by the use of transgenic mice.

Clinical Features

Basically, the two clinical forms of the disease, maedi and visna, can be distinguished in sheep infected with maedi–visna virus. However, visna is very rare; the much more frequent clinical picture is maedi.

“Maedi” is the Icelandic word for “dyspnoea”, breathlessness or respiratory distress, and describes the clinical symptoms characteristic of progressive interstitial pneumonia. The animals have emaciation and dyspnoea with the corresponding consequences, such as recumbency and lagging during migration with the herd, which are typical distinctive marks. The duration of maedi can last several months to years. The situation is similar in the case of visna (Icelandic noun for “wasting”, “shrinking”, “feebleness”, “weariness” or “fatigue”, or Icelandic verb for “to wither away” or “to fade away”, used as a term for enfeebled or weakened sick animals), even though it is not associated with a pneumonia, but with an encephalitis. The classic symptoms include paralysis and tremor as well as progressive weight loss (decay). In addition, mastitis (hard udder) is also occasionally observed.

Even the disease caused by caprine arthritis encephalitis virus is manifested in two different clinical forms: arthritis in adult goats, which is most often manifested in the carpal joints (front knee), and encephalitis in young animals. In addition, the infection frequently affects the udder, inducing mastitis.

Pathogenesis

Maedi–visna virus preferentially infects differentiating monocytes and macrophages. The degree of differentiation appears to be crucial for the susceptibility of the cells. In pathological and anatomical terms, maedi is predominantly characterized by a severe interstitial pneumonia and by a lymphadenopathy. Visna induces a demyelinating encephalomyelitis, similar to the brain disease caused by caprine arthritis encephalitis virus. After infection, a strong humoral and cellular immune response is induced, which, however, does not lead to the elimination of the virus or virus-infected cells.

Immune Response and Diagnosis

Maedi–visna virus infections are diagnosed by antibody detection using ELISA. Cultivation of the virus is possible by co-cultivation of lymphocytes from peripheral blood with primary macrophage cultures. The detection of viral RNA or proviral DNA by PCR is possible, but unusual. Because of cross-reactivities and the simple cultivation of maedi–visna virus, protein antigens of this virus are usually used to diagnose infections with caprine arthritis encephalitis virus.

Control and Prophylaxis

The control of infections with maedi–visna virus and caprine arthritis encephalitis virus is voluntary and not ruled by the state. It is mainly performed through regular serological monitoring of herds and by isolation and elimination of seropositive animals from the flock. No vaccines are available.

18.1.6.5 Equine Infectious Anaemia Virus

Epidemiology and Transmission

Equine infectious anaemia virus belongs to the lentiviruses. It is transmitted by various stinging insects such as brakes (*Tabanidae*), flies (*Stomoxys*) and midges (*Culicoides*). It infects horses, donkeys and other equines. These vectors transmit the virus only mechanically, as it cannot proliferate in the different insects. This insect-borne transmission explains the seasonal occurrence and the confinement of infection to specific climatic regions. This notion is implied in the term “swamp fever”. The virus was found worldwide; in many countries and regions of the American continent, equine infectious anaemia is still an enzootic disease. It had been eradicated from most European countries owing to successful control. Exceptions are Romania, Bulgaria and Italy. In 2010, equine infectious anaemia virus was reimported into some countries of central Europe by infected horses that had been illegally imported from southeastern Europe without prior serological testing. Apart from insects, the virus can also be transmitted by use of unsterile needles or devices in livestock.

Clinical Features

Equine infectious anaemia virus always induces a persistent infection in solidungulates. However, different clinical courses are known: the most frequent

is the acute form, which is characterized by undulating high temperature, severe anaemia and jaundice, as well as petechial haemorrhages. This form is usually fatal. The subacute and chronic forms show a milder and non-homogeneous clinical picture. All forms have in common the induction of a persistent infection.

Pathogenesis

The pathogenesis of the infection is characterized by viral replication in macrophages and lymphocytes. Furthermore, erythrocytes are destroyed by the induced immune response. Viral proteins are found in and on erythrocyte membranes. Whether these are attributed to infection of erythrocyte precursor cells or whether the red blood cells bind viral proteins secondarily remains to be clarified. Virus-specific antibodies bind to viral proteins in the erythrocyte membrane and induce the lysis of the cell by binding complement or by an antibody-dependent cell cytotoxicity reaction (▶ Chap. 7). Even this lentivirus is characterized by a high mutation rate. The external envelope protein gp90 changes its appearance by appropriate point mutations, whereby antibodies are no longer able to recognize and bind to the virus. This leads to a new infection cycle in the next phase, until new immune responses become effective and in turn erythrocytes are lysed. This cyclic infection behaviour is a standard example of immune escape and explains the fluctuating temperature.

Immune Response and Diagnosis

The diagnosis is easily made by detection of virus-specific antibodies against the viral capsid proteins using the immune diffusion method (agar gel precipitation test). This test was developed by Leroy Coggins at Cornell University in 1972. As the so-called Coggins test, it is still the diagnostic standard. Isolation and cultivation of the virus or detection of nucleic acid by PCR is possible, but unusual.

Control and Prophylaxis

Vaccines are not available. The infection is controlled by serological testing of horses and isolation of and killing infected animals.

18.1.6.6 Feline Immunodeficiency Virus

Epidemiology and Transmission

Feline immunodeficiency virus (FIV) causes a disease in cats which is similar to the illness that is provoked by HIV in humans (Sect. 18.1.5). FIV was originally isolated from a cat population which had chronic diarrhoea and stomatitis, as well as skin infections. Despite the fundamental similarity, FIV is far less important than HIV. However, it plays an increasingly important role as an animal model for AIDS research.

The virus is ubiquitous, and causes persistent infections in cats. The prevalence is 1 % of clinically normal cats. Transmission requires close contact among cats, and is especially efficient by biting during territorial disputes or hierarchical fights

within the population. In addition to the FIV of domestic cats, FIV-like viruses have been isolated from a variety of big and wild cats. However, no disorders have been observed in them so far.

Clinical Features

In principle, the disease caused by FIV is very similar to that of HIV infection in humans. An initial acute phase with fever and lymphadenopathy is followed by a long subclinical period that culminates in the immunodeficiency. It can also be divided into several stages, during which opportunistic infections become apparent, which eventually lead to the death of the animal. Because of this disease progression, infections with FIV are a disease of old cats, in contrast to those with feline leukaemia virus (Sect. 18.1.6.2). Encephalitis, as a special manifestation form of the disease, occurs in about 5 % of clinical cases.

Pathogenesis

The pathogenesis is characterized by infection of T-helper cells and macrophages. A selective reduction of the level of $CD4^+$ T lymphocytes occurs during infection, thus leading to a shift of the CD4/CD8 ratio (Sect. 18.1.5).

Immune Response and Diagnosis

FIV infection induces antiviral immunoglobulins that are detectable lifelong; however, they are not able to eliminate the virus. Detection of these antibodies by ELISA or immunochromatography indicates an infection with certainty. The provirus is detectable by PCR.

Control and Prophylaxis

Therapeutic attempts with agents such as azidothymidine, which are also used in HIV therapy, were satisfactory in experimental studies; however, they have invariably turned out to be disappointing in field studies. For a few years, a vaccine has been approved in the USA which is based on inactivated whole virus, and prevents infection. Inasmuch as the diagnosis of FIV infection is based on the detection of antibodies, the vaccine is not a marker vaccine and FIV infection is easy to control in animals through management measures, this vaccine has not been approved in Europe; hence, its use is basically not recommended.

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Viruses with a Double-Stranded DNA Genome

19

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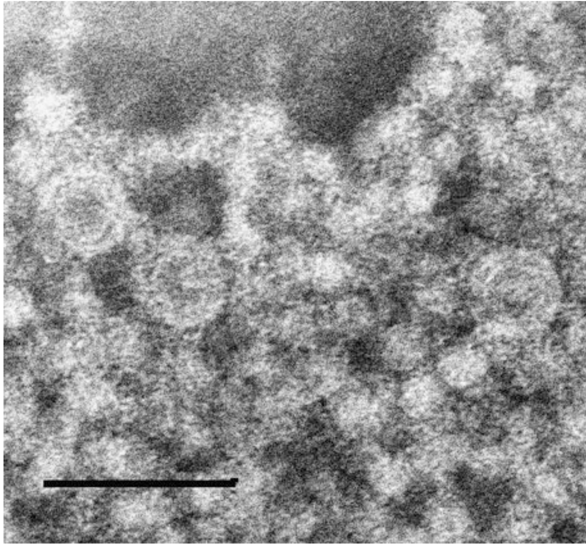
There are many viruses with a double-stranded DNA genome that are known to infect mammals. They are subdivided into seven virus families: *Hepadnaviridae*, *Polyomaviridae*, *Papillomaviridae*, *Adenoviridae*, *Herpesviridae*, *Poxviridae* and *Asfarviridae*. With the exception of the families *Poxviridae* and *Asfarviridae*, all families have members that can cause persistent infections in humans or animals. Hepadnaviruses, polyomaviruses, papillomaviruses and herpesviruses are causally related to human cancers. This suggests that double-stranded DNA viruses have many ways to regulate and influence cell division. Hepadnaviruses, which are described at the beginning of this chapter, are closely related to retroviruses. Many details of their replication cycle suggest that they have evolved from retroviruses.

19.1 Hepadnaviruses



The term "hepadnaviruses" characterizes a virus family whose principal prototype is human hepatitis B virus. This virus has also influenced the name of the family, as it has been characterized as a pathogen that contains a partially double-stranded DNA genome, and is the causative agent of the occasionally chronic form of liver inflammation (hepatitis). To distinguish this virus from other hepatitis viruses containing an RNA genome, the name "hepadnaviruses" was created, which is an acronym that stands for *hepa*titis *DNA viruses*. Other viruses with similar structure and a double-stranded DNA molecule have been discovered in various vertebrates. These viruses have also been classified into the family *Hepadnaviridae*.

19.1.1 Classification and Characteristic Prototypes

The currently known hepadnaviruses are divided into two genera (Table 19.1). Members of the genus *Orthohepadnavirus* infect mammals. These viruses include the prototypic hepatitis B virus, which causes acute and chronic forms of hepatitis in humans. Several variants of these viruses are known to differ in their antigenicity, i.e. in the ability of viral envelope proteins to be recognized immunologically. Similar viruses have been isolated from various primates (gorillas, chimpanzees, gibbons, and orang-utans), ground squirrels and American

Table 19.1 Characteristic prototypes of hepadnaviruses

Genus	Human virus	Animal virus
Orthohepadnavirus	Hepatitis B virus	Ground squirrel hepatitis virus
		Woodchuck hepatitis virus
		Woolly monkey hepatitis B virus
Avihepadnavirus		Duck hepatitis B virus
		Heron hepatitis B virus

woodchucks. The hepatitis B like viruses of primates exhibit very close homology to the human pathogenic hepatitis B virus at the DNA sequence level. The second genus, *Avihepadnavirus*, comprises virus types that infect birds (ducks, storks and herons). Avihepadnaviruses differ from orthohepadnaviruses because they lack the gene encoding the X protein, a non-structural protein that is thought to be involved in tumorigenesis. Animal pathogenic hepadnaviruses are important model systems for clarifying the pathogenesis of hepatitis B virus infection in humans; however, they do not constitute a problem related to infection for veterinary medicine. The structure of the genome of hepadnaviruses and the mechanism of replication are similar to those of caulimoviruses, a group of plant viruses whose particles, in contrast to those of hepadnaviruses, are not surrounded by an envelope. These include cauliflower mosaic virus and dahlia mosaic virus.

19.1.2 Structure

19.1.2.1 Virus Particle

Infectious virus particles are spherical with a diameter of 42 nm. In the case of hepatitis B virus, they are also known as Dane particles, named after David S. Dane, who discovered them in 1970. Three different-sized forms of the viral surface protein HBsAg – LHBsAg ("L" for "large"), MHBsAg ("M" for "medium"), and SHBsAg ("S" for "small") – are anchored in the viral envelope, which originates from the endoplasmic reticulum (ER) membrane. These isoforms differ by the presence of different amino-terminal sequences, whereas the carboxy-terminal domains are identical in all three versions. Monoglycosylated SHBsAg is the major surface protein of infectious hepatitis B virus particles. In relation to SHBsAg, LHBsAg and MHBsAg are present at a ratio of 1 % and 5 %, respectively. Avihepadnaviruses lack the respective MHBsAg version of the surface protein, and the ratio between SHBsAg and LHBsAg is similar to that for human hepatitis B virus. The viral envelope surrounds the icosahedral capsid (diameter 22–25 nm), which consists of 240 units of the viral capsid protein HBcAg (for "hepatitis B virus core antigen") and contains the DNA genome. In addition, some cellular proteins (protein kinases, chaperones) are also detectable in the virus particles.

Apart from infectious virions, there are also spherical (22 nm) and filamentous (200–300 nm long and 22 nm in diameter) particles which are not infectious and do not contain DNA. These particles are found in large quantities along with infectious virons in the blood of individuals acutely or chronically infected with hepatitis B virus. The spherical 22-nm particles, which are also called Australia antigen because they were initially found in serum samples of Aborigines, contain almost exclusively SHBsAg and only small amounts of MHBsAg, both variants in partially glycosylated form, which are embedded in an envelope (Fig. 19.1). The filaments consist of SHBsAg and MHBsAg. LHBsAg is present only in very low concentrations in the non-infectious virus particles.

HBsAg Particles Have Been Used as a Vaccine

The fact that large amounts of non-infectious HBsAg particles occur in individuals with chronic infections was exploited to develop the first vaccine against hepatitis B virus, which was introduced onto the market in the early 1980s. These particles were isolated and purified from the plasma of chronic hepatitis B virus carriers. These preparations were used to vaccinate especially people with a high risk of infection (medical staff, homosexuals). As a result, the vaccinated individuals produced antibodies against HBsAg, which conferred protection from infection. A few years later, this vaccine was replaced by the first genetically engineered vaccine to be used in humans. It is constituted of SHBsAg particles that have been isolated from recombinant yeast cells.

19.1.2.2 Genome Organization and Structure

The genome of infectious particles has an unusual structure. It consists of a partially double-stranded DNA containing approximately 3,200 base pairs. The so-called complete strand (minus strand) is not closed to a circular structure, but it contains a viral P protein molecule (also called terminal protein, TP) that is covalently bound to the 5′ terminus. The incomplete strand constitutes about 40–80 % of the genome. A viral P protein molecule is associated with its 3′ end as well, however in a non-covalent fashion (Fig. 19.2).

The genome of hepatitis B virus comprises, depending on the subtype, 3,100–3,300 base pairs (3,215 in subtype adr, 3,221 in subtype adw and 3,182 in subtype ayw). Just one strand is complete in Dane particles; it is the negative strand, which is transcribed during infection. The short (gapped) DNA strand (plus strand) consists of approximately 1,700–2,800 nucleotides. It does not encode viral proteins, and thus it is not transcribed. An additional feature of the hepadnavirus genome is the presence of direct repeats (DR1 and DR2), which have a length of 11 base pairs and are separated by about 225 nucleotides (Fig. 19.2). A 17–19-nucleotide-long 5′-capped RNA is located at the 5′ terminus of the incomplete strand, which is followed by the sequences of DR2. The P protein is covalently linked to the 5′ terminus of the negative strand, which ends with the DR1 sequences. The only consensus sequence for polyadenylation is located approximately 20 base pairs upstream of the 3′ terminus of the complete strand.

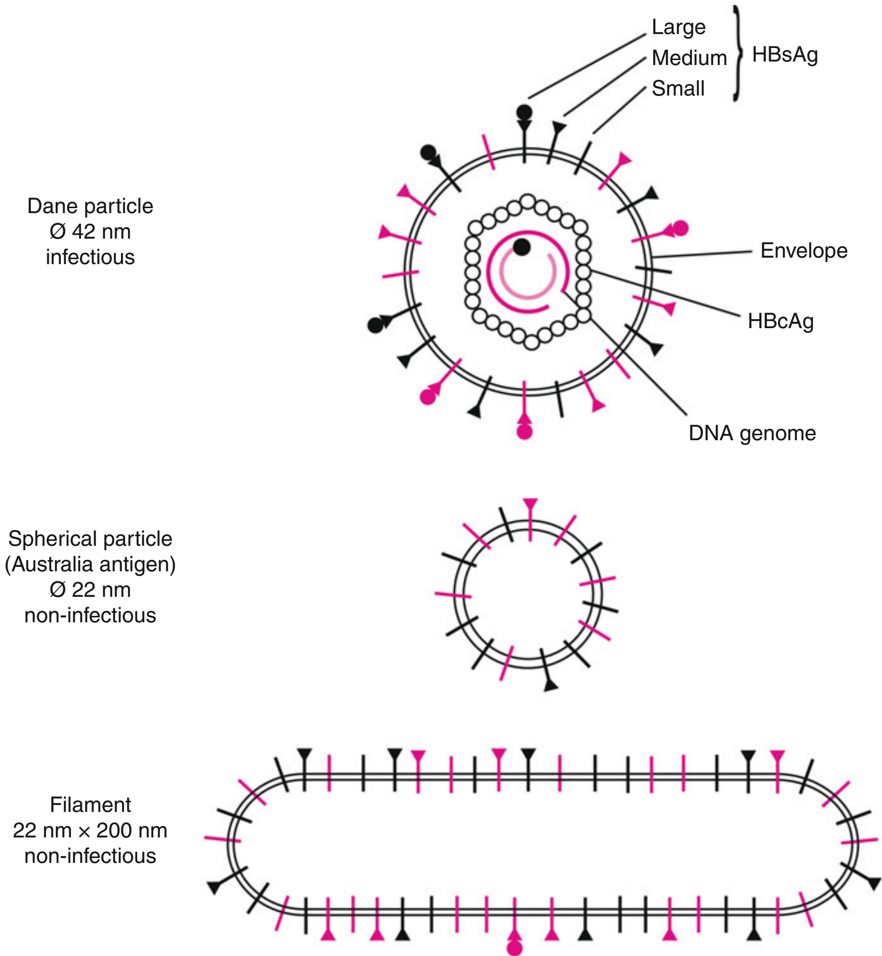


Fig. 19.1 Structure and composition of infectious and non-infectious particles of hepatitis B virus. An infectious hepatitis B virus is depicted in the *upper part*. It consists of a partially double-stranded DNA genome, which is associated with capsid proteins (HBcAg), forming an icosahedral nucleocapsid. This nucleocapsid is enclosed by an envelope, in which the viral surface proteins SHBsAg, MHBsAg and LHBsAg are embedded. The glycosylated envelope proteins are shown in *red* and the unmodified ones are shown in *black*. The non-infectious spherical and filamentous particles that do not contain viral genomes are depicted in the *lower part*. They are envelope vesicles that contain the various forms of viral surface proteins. Whereas spherical particles contain almost exclusively SHBsAg, filamentous particles also contain MHBsAg and small amounts of LHBsAg

Between DR1 and the poly(A) signal, there is a sequence of 60–70 base pairs which exhibits homology to the U5 region in the long terminal repeat of retroviruses. In general, hepadnaviruses show similarities to the family of retroviruses in respect of some sequence characteristics, the arrangement of genes on the viral DNA and the function of individual proteins (▶ Sect. 18.1).

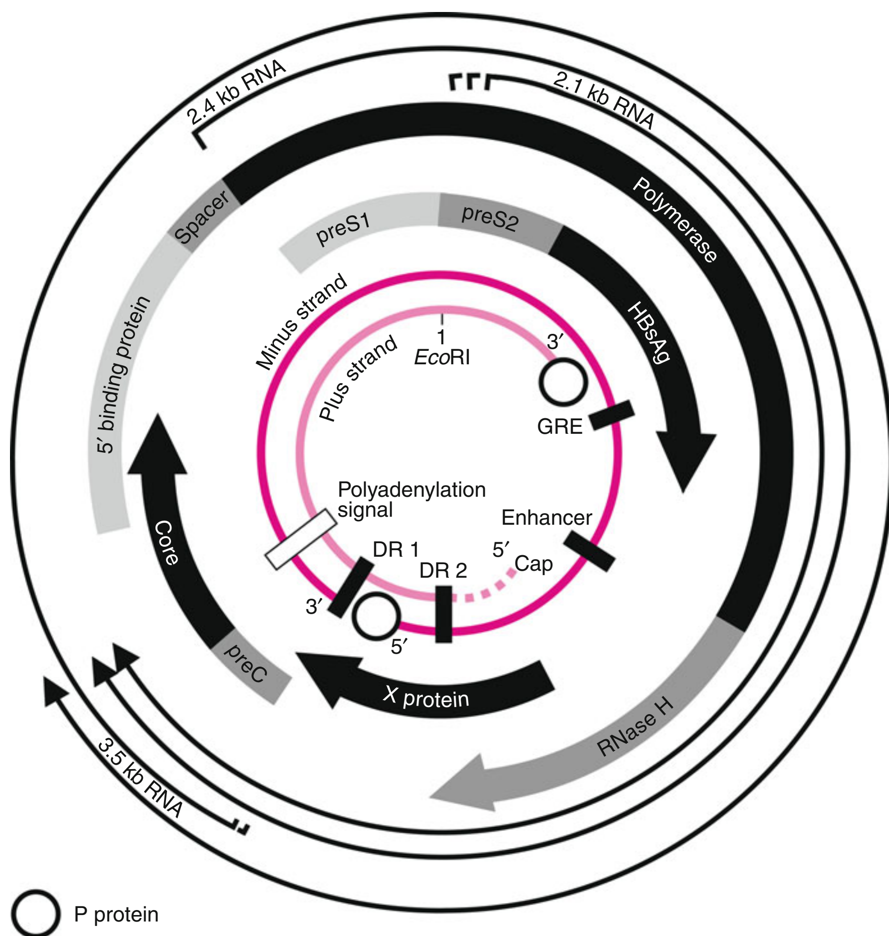


Fig. 19.2 Genome organization of hepatitis B virus (subtype ayw). In infectious virus particles, the genome is present as partially double-stranded DNA. The P protein is covalently bound to the 5′ terminus of the complete, non-circular closed minus strand by its terminal protein (*TP*) domain. This strand is transcribed during the replication cycle. A second P protein is non-covalently associated with the 3′ end of the incomplete plus strand. A short, 5′-capped RNA is associated with the 5′ terminus of this strand. Several regulatory elements are located in the genome: two direct repeats (DR1 and DR2), a signal for polyadenylation of messenger RNAs (mRNAs), i.e. poly(A), an enhancer, which increases the synthesis of the 2.1-kb mRNA that encodes SHBsAg, and a glucocorticoid response element (*GRE*), to which glucocorticoid receptors that are activated by hormone binding bind,. The positions of the open reading frames and the gene products encoded by them are indicated by *bold arrows*. So far, three different mRNAs and their respective start sites (3.5-kb, 2.4-kb and 2.1-kb RNAs) have been identified in infected liver cells. These mRNAs, which encode the different viral proteins, are represented in the *outer parts*. All mRNAs end at the single polyadenylation site. In addition, there is evidence for the existence of a 0.7-kb mRNA, which is thought to be responsible for the translation of the X protein (not shown). The genome contains a unique cleavage site for the restriction enzyme *Eco*RI. By arbitrary convention, the first adenine in the recognition sequence has been defined as position 1 for nucleotide numbering

The genome contains four open reading frames, which partially overlap, and can be read in different reading frames (Fig. 19.2). They include:

1. The gene encoding all three forms of the surface proteins SHBsAg, MHBsAg and LHBsAg.
2. The open reading frame encoding the capsid or core protein (HBcAg) and the secreted early antigen (HBeAg)
3. The open reading frame that encodes the P protein
4. The sequences encoding the hepatitis B X (HBx) protein

In addition to these viral genes, there are a number of regulatory sequences for control of transcription and the start of the various viral messenger RNA (mRNA) species (Fig. 19.2). Three different mRNA species have been found in vivo so far. Presumably, there is also a fourth transcript that comprises 700 nucleotides, and is used for translation of the HBx protein; however, it is not clear under what conditions this mRNA is synthesized. The transcripts have different start sites, but all end at the only existing polyadenylation signal. A start site is located six nucleotides upstream of DR1. Starting from this site, a pregenomic mRNA (pgRNA) is transcribed which has a length of about 3,500 nucleotides, extends throughout the entire genome and even overlaps with the sequences of the 5′ terminus over a range of about 120 nucleotides. The HBcAg and P proteins are translated from this mRNA. Another transcription initiation site has been identified roughly 30–40 nucleotides before the start of pgRNA. The use of this start site leads to the synthesis of an mRNA that also spans the entire genome and serves for the translation of HBeAg. A second mRNA species is 2,400 nucleotides in length; it begins approximately 38 nucleotides before the start of the reading frame of LHBsAg and serves for its translation. The start sites for the third mRNA species, which has a length of 2,100 nucleotides and is the most abundant, reside within the LHBsAg coding region. In this case, transcription can be initiated at three sites, which are located close together near the start codon of MHBsAg. Depending on the start site, either MHBsAg or SHBsAg is translated (Fig. 19.3) from these heterogeneous mRNAs. The mRNAs contain a *cis*-active signal, which is referred to as a post-transcriptional regulatory element, and lies after the stop codon of the surface proteins. It contains a stable hairpin loop structure in the region between nucleotides 1203 and 1515 which promotes the transport of the transcripts from the nucleus to the cytoplasm. The post-transcriptional regulatory element functions similar to the Rev response element of lentiviruses (▶ Sect. 18.1.3). However, unlike the Rev response element, no viral proteins bind to the post-transcriptional regulatory element, but solely cellular proteins associate with this RNA secondary structure and promote both transport of viral mRNAs to the nuclear pores and translocation through them.

A transcriptional enhancer sequence is located directly in front of the X gene and hence about 450 base pairs upstream of the start of the preC gene. This sequence resides within the X gene in hepatitis B virus subtypes that express a very long form of the X gene (adr subtype). Several cellular proteins bind to this enhancer in vitro. There is evidence that this element regulates – especially in liver cells – the enhanced transcription of viral genes. A sequence motif of 18 nucleotides in length

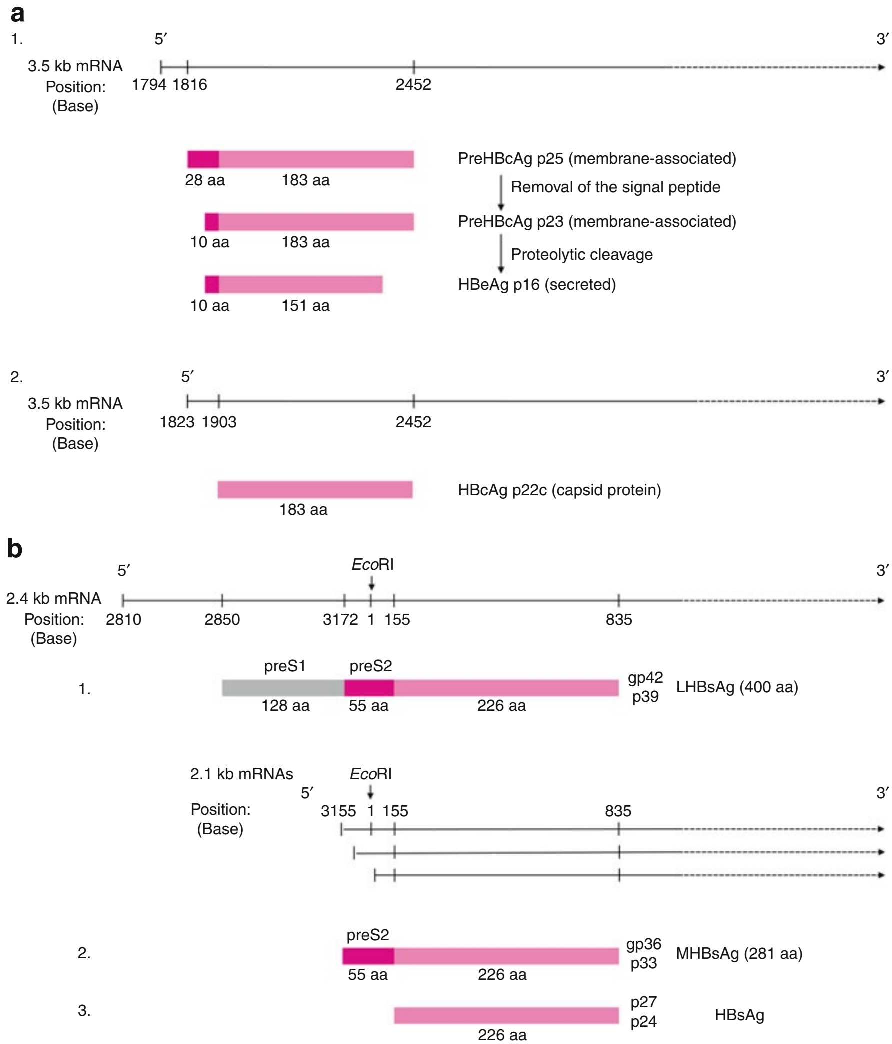


Fig. 19.3 Transcripts encoding the core/capsid and surface proteins of hepatitis B virus (subtype ayw). (**a**) The open reading frame "core" encodes the different variants of capsid protein HBcAg and the secreted HBeAg. HBeAg and related proteins are translated from the 3.5-kb preC mRNA (the *numbers* refer to the nucleotide position in the viral genome, starting from the *Eco*RI cleavage site as position 1). Another start codon is located in the frame 29 codons downstream, and is used to initiate the translation of HBcAg. The 29 amino-terminal amino acids constitute the precore domain and regulate the synthesis of the protein at the endoplasmic reticulum. The HBeAg protein arises after cleavage of the amino-terminal signal peptide and the carboxy-terminal domain. On the other hand, HBcAg is translated from the 3.5-kb mRNA as an 183 amino acid protein by using the start codon at position 1,903 (*lower part*). *aa* amino acids. (**b**) The LHBsAg protein is translated from the 2.4-kb mRNA (the *numbers* refer to the nucleotide position in the viral genome, with the *Eco*RI cleavage site defined as position 1). LHBsAg (p39 or gp42) starts at position 2,850 and contains, in comparison with MHBsAg, 128 additional amino acids at the

which resembles the recognition site of human glucocorticoid receptors (glucocorticoid response element, GRE) is located within the HBsAg gene. The expression of HBsAg is increased about fivefold in the presence of glucocorticoids.

19.1.3 Viral Proteins

19.1.3.1 HBcAg and HBeAg

The capsid is made from the viral core protein HBcAg, which has a molecular mass of 22 kDa (p22) (Fig. 19.3a). HBcAg is phosphorylated at serine residues probably by a cellular kinase during the viral replication cycle. It contains basic amino acid residues at the carboxy terminus, which are presumptively associated with the genome. HBcAg has the ability to assemble into particulate structures in the cell, and plays an important role in viral self-assembly.

In addition, a carboxy-terminal truncated variant of HBcAg (16 kDa) is synthesized, and this lacks 32–34 amino acid residues depending on the virus subtype. This truncation encompasses the basic amino acids that interact with the genome (Fig. 19.3a). This protein, known as HBeAg ("e" stands for "early", because it is detected very early in the blood during infection), is synthesized in a version that, in comparison with HBcAg, contains on average 29 additional amino acid residues at the amino terminus. It is translated from the preC mRNA beginning at an upstream start codon located in the same reading frame as that of HBcAg (Fig. 19.3). Thus, the resulting protein is longer than HBcAg, as its amino-terminal extension contains the 29 amino acids of the precore domain. This slightly variable "precore" domain functions as a signal peptide and ensures that protein synthesis occurs at the ER membrane. This domain is cleaved out during processing. The mature protein is transported to the cell surface via the Golgi apparatus, and finally released. A variant of HBeAg is found in the cell membrane. This protein is not a virion component, and can be detected in the blood of infected individuals. Table 19.2 gives an overview of the features and functions of the different viral proteins.

19.1.3.2 The Surface Protein HBsAg

Three different forms of the viral glycoprotein HBsAg are embedded in the envelope of orthohepadnaviruses (Fig. 19.1). The main protein of infectious virus particles is the SHBsAg form, which has a molecular mass of 24 kDa (p24) and consists of 226 amino acids. Some SHBsAg molecules are glycosylated at an asparagine residue (Asn146) (27 kDa, gp27). In addition, two other HBsAg variants are embedded in the viral envelope and contain additional amino acid sequences at

Fig. 19.3 (continued) amino terminus. MHBsAg (p33 or gp36) begins with the start codon at position 3,172 and is 55 amino acids longer than SHBsAg at the amino terminus. Both proteins are translated from a group of about 2.1-kb mRNAs that are characterized by heterogeneous 5′ termini (*lower part*). The start codon at position 155 is used for the translation of SHBsAg. The result is a 226 amino acid protein (p24) which is partially glycosylated (p27)

Table 19.2 Properties and functions of hepatitis B virus proteins

Protein	Molecular mass (kDa)	Modification	Function
SHBsAg (HBsAg)	24	–	Surface protein; induction of neutralizing antibodies; formation of particles
	27	Glycosylated	
MHBsAg (preS2-HBsAg)	33	–	Surface protein; induction of neutralizing antibodies; binding to serum albumin
	36	Glycosylated	
LHBsAg (preS1-HBsAg)	39	Myristoylated	Surface protein; induction of neutralizing antibodies; attachment to the receptor
	42	Glycosyated	
HBcAg	22	Phosphorylated	Capsid protein; interaction with the genome; particle assembly
HBeAg	16	–	Secreted protein, carboxy-terminal truncated form of HBcAg; a minor part is envelope-associated
P	90	?	DNA- and RNA-dependent DNA polymerase (reverse transcriptase); RNase H; terminal protein for initiation of replication
HBx (X protein)	17	–	Transactivator of viral and cellular promoters; binding to the tumour suppressor p53 and ultraviolet-damaged DNA-binding protein; stimulation of protein kinase C

the amino terminus. However, apart from that, their sequences are identical to the sequence of SHBsAg. They are termed MHBsAg (preS2-HBsAg, 33 kDa) and LHBsAg (preS1-HBsAg, 39 kDa). In comparison with SHBsAg, the variants MHBsAg and LHBsAg have by virtue of their preS2 and preS1 domains 55 or 108–128 (depending on the genotype) additional amino acids, respectively. An N-glycosylation signal is located at position 4 of the preS2 domain. In addition, O-glycosidically linked carbohydrate groups are found at threonine at position 37 in the preS2 domain of genotypes B to H. In their glycosylated form, the molecular masses of MHBsAg and LHBsAg are 36 and 42 kDa, respectively. The glycosylation signals of the preS1 domain are not used because this domain is oriented into the cytoplasm during translation; however, position 2 of LHBsAg is modified by myristoylation. It is believed that all HBsAg variants possess four transmembrane regions, and that both termini of SHBsAg and MHBsAg are situated at the particle surface. In contrast, 50 % of LHBsAg molecules seem to have preS1 domains that are oriented to the interior of the particle. Disulphide bridges between the SHBsAg-specific domains covalently link the surface proteins of both infectious virus particles and non-infectious virus particles; these cannot be broken by treatment with detergents. HBsAg proteins induce a protective humoral immune response. The most important immunogenic epitope is the “a” determinant; it is defined by two exposed domains of HBsAg (amino acids 120–163), and induces the formation

of neutralizing antibodies. Hepatitis B virus strains carrying mutations in this protein domain can undermine an induced immune protection. Such HBsAg variants are frequently not detected in common serological tests.

MHBsAg is not present in avihepadnaviruses; in contrast to orthohepadnaviruses, SHBsAg and LHBsAg are not glycosylated, but are present in phosphorylated forms. Another difference is that the surface proteins are not the linked by disulphide bonds in duck hepatitis B virus.

19.1.3.3 Polymerase (P Protein)

The P protein (molecular mass about 90 kDa) is non-covalently associated with the 3′ end of the incomplete DNA strand in infectious virus particles. The P protein can be divided into three domains. It is covalently linked to the 5′ terminus of the complete DNA strand by a tyrosine residue in the amino-terminal TP domain. This tyrosine residue acts as a primer for the initiation of DNA synthesis during genome replication. The TP domain is followed by the polymerase/reverse transcriptase, and the RNaseH domains.

19.1.3.4 HBx Protein

The tumorigenic capability of orthohepadnaviruses has been associated with the homodimeric X protein (17 kDa). The X protein is occasionally found in small amounts in liver biopsies of individuals infected with hepatitis B virus and in patients with a primary hepatocellular carcinoma. Such individuals produce antibodies against the HBx protein. If it is expressed in the liver of transgenic mice, it induces hepatomas. HBx is also necessary for the in vivo infectivity of woodchuck hepatitis B virus. The HBx protein acts as a transactivator of viral and various cellular promoters. It does not bind to the promoters, but indirectly enhances their function by interacting with cellular transcriptional activators, particularly members of the cyclic AMP response element (CRE) binding (CREB) and activating transcription factor (ATF) families and general transcription factors such as TATA-box-binding protein. Furthermore, HBx binds to p53 by its carboxy-terminal domain, thereby inhibiting the transactivator function of this pivotal tumour-suppressor protein. This interaction suppresses the expression of p53-dependent genes, which are mainly involved in DNA repair processes, in the regulation of the cell cycle and in the induction of apoptosis (► Chap. 6). Hence, the X protein functions like the T antigen of simian virus 40 (SV40) (Sect. 19.2.3), the E6 protein of human papillomaviruses (Sect. 19.3.3) and the E1B protein of adenoviruses (Sect. 19.4.3), which – albeit in different ways – impair the function of p53, thus inducing unrestrained cell division. In addition, HBx interacts with ultraviolet-damaged DNA-binding protein. This cellular protein is a component of the excision repair system that recognizes, removes and replaces incorrectly pairing or damaged nucleotides, which are caused, for example, by ultraviolet rays. Among other things, discussion is ongoing concerning a direct interaction with different cellular components as well an activation of protein kinase C and its associated signalling pathway, whereby the activity of various transcription factors may be influenced.

Other data show that HBx activates nuclear factor κB (NFκB) by reactive oxygen intermediates, and that it also enhances the activity of cyclin-dependent kinase 2, thus increasing the cell division rate.

19.1.4 Replication

Duck hepatitis B virus binds to its receptor, a membrane-associated form of carboxypeptidase D (gp180), by the preS1 domain of LHBsAg. In the next step, virus particles enter the cell by receptor-mediated endocytosis, but the usual acidification of the endosome content is not necessary for their release. A similar entry mechanism is assumed for the human pathogenic hepatitis B virus. However, the cellular receptor has not yet been definitively identified, but the amino-terminal $preS_1$ domains of LHBsAg seem also to be responsible for attachment. Among others, annexin V (formerly known as endonexin II), the asialoglycoprotein receptor and a membrane-associated serine protease inhibitor have been described as putative receptors. Possibly, attachment is additionally mediated by serum albumin, which binds to the preS2 sequences of MHBsAg and to a receptor on hepatocytes. Serum albumin binding is also held responsible for autoimmune mechanisms that frequently occur in association with hepatitis B virus infection. It is believed that serum albumin slightly modifies its structure by interacting with virus particles, and thus is regarded as a foreign antigen.

Figure 19.4 illustrates the pathway followed by hepatitis B virus in an infected cell. After the virus has penetrated into the cell, the capsids harbouring the viral genome are transported along the microtubules to the nuclear pores. This process is mediated by a nuclear localization signal at the amino-terminal domain of HBcAg and which interacts with the nuclear import receptors importin α and importin β. The non-covalently closed viral genomes (relaxed circular DNA) are released at nuclear pores, and are then translocated into the nucleus. The incomplete DNA strand is then converted to a double strand by the DNA polymerase activity of the P protein. During this process, the 5′-capped RNA oligonucleotide is degraded, the TP is removed and the gap is closed. This leads to a double-stranded, covalently closed, circular DNA, which associates with histones to form nucleosomes and is present in a supercoiled structure. In rare cases, a part of or the entire hepatitis B virus genome is integrated into the genome of the cell in this infection stage.

In the nucleus, the now episomal viral genome is transcribed by the cellular RNA polymerase II. All synthesized mRNA molecules have the same 3′ end – common poly(A) site – but different initiation sites and thus different lengths (Fig. 19.2). These include the 3.5-kb precore transcripts (preC mRNA) for the translation of HBeAg and the pgRNAs, from which the HBcAg and P proteins are translated, the 2,400-nucleotide-long mRNA for the synthesis of LHBsAg and the about 2.1-kb transcripts for the synthesis of MHBsAg and SHBsAg. Following modification of 5′ ends with cap structures and polyadenylation of 3′ termini by cellular enzymes, the different RNA molecules are transported into the cytoplasm and translated.

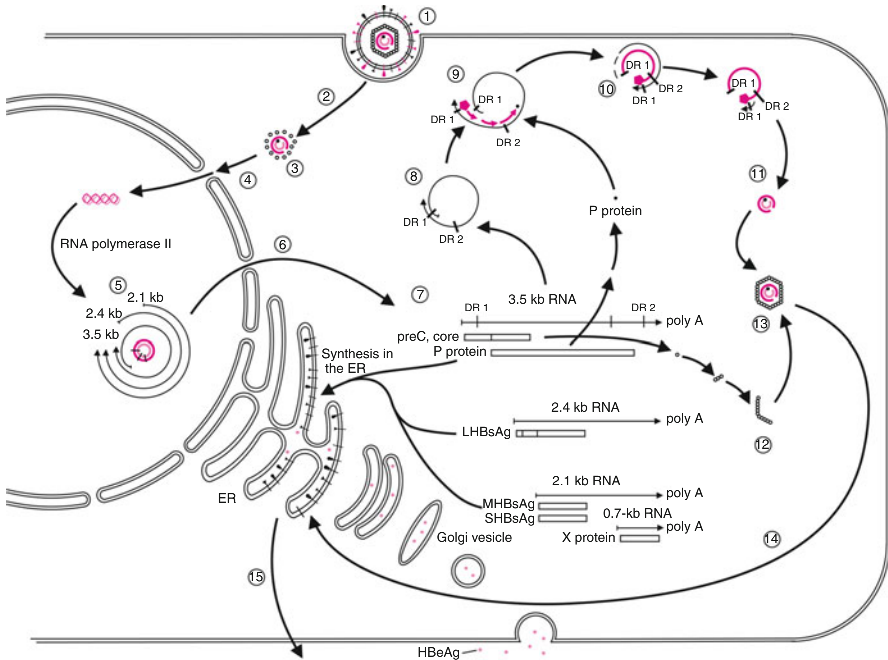


Fig. 19.4 Replication cycle of hepatitis B virus in a cell. *1* attachment. *2* receptor-mediated endocytosis. *3* transport of viral capsids to nuclear pores and translocation of the viral genome (relaxed circular DNA) into the nucleus. *4* completion to form covalently closed, circular, supercoiled DNA. *5* transcription. *6* export of various mRNA species from the nucleus to the cytoplasm. *7* translation of mRNA: HBsAg and HBeAg at the endoplasmic reticulum followed by transport via the Golgi apparatus; HBcAg, polymerase and X protein in the cytoplasm on free ribosomes. *8* use of the 3.5-kb pregenomic mRNA (pgRNA) as a pregenome. *9* initiation of the synthesis of the first strand of DNA by the viral polymerase (priming by P protein). *10* degradation of the RNA moiety in the hybrid molecule by the RNase H activity of the P protein. *11* Synthesis of the second strand of viral DNA after primer transfer. *12* aggregation of HBcAg. *13* packaging of the incomplete genome with HBcAg aggregates. *14* and *15* release of infectious particles which have been previously enclosed with an HBsAg-containing envelope membrane. *ER* endoplasmic reticulum

The synthesis of the surface proteins LHBsAg, MHBsAg and SHBsAg occurs at the ER membrane, where the nascent amino acid chains are translocated into the lumen and anchored in the membrane by their four transmembrane domains. The precore mRNA serves for the translation of the precore protein (precore HBcAg), which also contains a signal peptide at the amino terminus. After removal of the signal peptide by a signalase, this protein is released into the lumen of the ER, and after proteolytic cleavage of the carboxy-terminal domain, it is finally secreted as HBeAg by the Golgi vesicles. The signal peptide is not excised from all molecules. Therefore, some proteins remain associated with the cytoplasmic membrane in the form of precore HBcAg and precore HBeAg (Fig. 19.3a). The capsid protein HBcAg and the P protein are translated from open reading frames of the pgRNA.

The start codon of the P protein is located more than 500 nucleotides downstream of the 5′ terminus of the RNA. It is not known how this internal translation initiation occurs. In human hepatitis B virus a special mRNA seems to be synthesized for translation of the X protein. It starts from a promoter located upstream of the start of the X reading frame, and consists of approximately 700 nucleotides. However, whether this mRNA is synthesized during the infection and replication cycle is unclear. In human hepatitis B virus the X protein does not appear to have an essential function in replication. This is in contrast to hepatitis B viruses of woodchucks and ground squirrels, where the transactivator function of the X protein is essential for infection.

Probably, multiple P protein molecules bind to a stable stem-loop structure (also referred to as an ε-signal) during initiation of replication; this is located at the 5′ terminus of the pgRNA between the DR1 and DR2 regions. The amino-terminal TP domain of the P protein provides the OH group of a tyrosine to prime reverse transcription; thereby a molecule of the P protein remains covalently linked to the 5′ end (Fig. 19.5). A short DNA molecule is synthesized which is complementary to the sequences spanning the region between the ε-signal and the 5′ end of the pgRNA. This initiation complex is transferred to the ε-structure at the 3′ end of the pgRNA. Since the pgRNA contains identical nucleotide sequences at both the 5′end and the 3′ end, the newly synthesized DNA segment can hybridize with them. Similar processes are found during reverse transcription of retroviruses (► Sect. 18.1.4). After transfer this complex serves as a primer for the synthesis of a continuous DNA strand which encompasses the entire pgRNA, a process that is catalysed by the reverse transcriptase activity of the P protein. In duck hepatitis B virus, the P protein can exerts its polymerase activity only when it is structurally refolded by cellular chaperones (Hsc70, Hsp40). This process appears to unnecessary in human hepatitis B virus.

As soon as the initiation complex of pgRNA and P protein constitutes the recognition structure for aggregation of HBcAg capsid proteins, viral capsid precursors are formed, and are subsequently transported from the nucleus into the cytoplasm. During this process, the RNA moiety of the DNA–RNA hybrid is degraded by the RNase H activity of the P protein. However, a short fragment of capped RNA at the 5′ terminus is not degraded, and its 3′-OH end is used as a primer for the synthesis of the complementary DNA strand. The synthesized DNA strand adopts the form of a ring which cannot be closed because of the covalently linked protein at the 5′ end. The complementary DNA strand spans the gap of the first strand. The still nascent viral genome, which is already associated with capsid proteins, interacts with cytoplasmic chaperones and protein kinases. These induce phosphorylation and maturation of capsids. Owing to these structural changes the genome-containing capsids become surrounded by HBsAg-containing ER membranes. Thereby, the virus particles bud into the lumen of the ER and are transported through the Golgi vesicles to the cell surface, where they are finally released. The polymerization of the second DNA strand continues only until the nucleotides which have been incorporated in the capsids during packaging become depleted. A subset of viral capsids remains non-enveloped.

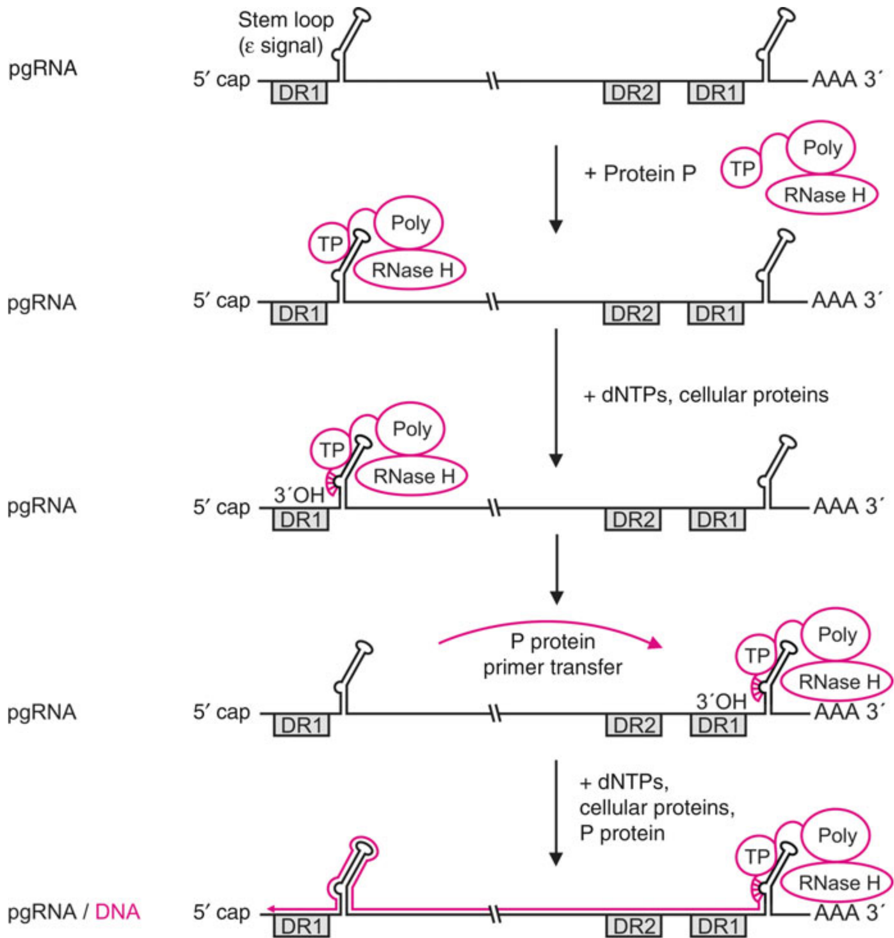


Fig. 19.5 Genome replication of hepadnaviruses. Processes during P protein primed initiation of DNA synthesis. Several P protein molecules bind to the ε-hairpin structure that is adjacent to the DR1 element at the 5′ end of pgRNA (for illustrative reasons, only one P protein is indicated). A tyrosine residue at the amino-terminal TP domain of the P protein serves as a primer for initiation of DNA synthesis (*red*), which is catalysed by the reverse transcriptase activity of the polymerase domain (*poly*) of the P protein. Subsequently, the complex of the P protein and of the short DNA fragment is transferred to the ε-hairpin structure at the 3′ end of the pgRNA. The viral polymerase catalyses the synthesis of a continuous single-stranded DNA molecule; it is complementary to the entire pgRNA. Subsequently, the RNA moiety of the DNA–RNA hybrid is degraded by the RNase H activity of the P protein, which resides in the carboxy-terminal domain (not shown)

Analogously to the processes that occur in the early stages of viral replication, these capsids are transported to the nuclear pores, through which their genomes enter the nucleus, where they are converted into covalently closed, circular DNA viral genomes. This leads to the accumulation of viral genomes in the nucleus, reaching up to 100 copies per infected cell.

19.1.5 Human Pathogenic Hepadnaviruses

19.1.5.1 Human Hepatitis B Virus

Epidemiology and Transmission

Hepatitis B virus is transmitted by blood (also at delivery), blood products and sexual intercourse. Humans are the sole reservoir of the pathogen, but experimental transmission to chimpanzees is possible. Hepatitis B virus particles are present in different concentrations in the blood of acutely infected patients and of chronic virus carriers. One millilitre of blood contains up to 10^9 infectious virions, as well as 10^{13} spherical (corresponding to about 50 μg/ml) and 10^9 filamentous non-infectious HBsAg particles. The blood is already infectious before the onset of liver inflammation. Pregnant women with chronic hepatitis B infections, or those who become ill because of an acute infection at the time of delivery, can vertically transmit the virus (prenatally, perinatally or postnatally) to their offspring. This is especially true for the highly endemic regions in East Asia (Taiwan) and Central Africa and West Africa. In such regions, 20–80 % of the population are infected with hepatitis B virus, whereby in most cases (90 %) the infection is acquired perinatally. The virus can also be transmitted to infants through breast milk. In developed countries the situation is different: approximately 0.5 % of the population are carriers of HBsAg and 5–10 % have antibodies specific for hepatitis B virus, indicating a past infection. Overall, it is reckoned that 350–400 million people are chronically infected worldwide. Hepatitis B virus is only sporadically endemic in developed countries. A particular problem is the transmission of hepatitis B virus to medical personnel, which especially occurs by contact of blood containing hepatitis B virus with the mucosa or with non-intact skin or by injuries caused by contaminated needles.

The mutation rate of hepatitis B virus is similar to that of the retroviruses. The reason is because the enzymes involved in hepatitis B virus genome replication, the viral P protein and the cellular RNA polymerase II, do not possess 3′→5′ exonuclease (proofreading) activities to check and improve the accuracy of polymerization. However, because of the very compact organization of the hepatitis B virus genome, all the genes overlap. Therefore, mutations usually give rise to changes in two viral genes, affecting the infectivity and replication capability of the resulting mutant strains. Phenotypically, hepatitis B viruses can be classified into four distinct serotypes. The common determinant of all serotypes is denoted by “a”. It is defined by two exposed domains of HBsAg (amino acids 120–163) and is the most important immunogenic epitope; it induces the formation of neutralizing antibodies. In addition, there are two pairs of subtype determinants which largely behave allelically and are mutually exclusive: d, y and r, w. They give rise to the serologically distinguishable subtypes adr, adw, ayr and ayw. The determinant w can be divided into four additional variants: w, w2, w3 and w4. Furthermore, there are also the less frequent determinants q, x and g. Their reciprocal combination results in the mainly occurring subtypes of hepatitis B virus, which can be assigned to specific geographical regions: ayw, ayw2, ayw3, ayw4, ayr, adw4, and adr. Unusual variants such as adwr, adyw and awr can also be occasionally

observed in individual virus isolates, commonly in Southeast Asia. The determinants d/y are located between amino acids 284 and 323 of SHBsAg. They seem to depend on the three-dimensional folding of the protein chain and the correct formation of disulphide bridges. Point mutations may alter the various determinants and their immunological recognition by the corresponding antibodies. Such mutations may undermine the vaccine-induced immune protection and contribute to immune evasion by the mutant viruses.

The current classification of hepatitis B viruses into eight different genotypes (A to H) has proven to be reasonable, in particular, for epidemiological studies. The genome sequences of the various genotypes differ from each other by at least 8 %. Genotypes A, B, C and F are further divided into additional subgenotypes. The different genotypes exhibit a specific geographical distribution: virus isolates of genotype A predominate in central Europe, whereas genotype D predominates in southern and eastern Europe, Russia, India and North Africa. Genotypes B and C are isolated preponderantly in China and Alaska as well as in Southeast Asia and Australia. Genotype E is prevalent in West Africa, genotype F is prevalent in South America, and genotypes G and H are mainly found in Central America.

Clinical Features

Hepatitis B virus infects the cells of the liver and can cause acute hepatitis. Depending on the infection doses, the incubation period lasts 2–6 months. Approximately 65 % of infections are asymptomatic in adolescents and adults, whereas 35 % of those infected develop acute hepatitis (liver inflammation). The main symptom is jaundice (icterus). Other symptoms are enlargement of the liver and spleen as well as – very rarely – blood disorders and skin eruptions. Hepatitis usually lasts 2–3 weeks. If HBsAg is still detectable in the blood after 6 months, a chronic infection can be assumed. This form is found in about 5–10 % of patients in relation to all hepatitis B infections. Approximately 90 % of perinatal infections and about 50 % of infections in infants lead to chronic liver inflammations which are commonly not associated with an acute disease. About 60 % of chronically infected individuals remain asymptomatic "healthy" virus carriers. Histopathologically, the symptomatic chronic hepatitis B virus infection is divided into chronic aggressive (replicative) hepatitis and chronic persistent hepatitis. Chronic aggressive hepatitis can spontaneously change into chronic persistent hepatitis. Liver cirrhosis and hepatocellular carcinoma are complications or sequelae. It is believed that particularly children of chronically infected mothers develop an immunological tolerance to hepatitis B virus which can induce a lifelong virus carrier state. This immunological tolerance is mainly attributed to the placenta-permeable HBeAg. Evidence supporting this notion has arisen from experiments with transgenic mice.

Pathogenesis

Acute Hepatitis B Virus Infection

Starting from the entry site, the hepatitis B virus enters the liver via the bloodstream. In this organ, hepatocytes containing high concentrations of HBsAg can be found, and are also referred to as milk glass cells. Hepatitis B virus is not

cytopathic. The destruction of liver cells is caused by $CD8^+$ lymphocytes (cytotoxic T cells), which can be detected in near infected liver cells. These lymphocytes are directed against hepatitis B virus peptides, which are presented to the cellular immune system in complex with MHC class I antigens on the surface of infected hepatocytes. In addition, cytotoxic T cells secrete cell-damaging cytokines such as TNF-α. Thus, acute hepatitis is an immunopathologically induced inflammatory reaction which actually constitutes a protective response of the organism. Antigen–antibody complexes that circulate in the blood during infection also play an important role in the pathogenesis: they are deposited in arterioles and are responsible for inflammatory processes in these regions, and for the manifestation of periarteritis nodosa and also of the exanthemas that are occasionally associated with hepatitis B virus infections. The significance of the occurrence of hepatitis B viruses in macrophages and blood monocytes as well as in pancreas, semen and other cells is not understood. It has been shown that myelopoiesis is impaired, and that hepatitis B viruses also infect immature blood cells. The latter are probably the reservoir for the virus in persistently infected patients.

An acute, but healing hepatitis has the following course. Hepatitis B viruses and increased levels of class I interferons can be detected in the serum as early as during the incubation period. The high interferon concentration is also responsible for the fever and the severe malaise at the onset of the disease. Interferons induce the transcription of MHC genes, increasing the amount of MHC class I antigens on the cell surface. This mechanism supports the recognition of cells infected with hepatitis B virus by the cellular immune system. It has also been demonstrated that interferon-α (IFN-α) and interferon-β (INF-β) reduce viral gene expression in both transgenic mice and human cells. What antiviral mechanisms play a role in this process remains to be elucidated. There is evidence for an involvement of antiviral MxA proteins, which are otherwise important for the non-adaptive defence mechanisms against RNA viruses (▶ Chap. 8, ▶ Sects. 15.3 and ▶ 16.3).

Global Vaccination Programmes Will Contribute to Reducing the Number of Virus Carriers and Hence the Incidence of Cirrhosis and Hepatocellular Carcinoma in the Population

The World Health Organization has been actively trying to counteract the high prevalence of hepatitis B virus infection in developing countries for several decades. In these regions, the very high prevalence rate in the population is 20–80 %, which consequently entails a large number of chronic virus carriers. Primary hepatocellular carcinoma is the commonest cancer disease in such countries. Extensive vaccination programmes for the population should provide adequate protection against hepatitis B virus infection, and interrupt the infection chain in these countries. The most important measures are the passive vaccination of newborn infants of mothers who test positive for hepatitis B virus, and the simultaneous active immunization with recombinant HBsAg particles. This approach has already led to

a decrease in cancer incidence. Vaccination against hepatitis B has been incorporated into routine vaccinations of infants and young children also in central Europe and the USA. This measure will eventually contribute to the eradication of hepatitis B virus. This seems possible because hepatitis B virus infects only humans and there are no animal reservoirs.

Chronic Hepatitis B Virus Infection

The two forms of chronic hepatitis emerge when the immune system cannot control the viral infection. In contrast to people who can successfully eliminate the virus after an acute hepatitis B virus infection, patients with a persistent hepatitis B virus infection exhibit a qualitatively and quantitatively significant lower cellular immune response. Different scenarios can be taken into account to explain this discrepancy:

1. In children of chronically infected mothers who have been perinatally infected, most often a chronic, persistent infection is established. Hepatitis B virus is not transmitted prenatally to the developing child, but the HBeAg molecules that are present in the blood of chronically infected pregnant women pass through the placenta and enter the fetal circulation. In this phase, the fetal immune system does not recognize HBeAg as a foreign protein. The embryo does not develop immunity, but develops an immunological tolerance. This means that the peptide epitopes of HBcAg and HBeAg are not immunologically recognized as foreign even after birth in the perinatally infected child. This has the consequence that the humoral and cellular immune responses to HBcAg and HBeAg cannot be developed.
2. In a perinatal infection and in infection in infancy, the virus meets a biologically immature organism, which can develop an immune tolerance. In adolescents and adults, an interferon deficiency may contribute to the establishment of chronic infections. It has also been found that synthesis of large amounts of HBcAg during infection specifically suppresses the interferon-induced expression of MxA genes.
3. Very high quantities of non-infectious HBsAg particles, as found in some patients during the acute phase of the disease, also play a role in the development of chronic persistent infections. These particles can intercept neutralizing, HBsAg-specific antibodies. The concentration of non-infectious HBsAg particles is particularly high among men. Male sex hormones bind to glucocorticoid receptors in the cells. These complexes bind to the GRE (Fig. 19.2) in the hepatitis B virus genome, significantly increasing the transcription of SHBsAg mRNA (2.1-kb mRNA). Accordingly, this leads to an increased production of this protein, to greater amounts of non-infectious HBsAg particles and to an increased incidence of chronic hepatitis. This may explain why more men than women acquire primary liver cell carcinoma.
4. Virus variants that do not synthesize HBeAg are detectable in up to 20 % of chronically infected individuals, especially in southern Italy and Southeast Asia.

In these cases, the disease is characterized by high transaminase levels and large amounts of hepatitis B virus DNA as well as severe disease and a rapid transition to liver cirrhosis. These HBeAg-negative hepatitis B viruses arise by mutations in the preC region of the genome which generate stop codons in the coding sequences. It is known that these mutants are transmissible and can replace the wild-type virus in the liver.

5. Hepatitis B virus variants carrying mutations in the HBsAg gene have emerged especially in southern Italy. They alter the protein and antigenic determinants in such a way that neutralizing antibodies directed against the wild-type protein cannot bind (or can only ineffectively bind) to these viruses (Sect. 19.1.3). In addition to mutations in SHBsAg, there are also mutations in the $preS_1$ and $preS_2$ regions of LHBsAg and MHBsAg. They have hardly been investigated, and it is uncertain whether these virus variants cause atypical patterns of hepatitis B virus infection.

Primary Liver Cell Carcinoma

About 250,000 people die every year of a primary liver cell carcinoma (also referred to as hepatocellular carcinoma), which occurs in patients with chronic persistent infections. The hepatitis B virus is the most important risk factor for its development. The longer a chronic hepatitis B virus infection persists, the higher is the probability of the development of a primary liver cell carcinoma. Its incidence correlates with the geographical regions with a high prevalence of chronic hepatitis B virus infections. The overall risk of developing a hepatocellular carcinoma is increased approximately 200-fold in individuals with a chronic hepatitis B infection.

Hepatocellular carcinoma develops gradually at the molecular level, whereby the formation of preneoplastic lesions suggests the involvement of several different genetic events:

1. A key event in the causal chain leading to hepatocellular carcinoma is the integration of the hepatitis B virus genome at random sites in the cellular genome. It preferentially occurs in chronic carriers who produce large amounts of virus over long periods. In this process, the circular hepatitis B virus DNA is usually cleaved in the gap region of the incomplete DNA strand – the gene sequence encoding HBsAg is only rarely affected by this event – so in cancer cells HBsAg and frequently also the X protein are synthesized even after integration of viral sequences. The production of infectious virions does not occur owing to the destruction of the integrity of the viral genome that is associated with the integration. Furthermore, this process is frequently accompanied by deletions in the hepatitis B virus genome, so no infectious virus particles can be produced. The long incubation period until the onset of liver carcinoma and its monoclonality suggest that the necessary processes are slow and rare. The unspecific integration at arbitrary sites in the chromosomal DNA can result in the destruction of cellular genes and, consequently, in the failure of essential functions, which most likely will lead to the death of the cell. Ina transformed cell, the viral integration into the cellular DNA of must bring about

not the death of the affected cell, but rather the ability to divide infinitely. This can occur by interfering with different cell functions, e.g. the integration event can occasionally alter the gene expression of a cyclin gene, which can lead to an increased cell division rate.

2. The HBx protein, which is frequently expressed in tumour cells, also plays an important role in the development of hepatocellular carcinoma. This protein transactivates cellular promoters, and exerts a stimulating effect on cell division via the protein kinase C pathway. In addition, the X protein induces the transcription of the 2.1-kb mRNA by binding to the enhancer region of the viral genome, which is primarily responsible for the translation of the small form of HBsAg. As previously mentioned, the higher cancer rate in males can also be explained by the strong stimulation of HBsAg expression by binding of activated glucocorticoid receptors to the GRE sequences. The high concentrations of the protein in the form of non-infectious particles can intercept neutralizing antibodies, and thus lead to the establishment of a chronic progressive form, which is a prerequisite for the development of hepatocellular carcinoma. Moreover, the X protein inhibits the cellular tumour suppressor p53. This leads to inhibition of apoptosis and also to premature entry of the cell into the S phase of the cell cycle, in which the cellular genome is replicated (▶ Chap. 6). This and the concomitant inhibition of ultraviolet-damaged DNA-binding protein suggest that spontaneously arising DNA damage, or damage caused by mutagenic agents, is not correctly repaired, resulting in an increased mutation rate in the affected cells. Owing to infection-induced damage, liver cells are subjected to a high regeneration and division rate, which should increase the probability of occurrence of additional DNA damage and mutations.
3. Some cofactors also seem to contribute to carcinogenesis. These include aflatoxins. These are difuran coumarin derivatives which possess a high mutagenic potential. They are metabolic products of *Aspergillus flavus* which accumulate in spoiled food. If such products are consumed, aflatoxin is converted into an active product in the liver which binds to DNA. This leads to increased numbers of mutations, which can also affect the cellular tumour suppressor p53. Mutations of p53 that impair the tumour-suppressor function have been predominantly found in codon 249 in patients with frequent exposure to aflatoxins. In this way, aflatoxins exacerbate the inactivating effect of the X protein on p53. Chronic inflammatory processes associated with high alcohol consumption and liver cirrhosis are additional cofactors that contribute to tumorigenesis.
4. In addition, chromosomal deletions, DNA and gene amplifications and rearrangements of cellular DNA have been observed in liver tumour cells. In hepatitis B virus of woodchucks, which induces chronic hepatitis in these animals, an activation of the cellular proto-oncogene c-*myc* has also been reported. Many infected animals (16–30 %) develop a persistent infection, which ultimately always develops to hepatocellular carcinoma after a short time.

Duck Hepatitis B Virus: – An Important Model System

Another model system for molecular biological investigation of hepatitis B virus is infection of Chinese domestic ducks and American ducks with duck hepatitis B virus. This system is characterized by a vertical transmission from the mother duck to the eggs. The virus replicates in the yolk sac tissue of the developing embryo and is probably transmitted to hepatocytes from the sixth day of embryonic development. Experimental infections can be induced in 1-day-old ducklings by intravenous or intrahepatic injection of cloned viral DNA. Experimental infections can also be induced by *in ovo* injections, by which, however, infection is possible only during a very limited time window. The fact that primary hepatocytes which are held longer than 1 week in culture can no longer be infected additionally suggests that the degree of cell differentiation plays an important role in determining the permissiveness of the cells. Whether ducks develop acute liver diseases is still uncertain. Symptoms have hardly been found. The development of cirrhotic symptoms and hepatocellular carcinomas has been described only in a certain type of Chinese ducks. In this case, the DNA of duck hepatitis B virus is integrated in the cellular genome. The fact that avihepadnaviruses do not possess a gene coding for the X protein indicates that X-protein-independent processes must be responsible for tumour formation.

Immune Response and Diagnosis

Antibodies against epitopes of all forms of HBsAg are neutralizing, and largely block dissemination through the blood; they also protect against re-infection. In addition, the formation of antibodies is also directed against HBcAg and HBeAg proteins, which, like the amino-terminal domains of LHBsAg and MHBsAg, contain important T-cell epitopes, which in association with MHC class I proteins are presented on the surface of infected cells, triggering their destruction by cytotoxic T cells. Cytotoxic T cells against HBcAg can already be detected in large quantities in the incubation period and during acute infection, especially in liver tissue. Natural killer cells, which can lyse infected hepatocytes, have also been found in chronic infections.

The serological diagnosis of acute hepatitis B includes the determination of transaminase levels, the presence of HBsAg and HBeAg and the concentration of IgM antibodies against HBcAg. Healing is indicated by a continuous decrease of HBsAg and HBeAg levels in the blood – both antigens are finally no longer detectable – and the presence of IgG antibodies, first against HBeAg and later against HBsAg (Fig. 19.6). IgG antibodies against HBcAg remain detectable for life. The diagnosis of chronic forms results from the detection of HBsAg beyond a period of 6 months. The presence of HBsAg is, like the detection of viral DNA in serum by PCR, a measure of the activity of infection and the intensity of viral replication.

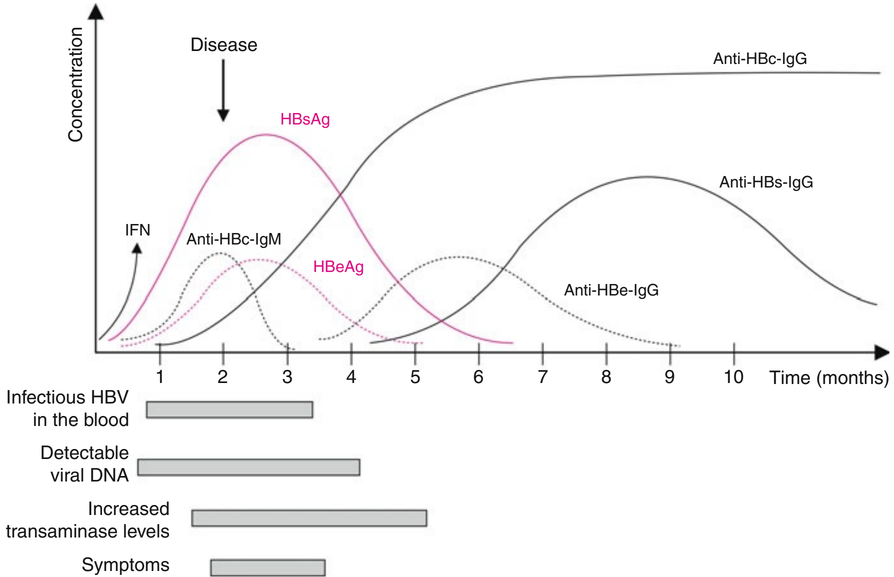


Fig. 19.6 Serological parameters of an acute hepatitis B virus infection. The *downward-pointing arrow* indicates the onset of the disease. At that time, HBsAg, HBeAg and IgM antibodies against HBcAg are present in the blood. IgG antibodies against HBcAg are produced soon after. IgG antibodies against HBeAg and against HBsAg can be detected several months after onset of the disease. *HBV* hepatitis virus B, *IFN* interferon

If HBeAg is detected in the blood, it is always an important indication of the presence of infectious hepatitis B virus in the blood. Furthermore, the content of hepatitis B virus DNA serves as an indicator of the risk of transmission. The prevalence in the population is determined by detecting antibodies against HBcAg. These antibodies are present for life in all people who have been infected with hepatitis B virus, i.e., in acutely or chronically infected people or in patients who have recovered from hepatitis B (Fig. 19.6). Immunoglobulins against HBsAg are produced relatively late and their concentration can decrease during infection. There are no cell lines that allow cultivation of hepatitis B viruses in vitro. Although human hepatocellular carcinoma cell cultures which carry the hepatitis B virus DNA integrated into the cellular genome and continuously produce HBsAg (e.g. HepG2 cells) have been established, these cells do not produce infectious viruses. However, this in vitro system at least allows the influence of potentially effective therapeutic agents on HBsAg production to be tested. For example, IFN-α and IFN-β inhibit the proliferation of hepatitis B virus and induce a state of resistance in HepG2 cells, a result that has been confirmed in vivo.

Therapy and Prophylaxis

Patients with clinically manifested chronic hepatitis B are treated with INF-α. The PEGylated form of INF-α has a particularly good effect: it has a long-lasting depot

effect of roughly 7 days, and also reduces the replication of the viral genome in vitro. It significantly reduces the amount of hepatitis B virus in the blood in more than 30 % of cases, and at the same time, antibodies against HBeAg become detectable. However, INF-α does not usually achieve the complete elimination of the pathogen. Some genotypes, especially genotype C viruses, are largely resistant to INF-α therapy. Besides lamivudine (3TC, 2′,3′-dideoxy-3′-thiacytidine; ▶ Chap. 9), a very good inhibitor of the pyrophosphorolysis activity of viral reverse transcriptase, the nucleoside analogues entencavir, telbivudine and adefovir have been approved as antiviral drugs. Although the application of these virostatic agents leads to a reduction of the viral load, they do not completely eliminate the pathogen. Instead, resistant variants emerge rapidly. For better control of infection during therapy, a quantitative PCR is performed to determine the amount of viral DNA in plasma. The occurrence of resistance to lamivudine and other inhibitors of reverse transcriptase can be determined by subsequent sequencing; e.g. the appearance of the YMDD motif in the P protein indicates resistance to lamivudine. After cessation of therapy, reactivation can be observed, which leads to a rise in viral load (relapse). Therefore, the aim of long-term antiviral therapy is primarily a significant reduction of the viral load as well as normalization of both transaminase levels and structure of the liver tissue.

Prophylactic vaccinations are performed with recombinant SHBsAg, which is produced by genetic engineering methods in yeast (*Saccharomyces cerivisiae*). General vaccination of all infants and non-vaccinated adolescents is recommended in most countries. The success of vaccination is checked by antibody detection only in the case of medical personnel. A very small proportion of vaccinees show an insufficient immune response (so-called non-responders) because of genetic reasons. In such cases, an immune response against the vaccine can be partially achieved by administration of interleukin-2. However, vaccinated individuals exhibiting no measurable antibodies against HBsAg also seem to be protected from the disease, as a T-cell response can frequently be observed in these people. Newborns of chronically infected mothers immediately receive both active and passive immunization. The same applies in the case of suspected hepatitis B virus transmission by injuries and contaminated blood if no immune protection exists. The transmission through blood transfusions and blood products can be forestalled by diagnostic examination of donors. Indeed, the incidence of transfusion-associated hepatitis B has significantly diminished since the introduction of this measure. However, the methods are still not sensitive enough to exclude the possibility of transmission absolutely (the risk is 1:250,000).

19.1.5.2 Hepatitis D Virus

Mario Rizzetto discovered hepatitis D virus in patients with chronic hepatitis B in 1977. It was originally regarded as a new antigen of hepatitis B virus, and it was termed delta antigen. Only later it turned out that it is a pathogen with a single-stranded RNA genome encoding the delta antigen. The genus *Deltavirus* was created for this virus, but it has not yet been assigned to a virus family. The phytopathogenic virusoids are similar to hepatitis D virus in terms of genome organization and

replication. Hepatitis D virus infection occurs only in association with an acute hepatitis B virus infection, or in patients who have already developed chronic hepatitis B. Thus, hepatitis D virus is infectious only in cooperation with hepatitis B virus, and requires the HBsAg proteins of hepatitis B virus for the generation of infectious progeny virus. Hepatitis B virus provides the surface structures that enable hepatitis D virus to enter cells, and thus acts as a helper virus; however, an active replication of hepatitis B virus is not necessary. Because of this relationship, we discuss the biology of hepatitis D virus along with that of hepatitis B virus.

Structure and Viral Proteins

Hepatitis D virus particles are spherical and have a diameter of 34–36 nm. They are made up of the surface proteins of hepatitis B virus (SHBsAg, MHBsAg, LHBsAg), which are embedded in an envelope. This encloses the single-stranded, circular RNA genome of hepatitis D virus, which is associated with the hepatitis delta antigen (HDAg). Two variants of HDAg are translated from a single open reading frame, a large version (LHDAg, 27 kDa) and a small version (SHDAg, 24 kDa), which are the only gene products encoded by hepatitis D virus, and are present in approximately equimolar quantities in viral particles.

SHDAg and LHDAg are phosphorylated. In infected cells, these proteins are located exclusively in the nucleus. They seem to be cytotoxic, and are released into the environment during death of infected cells. Both proteins are found in approximately equal amounts in the blood of infected patients.

SHDAg and LHDAg have nearly the same sequence, but they differ by only 19 additional amino acids at the carboxy terminus of LHDAg. The carboxy-terminal amino acids cysteine, arginine, proline and glutamine constitute a consensus sequence for the modification of LHDAg with farnesyl acid or prenyl acid and are essential for the morphogenesis of hepatitis D viruses. Presumably, LHDAg molecules attach to the membrane of Golgi vesicles by their fatty acid modified protein domain, where they interact with the cytoplasmic amino acid sequences of HBsAg proteins of hepatitis B virus, which are also anchored in the ER membrane. The amino-terminal region in SHDAg and LHDAg contains a coiled-coil oligomerization domain (amino acids 12–60), which mediates the conversion of HDAg proteins into dimers and higher-order complexes. This is followed by a nuclear localization signal (amino acids 68–88) and an arginine-rich RNA-binding domain, which is divided into two sections (residues 97–107 and 136–146). SHDAg is required for genome replication, whereas LHDAg inhibits this process when present in a large excess compared with SHDAg.

Genome and Replication

The genome is a circular, single-stranded, negative-sense RNA and consists of 1,672–1,683 nucleotides depending on the isolate. Approximately 70 % of the RNA sequence forms intramolecular base-pairing. This confers high stability on the genome, which exhibits a rod-shaped appearance in the electron microscope. The genome sequence is highly variable; there are eight genotypes, whose sequences differ by up to 40 %.

The surface components of hepatitis D virus are identical to those of hepatitis B virus; therefore, hepatitis D virus uses the same mechanism of infection as hepatitis B virus. Finally, the RNA genome complexed with SHDAg and LHDAg proteins enters the nucleus, where the cellular RNA polymerase II transcribes the RNA genome into an 800-nucleotide-long mRNA that encodes the SHDAg protein (Fig. 19.7). The activity of SHDAg, along with the RNA polymerase II, is necessary to transcribe the viral RNA genome into a complementary antigenome. SHDAg binds directly to RNA polymerase II, thus impeding the interaction of the 66-kDa subunit of negative elongation factor (NELF) with the enzyme. This process is probably based on sequence homology between SHDAg and NELF. NELF usually regulates, along with other cellular factors, the activity of RNA polymerase II by reducing elongation. The genome of hepatitis D virus replicates by a process that resembles the rolling-circle mechanism, as found in the lytic replication cycle of herpesvirus DNA (Sect. 19.5.6). This replication mechanism produces concatemers comprising multiple copies of antigenomes, which are subsequently cleaved into individual units. Autocatalytic cleavage is responsible for this process. Both the antigenome and the RNA genome have sequences that act as endonucleases; these sequence-specifically cleave the genomic RNA and the antigenome between nucleotides 685 and 686 and nucleotides 901 and 902, respectively. Subsequently, the cleaved RNA molecules are religated (Fig. 19.7). These endonuclease and ligase activities are exerted by about 85 nucleotides that surround the cleavage sites. In this, the genome of hepatitis D virus is similar to the plant pathogenic viroids that have a similar ribozyme activity, which is known as the hammerhead ribozyme. However, the catalytic domain in the RNA sequence of hepatitis D virus is dissimilar to the ribozymes of plant viroids, but resembles that of the cytoplasmic polyadenylation element binding protein 3 (CPEB3) ribozyme, a conserved mammalian base sequence in an intron of the CPEB3 gene.

In a subset of antigenomes, the adenosine residue in the stop codon (UAG) that terminates the translation of SHDAg is deaminated to inosine by the cellular adenosine deaminases ADAR1 and ADAR2 (adenosine deaminases that act on RNA). Both the edited and the unedited antigenomes are transcribed into genomic RNA strands by RNA polymerase II. Similarly as described above, concatemers are also formed here which contain multiple genome copies and are autocatalytically cleaved into monomers by the ribozyme activity of the RNA molecule. From the RNA genome strands which have been transcribed from the edited antigenomes, mRNAs are produced in which the original stop codon UAG has been converted to UGG that encodes the amino acid tryptophan. In this way, SHDAg is extended by 19 amino acids, creating LHDAg, which is required for packaging of RNA genomes and morphogenesis of virus particles. Some other open reading frames that are not conserved in different isolates of hepatitis D virus are found in the genomes and in the antigenomes, but they are not expressed.

Both SHDAg and LHDAg interact with the RNA genome. The large form of the protein binds to the membrane of the Golgi vesicles by its fatty acid modified

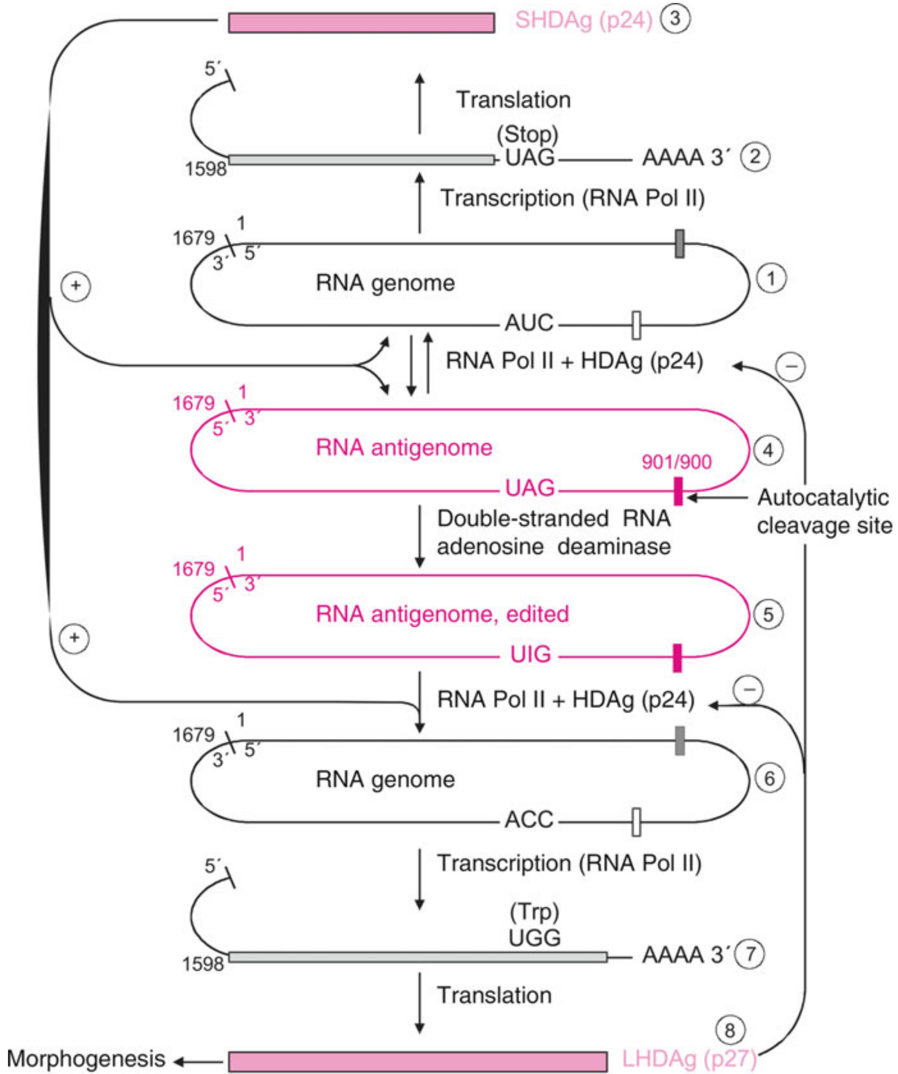


Fig. 19.7 Structure and replication of the hepatitis D virus genome. The genome of hepatitis D virus consists of a single-stranded RNA (*1*), which forms a high percentage of intramolecular base pairings (nucleotide numbering starts at the unique *Hin*dIII cleavage site, which is formed when the RNA is converted into double-stranded DNA). An mRNA (*2*) is transcribed from the genomic RNA (*1*), from which SHDAg (p24) is translated (*3*). It interacts with the RNA polymerase II, causing the formation of complementary antigenomes (*4*), which in turn serve as templates for the synthesis of genomes. In an editing step, the adenosine in the UAG stop codon is deaminated to inosine (*5*) in a subset of the antigenomes. The edited antigenomes are also converted into genomic RNA molecules (*6*), from which an mRNA (*7*) is transcribed which encodes LHDAg (27 kDa) (*8*). This impedes genome replication and is important for the morphogenesis of hepatitis D virus. *Pol* polymerase

carboxy-terminal domain. The various forms of HBsAg molecules are also embedded in Golgi vesicles. The RNA-SHDAg-LHDAg complexes are surrounded with the HBsAg-containing membrane; the resulting particles bud into the lumen of the Golgi vesicles, which transport them to the cell surface, where they are secreted.

Epidemiology, Clinical Features and Pathogenesis

Hepatitis D virus is found worldwide; there are geographically delimited regions where infections with this virus occur more frequently. These include southern Italy, Spain, Turkey, Taiwan and Samoa. To date, eight genotypes (I–XIII) have been found, and their RNA sequences differ by up to 40 %. The transmission paths of hepatitis D virus are identical with those of hepatitis B virus. The incubation period is between 3 and 7 weeks. If an acute hepatitis B infection is accompanied by a hepatitis D infection, the disease manifests itself with a wide spectrum of severity levels, ranging from a relatively mild illness to the symptoms of fulminant hepatitis. There is evidence that the various genotypes of hepatitis D virus differ in their virulence, whereas the genotype of hepatitis B virus, which serves as a helper virus, does not affect the severity of the disease. Acute co-infection of genotype I of hepatitis D virus is characterized by increased persistence and mortality rates in comparison with the singular hepatitis B infection. By contrast, co-infection with genotypes II and IV of hepatitis D virus seem to have a milder form, as persistence of the pathogens is not established, and they are completely eliminated. The different disease forms may possibly be attributed to specific nucleic acid sequences in the RNA genome of genotype I which are bound and very efficiently packaged by HBsAg molecules.

If patients with chronic hepatitis B are infected with hepatitis D virus, chronic hepatitis D is usually established. Approximately 60–70 % of such individuals develop liver cirrhosis. Since acute infections with hepatitis D virus only rarely occur in European countries owing to vaccination against hepatitis B virus infection, the associated symptoms of a fulminant hepatitis are observed very seldom. Instead, cases of liver cirrhosis are more apparent in patients with chronic and persisting infections who were infected 20–30 years ago.

It is unclear whether the cells are damaged by the hepatitis D virus infection or by immunopathological mechanisms. The latter is supported by the finding that the cellular alterations in the liver usually appear only when viral replication has already subsided, and antibodies against HDAg become detectable. However, treatment of patients with immunosuppressive drugs has no effect on disease progression. The infected liver cells show increased vacuolization. These cells exhibit a delayed growth rate in vitro or die if HDAg is synthesized. Apoptotic processes do not seem to be responsible for that. HDAg is able to interact with cellular RNAs and proteins. An interaction of the large subunit of NELF as part of the RNA polymerase II and the nucleolar phosphoprotein B23 which influences their function has been demonstrated experimentally.

Another hypothesis proposes that the viral RNA has similarities to cellular 7S RNA, which is a component of the signal recognition particle. This might impair the synthesis of cell membrane proteins. On the other hand, there are cell lines which harbour a hepatitis D virus genome integrated in the cellular genome and continuously synthesize viral RNA and HDAg without apparent changes.

Immune Response and Diagnosis

The RNA genomes of hepatitis D virus can be detected in the blood of patients by reverse transcription PCR. During infection, antibodies against HDAg can be detected in the blood of patients by ELISA. However, they are not virus-neutralizing, and do not protect against reinfection, as neither SHDAg nor LHDAg is exposed on the surface of virus particles. HDAg-specific cytotoxic T lymphocytes and T-helper cells can be found in the liver during infection.

Therapy and Prophylaxis

An antiviral therapy is currently not available. IFN-α is used to reduce the amount of hepatitis B virus, but whether this treatment is statistically successful even for hepatitis D has not been conclusively demonstrated because of the relatively small therapy and comparison groups. Since hepatitis D virus requires hepatitis B virus as a helper virus, the hepatitis B vaccine also protects against infection with hepatitis D virus. The significant decrease of hepatitis D virus infections, which has been observed especially in Europe since the 1990s, can presumably be ascribed to the systematic application of the hepatitis B vaccine.

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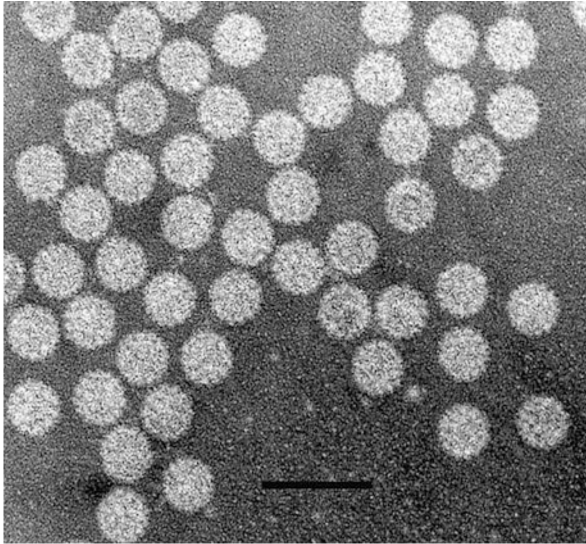
19.2 Polyomaviruses



The family *Polyomaviridae* contains viruses with small capsids, which are not enveloped, and contain a circular double-stranded DNA genome. Polyomaviruses were originally classified along with papillomaviruses, which are also characterized by these molecular features, into the former family *Papovaviridae* by Joseph Melnick in 1964. However, since significant differences in genome structure, replication and the molecular biology of polyomaviruses and papillomaviruses have been found in recent years, these viruses are now classified into two separate virus families.

19.2.1 Classification and Characteristic Prototypes

The first prototype of this virus family, murine polyomavirus, was discovered and described as tumorigenic by Ludwik Grosz in 1953. He found that cell extracts of leukaemic mice caused various types of cancer (leukaemias and parotid tumours) after transfer into healthy animals. Murine polyomavirus was isolated by Sarah Stewart and Bernice Eddy (1957–1958) after inoculation of mouse fibroblast cultures in which they observed a cytopathic effect. They ascertained 24 different tumour types after transmission of the virus into newborn mice and hamsters. The word "polyoma" relates to the property of being able to produce tumours in many different organs. In addition to SV40, which was isolated from rhesus monkeys by Benjamin Sweet and Maurice Hilleman in 1960, the human pathogenic BK and JC polyomaviruses also belong to the SV40-like viruses, which are widespread and persist after infection. Diseases associated with these viruses are found almost

Table 19.3 Characteristic prototypes of polyomaviruses

Genus	Human virus	Animal virus
Polyomavirus	BK polyomavirus[a] JC polyomavirus[a] KI polyomavirus WU polyomavirus	Simian virus 40 Bovine polyomavirus Budgerigar fledgling disease polyomavirus
	Merkel cell polyomavirus	B-lymphotropic polyomavirus Murine polyomavirus Hamster polyomavirus

KI Karolinska Institute, *WU* Washington University School of Medicine
[a]These viral species were named using abbreviations of the names of the patients from which they were originally isolated

exclusively in immunosuppressed patients. In 2007, the groups of Tobias Allander at the Karolinska Institute (Stockholm, Sweden) and David Wang at the Washington University School of Medicine (St. Louis, USA) isolated two additional SV40-like polyomaviruses from children with respiratory infections: the KI and WU polyomaviruses. Both viruses seem to be widespread and have been detected in both sick and healthy children. Whether infections with KI and WU polyomaviruses are associated with diseases in humans is still unclear. In 2008, Huichen Feng and co-workers described another human pathogenic polyomavirus, Merkel cell polyomavirus. Merkel cell polyomavirus DNA is integrated into the genome of tumour cells of a rare skin cancer, neuroendocrine Merkel cell carcinoma. Today, all known polyomaviruses are classified into just one genus (Table 19.3). There are several proposals to change the taxonomy, and it has been proposed to classify the viruses into three genera, i.e., *Orthopolyomavirus*, *Wukipolyomavirus* and *Avipolyomavirus*.

19.2.2 Structure

19.2.2.1 Virus Particle

The infectious virus particles are capsids without an envelope, and have an icosahedral structure with a diameter of 45 nm (Fig. 19.8). They are constituted of three structural proteins: VP1, VP2 and VP3. VP1 is the main component. Each particle contains 72 capsomers consisting of pentameric VP1 complexes. VP2 and VP3 are present only in small amounts in the capsids and are required for the orderly assembly of the various components of infectious virions. Furthermore, small amounts of the agnoprotein LP1 have been found in the particles of budgerigar fledgling disease polyomavirus. The particles of BK and JC polyomaviruses have haemagglutination ability owing to their interaction with neuraminic acid residues on the cell surface. SV40 does not possess this property. The viral genome is contained within the icosahedral capsid.

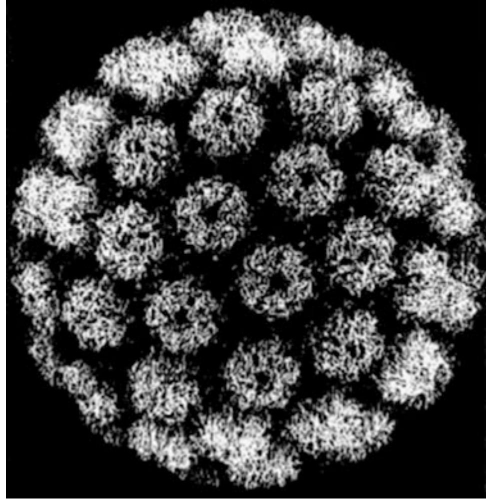


Fig. 19.8 The structure of a simian virus 40 (SV40) particle as the prototype of polyomaviruses. The viral capsid consists of 72 pentamers of VP1. Twelve of these constitute the vertices of the icosahedron and are associated with five additional pentamers. The circular, double-stranded DNA genome and small amounts of proteins VP2 and VP3 reside inside the particle (From Cole 1996)

19.2.3 Genome Organization and Structure

Polyomaviruses have a covalently closed, and thus circular, double-stranded DNA genome with a length of approximately 4,700–5,400 base pairs (bovine polyomavirus 4,697 base pairs, KI polyomavirus 5,040 base pairs, BK polyomavirus 5,130 base pairs, JC polyomavirus 5,153 base pairs, SV40 5,243 base pairs, WU polyomavirus 5,229 base pairs, Merkel cell polyomavirus 5,387 base pairs; Fig. 19.9). The genome is a supercoiled DNA molecule, which is associated with the four cellular histones (H2A, H2B, H3, H4) into nucleosome structures. Twenty-four to 26 nucleosomes per genome are found. The DNA sequences of BK and JC polyomaviruses have a reciprocal homology of about 75 %, and exhibit a homology of nearly 70 % to the SV40 genome.

In terms of gene expression, the circular, double-stranded DNA genome can be divided into two regions (Fig. 19.9a). The early region encodes proteins which are referred to as T antigens (tumour antigens). In SV40-like viruses, early RNA synthesis starts from a promoter, leading to the synthesis of a precursor transcript, from which three mRNAs are formed by using alternative splice sites. These mRNAs are used for the synthesis of the early leader protein as well as the large and the small T antigens. Polyomaviruses synthesize even three RNA species, from which the three different T antigens are translated (small, middle, and large T antigens).

The late region of the genome encodes the structural proteins VP1, VP2 and VP3. Furthermore, the genetic information for the small LP1 protein (agnoprotein) is located in the leader sequence at the 5′ terminus of the late mRNA species of SV40-like viruses. Even in this case, the reading frames overlap with each other and the different proteins are translated from the

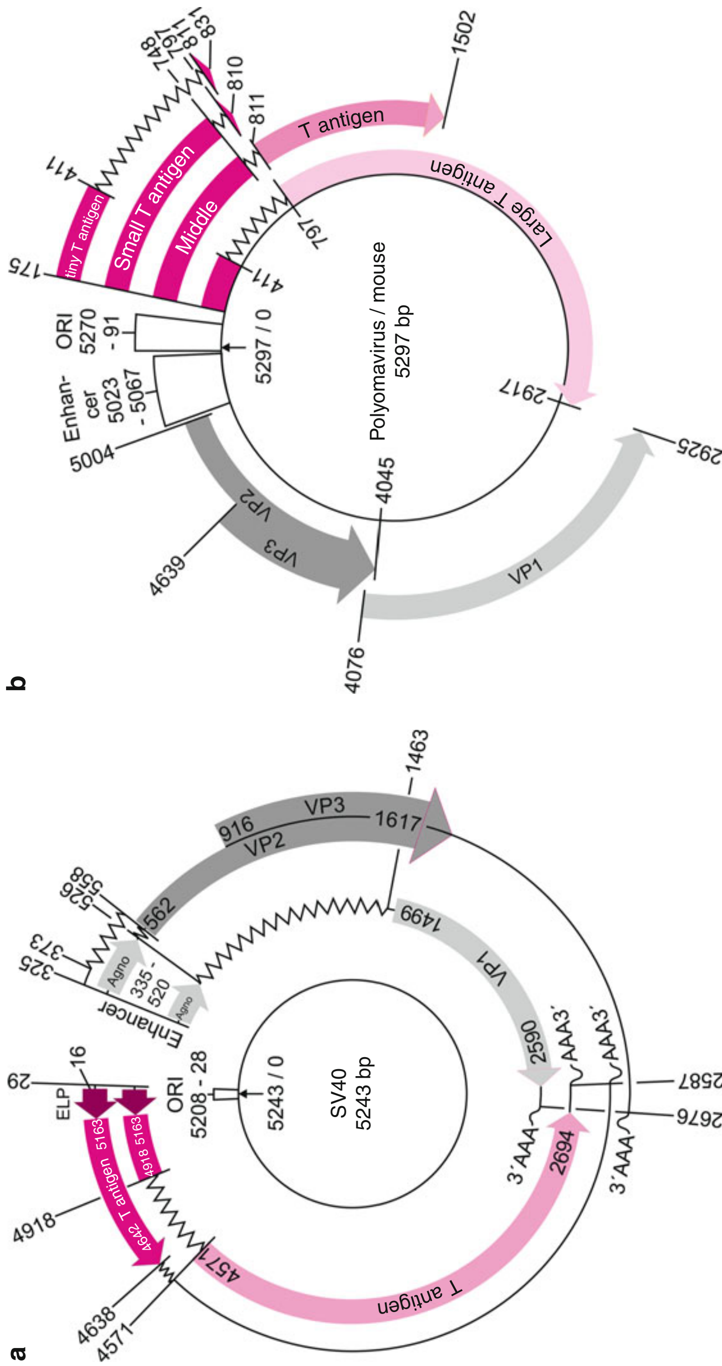


Fig. 19.9 Genome organization of polyomaviruses. (**a**) SV40. (**b**) Murine polyomavirus. The origin of replication sequences and the regulatory elements of the non-coding region are depicted in the upper parts of (**a**) and (**b**). The early genes of SV40 are transcribed anticlockwise, whereas the converse applies to murine polyomavirus. The reading frames coding for proteins are *shaded*. Early proteins are depicted in *red*/*bright red* (early leader protein, *ELP*, large T antigen, middle T antigen, small T antigen and "tiny" T antigen). The late structural proteins VP1, VP2, VP3, and the agnoprotein are depicted in *grey*. The positions of introns and exons are also indicated. *ORI* origin of replication

mRNAs which arise by alternative splicing, whereby additionally different start sites are used for transcription. The early and late regions of the genome are transcribed in opposite directions. Accordingly, different DNA strands are used as a template for transcription in the early and the late phase of infection. A short non-coding region of up to 400 base pairs is located between the start sites of transcription of the early and late regions of polyomaviruses (Fig. 19.9a). It contains regulatory sequences, including the origin of replication, the promoters, a GRE and an enhancer region. It is bound by the viral T antigen and several cellular transcription factors for regulating and enhancing viral transcription (Fig. 19.10). In SV40-like viruses, the reading frame encoding the early leader protein is located in this region.

SV40 Has Proven to Be an Important Model System for the Molecular Biology of Eukaryotic Cells

Supercoiled DNA with nucleosome structures was found for the first time by analysing the genome of polyomaviruses, namely SV40. Later, it was found that even the cellular DNA is associated with histone proteins and arranged into nucleosomes. The nucleosome structure of SV40 DNA – also known as "minichromosome" – served originally as a model system for analysing comparable structures of the cellular genome, and the study of DNA replication in eukaryotic cells. Even the existence of alternative RNA splicing was first found in SV40, namely by examining the transcription of the early region encoding the T antigen. Hereby, different and overlapping reading frames are used for the synthesis of various proteins. In particular, the coding capacity of small viral genomes can be augmented very effectively. Later, similar mechanisms were also found in eukaryotic systems. Furthermore, the SV40 enhancer was discovered as the first *cis*-acting regulatory DNA element which increases transcription of specific promoters in an orientation-independent manner.

19.2.4 Viral Proteins

19.2.4.1 Early Proteins

The early proteins of polyomaviruses comprise the various T antigens, which according to their molecular masses are termed large, middle (not in SV40-like viruses) and small T antigens. Another form has been found in murine polyomavirus, the tiny T antigen. Its function is unknown. An overview of the molecular characteristics of the proteins is given in Table 19.4. Presumably, the proteins of the human pathogenic BK and JC polyomaviruses play a similar role in replication as those of SV40, which is considered a well-studied model virus. Therefore, the discussion of the molecular biology will be focused on the known data for SV40, unless otherwise mentioned.

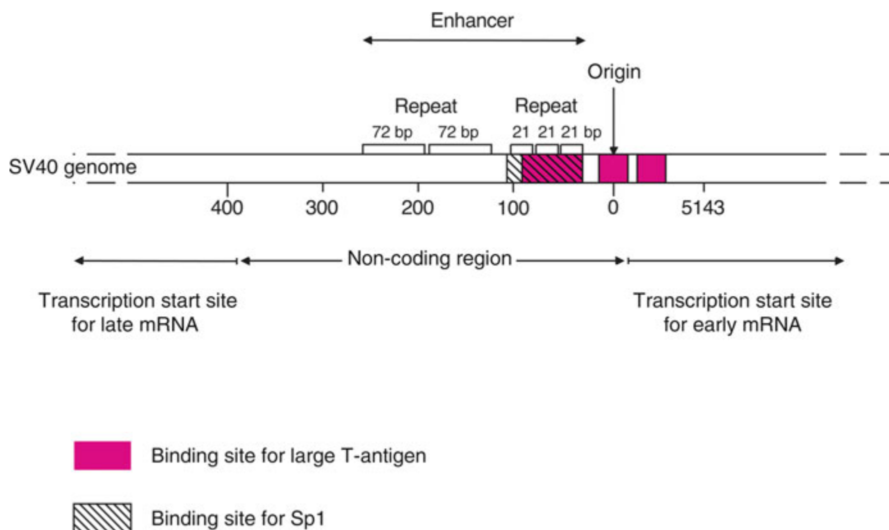


Fig. 19.10 Arrangement of sequence elements in the regulatory region of SV40. The origin of DNA replication is marked by the *arrow* arbitrarily; it represents the start of nucleotide numbering towards the genes encoding the late proteins (structural proteins). The start site for early mRNA synthesis is located in near the origin of replication. Transcription of late genes occurs in the opposite direction. A non-coding region is located between the transcription initiation sites. It contains the enhancer, which is composed of two repeats of 72 base pairs and three repeats of 21 nucleotides. The binding site for the large T antigen is located in this region, which overlaps with the binding site for the cellular transactivator Sp1

Large T Antigen

The large T antigen of SV40 is one of the best characterized viral proteins. It is multifunctional and has a length of 708 amino acids and a molecular mass of 90 kDa. Most of its activities are executed in the nucleus (Fig. 19.11). Inasmuch as the large T antigen is synthesized in the cytoplasm of the cell, it must be transported into the nucleus to exert its functions. This process is mediated by a nuclear localization signal, which consists of a sequence of mainly basic amino acids (amino acids 126–132 in SV40). In contrast, a small percentage of the synthesized large T antigen (about 5 %) is in the cytoplasm or is closely associated with the cell membrane. Anchoring occurs via the amino-terminal fatty acid modification of the membrane-associated T antigen. Other subsets of this protein are phosphorylated in various degrees at several serine and threonine residues. Further modifications are poly-ADP-ribosylations, glycosylation and acylations. The various chemical modifications influence the activity of this protein. In this way, these relatively small viruses with very low coding capacity are able to produce protein variants with different function by various modifications, in addition to the use of alternative splicing.

Some of these functions are of crucial importance for viral replication. The large T antigen exerts the following activities:

1. It binds to the viral DNA at the origin of replication.

Table 19.4 Comparative overview of the functional properties of proteins of different polyomaviruses

	Length and molecular mass				
Protein	**SV 40**	**BK polyomvirus**	**JC polyomvirus**	**Murine polyomavirus**	**Modification, function**
Early proteins					
Large T antigen	708 aa, 90 kDa	695 aa, 90 kDa	688 aa, 88 kDa	785 aa, 100 kDa	Phosphorylated, N-myristoylated, O-glycosylated, adenylated, poly-ADP-ribosylated, palmitoylated; regulation of transcription; initiation of replication; transformation
Small T antigen	174 aa, 20 kDa	172 aa, 20 kDa	172 aa, 20 kDa	195 aa, 22 kDa	Accumulation of viral genomes
Middle T antigen	–	–	–	421 aa, 35 kDa	Interaction with pp60src
Tiny T antigen	–	–	–	78 aa	Unknown
Early leader protein	2.7 kDa	4.3 kDa	4.3 kDa	–	Early leader protein
Late proteins					
VP1	362 aa, 45 kDa	362 aa	354 aa	385 aa	Main capsid protein, pentamer
VP2	352 aa, 38 kDa	351 aa	344 aa	319 aa	Capsid component
VP3	234 aa, 27 kDa	232 aa	225 aa	204 aa	Capsid component
LP1/ agnoprotein	62 aa	66 a	71 aa	–	Late protein; shuttle protein; phosphorylated by protein kinase A; present in low amounts in virions

The molecular masses of early proteins are average values. Because of numerous and different modifications of early proteins, the values differ considerably in the different proteins subfractions.
aa amino acids

2. It has ATPase and helicase activity and separates the DNA strands at the origin of replication by breaking the hydrogen bonds between complementary nucleotides.
3. It is involved in the regulation of transcription of early genes, as it represses the transcription of early genes by binding to the promoter region, thus regulating its own synthesis.
4. It has a transactivating effect on the promoter of late mRNA.

In addition to these activities which are important for the virus, the large T antigen also influences cellular functions. It stimulates the cellular DNA and

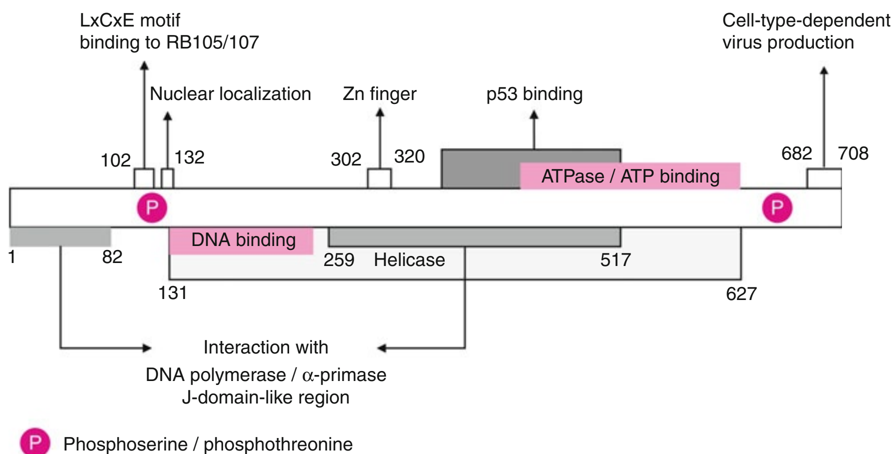


Fig. 19.11 Location of the functionally active domains of the large T antigen of SV40. The *numbers* refer to the amino acid positions, starting from the amino terminus

ribosomal RNA synthesis. These processes are mediated by different protein domains. Furthermore, the T antigen acts as a chaperone. The amino-terminal domain has homology to the J domain of the DnaJ chaperone family of *Escherichia coli*, and can replace it functionally. DnaJ chaperones bind by means of the J domain to members of the DnaK family. In eukaryotic cells, the Hsp70 chaperones are functionally similar to the DnaK folding catalysts of bacteria. Hsc70 protein is a member of the Hsp70 chaperones. The large T antigen interacts with it by the J domain, inducing it to hydrolyse ATP. Thereby Hsc70 changes its conformation – a process that prevents and reverses the incorrect aggregation and folding of proteins. Whether the interaction of the T antigen with Hsc70 is responsible for some of the many functions of the T antigen has not yet been conclusively elucidated. The J domain of the T antigen is not responsible for replication of the viral genome, but is responsible for many of the activities of this multifunctional protein, e.g. for its capability of transforming cells and stimulating cell division, inhibiting apoptosis or releasing E2F transcription factors from their complexes with RB105/RB107. Moreover, it binds to the cell-cycle-regulating proteins cyclin, tubulin and cyclin-dependent kinase 1.

However, the ability the large T antigen of SV40 and murine polyomavirus to induce cell immortalization has rendered it famous. It enables the viruses to induce unrestrained growth in primary cells in tissue culture and carcinogenesis in hosts which under natural conditions are not infected by the virus. SV40 causes tumorigenesis in newborn hamsters and mice. The large T antigen exerts its effect by binding to cellular tumour-suppressor proteins. Examples are the cellular tumour-suppressor proteins p53 and RB105/RB107, which are involved in regulation of the cell cycle and cell division. The J domain of the T antigen is responsible for the interaction with RB105. The RB105 proteins are present in a complex with the

transcription factor E2F. If RB105 is phosphorylated by cyclin-dependent kinases in the G_1 phase of the cell cycle, this causes the release of the E2F components, which subsequently enter the cell nucleus and influence the transcription of specific cellular genes (▶ Chap. 6). The interaction of the T antigen with a pocket-like domain in the centre of RB105 leads to the complex with E2F being released independently of cell-cycle-dependent phosphorylation. Thereby the T antigen induces the transition from the G_1 phase to the S phase (Sect. 19.4.3). The strong ability of T antigens to immortalize cells has not yet been found in human BK polyomavirus and JC polyomavirus.

Small T Antigen

The small T antigen of SV40 has a molecular mass of approximately 20 kDa and is 174 amino acids long. The mRNA synthesis of the small and the large T antigens is regulated by the same promoter, their 5′ ends are collinear and the first 82 amino acids of both SV40 proteins are identical. A part of the early mRNA is spliced, and the translation of the second exon is continued in another reading frame. The result is the large T antigen. The sequences of the small T antigens end at a stop codon which is located in the intron and is removed by splicing from the mRNA of the large T antigen (Fig. 19.9a). The small T antigen is equally localized in the cytoplasm and in the nucleus. Its role in the viral replication cycle is not clearly understood. Since it is identical to the T antigen in the amino-terminal region, it contains a part of the DnaJ domain and possesses the corresponding functions. The small T antigen specifically binds to several cellular proteins and seems to be important for the accumulation of viral DNA in the infected cell.

Middle T Antigen

The middle T antigen is produced only in murine polyomavirus and related virus types (hamster polyomavirus). The synthesis of the middle T antigen is controlled by the same promoter as for the large T antigen. Therefore, the first 78 and 195 amino acids of the middle T antigen (421 amino acids long) are identical to the sequences of the large and small T antigens, respectively. By use of splice donor and acceptor sites different from those that lead to the synthesis of the large T antigen, the protein synthesis occurs in a different reading frame (Fig. 19.9b). Most of the middle T antigen molecules are associated with the cell membrane. Small amounts are located in the perinuclear space and in the cytoplasm. Apparently, the middle T antigen of murine polyomavirus induces cell transformation together with the large T antigen. It interacts with the cellular proto-oncogene pp60src. However, the exact mechanism of action of the middle T antigen and its contribution to transformation are still largely unknown.

Tiny T Antigen

Another small version of the T antigen was found in polyomaviruses. It is the tiny T antigen, which is identical with the 78 amino-terminal residues of the other three T antigens. Its function is unknown.

19.2.4.2 Late Proteins

Structural Proteins

The main component of viral capsids is VP1, which is synthesized in the late phase of the replication cycle. This protein also mediates attachment of viruses to defined receptors on the cell surface. Neutralizing antibodies are directed against epitopes of VP1. In the pentameric VP1 complexes of the capsomers, the amino terminus of the protein is supported inside the capsid, whereas the carboxy-terminal "arm-like" domains interact with neighbouring capsomers via non-covalent bonds, thus interlinking the individual aggregates into a network on the particle surface (Fig. 19.8), as ascertained by Robert C. Liddington and colleagues by X-ray structural analysis of SV40 particles. SV40 VP1 contains 362 amino acids, and is encoded in the 3′ half of the mRNA precursor (Fig. 19.9a).

VP2 and VP3 are translated from a bicistronic mRNA, whereby the 234 amino acids of the SV40 VP3 correspond to the carboxy-terminal region of the VP2 protein. VP2 uses a different translation start, so it has 118 additional amino acids at the amino terminus (352 amino acids in SV40) (Fig. 19.9a). In JC polyomavirus and murine polyomavirus, the VP2 proteins start 118 and 115 residues before the start site of VP3, and thus are 341 and 319 amino acids long, respectively (Fig. 19.9b). Since the assembly of the various components into particles is performed in the nucleus, all viral structural proteins have nuclear localization signals.

Non-Structural Proteins

The late-produced agnoprotein (LP1 protein) is synthesized by SV40-like viruses. It is encoded in the leader sequence at the 5′ terminus of the late mRNA species and is a small, basic polypeptide (approximately 8 kDa), and has a length of 62, 66 and 71 amino acids in SV40, BK polyomavirus and JC polyomavirus, respectively. Whereas the amino-terminal domains have a high degree of homologous amino acids, the carboxy-terminal region (amino acid residues 50–71) is significantly different. Agnoprotein-deficient mutants of SV40 are infectious, but multiply substantially more slowly than wild-type viruses. After its synthesis, the agnoprotein is phosphorylated probably by protein kinase C, and is present in this form in the cytoplasm. By contrast, non-phosphorylated molecules are located in the nucleus. It is thus assumed that the agnoprotein is a shuttle protein, which, depending on its phosphorylation status, is transported from the cytoplasm into the periplasmic space of the cell nucleus and back. It interacts with the capsid protein VP1 in the periplasmic space, and it probably plays an important role in morphogenesis. It is suspected that different agnoprotein versions are formed by alternative splicing in budgerigar fledgling disease polyomavirus, one of which, agnoprotein 1a, is present in small amounts as a fourth structural protein in the capsids.

19.2.5 Replication

Polyomaviruses bind to cellular receptors through their VP1 proteins. For this purpose, SV40 uses the membrane-anchored MHC class I antigens.

Other polyomaviruses bind to terminal *N*-acetylneuraminic acid residues, which are present as a modification of cell surface proteins. In addition, there is also some evidence that these viruses are able to interact with lipid components of the cytoplasmic membrane containing sialic acid like gangliosides. JC polyomavirus preferably interacts with the serotonin receptor $5HT_{2A}$, binding to terminal sialic acid residues, which are linked to the adjacent carbohydrates by α-(2,6) glycosidic bonds. Murine polyomavirus binds to α-(2,3)-linked sialic acid of integrin $\alpha_4\beta1$. Also, BK polyomavirus binds preferentially to sialic acid residues linked by α-(2,3) glycosidic bonds. The protein domain to which the carbohydrate groups are attached has not been definitively identified. A similar use of *N*-acetylneuraminic acid residues as receptors is also known in paramyxoviruses and orthomyxoviruses (▶ Sects. 15.3 and ▶ 16.3).

The particles enter the cell by receptor-mediated endocytosis, are then present in clathrin-rich vesicles and, bypassing the lysosomal pathway, are probably transported along the microtubules to the nucleus, where the viral genome is released. This is followed by the early transcription of the mRNA species which encode the various T antigens (Fig. 19.9). The mRNA is synthesized by the cellular RNA polymerase II, as demonstrated by the inhibition of transcription through α-amanitin. Viral proteins are not necessary for early transcription. The mRNAs encoding the different T antigens are formed by alternative splicing from a common precursor RNA molecule, whereby the 5′ end of SV40-specific RNA is located 70 nucleotides upstream of the initiation codon (nucleotide 20 in polyomavirus). If the large T antigen has accumulated in sufficient quantities in the cell owing to translation of the respective mRNA, it binds to the early promoter and represses early transcription. The early promoter of SV40 is very similar to cellular transcription regulators (Fig. 19.10) and is located about 30 nucleotides upstream of the actual transcription start. A GC-rich region ("21 base pair repeat") is situated 40 nucleotides before the transcription start. The cellular Sp1 transcription factor binds to this region. In addition, the adjacent enhancer promotes early RNA synthesis.

The cell must be in the S phase of the division cycle for successful replication of the viral genome; therefore, viruses have evolved mechanisms to influence these cellular processes. The large T antigen, which is synthesized early during infection, is responsible for this; it interacts with the cellular proteins p53 and RB105/RB107, and inhibits their regulatory functions. This activates, among other things, cyclin-dependent kinase 1, which phosphorylates the T antigen at the threonine at position 124. Only the modified T antigen binds to the origin of replication, opens and separates the DNA duplex in this region and leads to the formation of a replication bubble. In the area of this bubble, the cellular protein complex composed of single-stranded binding proteins, DNA primase and DNA polymerase-α bind to the DNA, and start the synthesis of RNA primers. At the 3′-OH ends of the leading strand, nucleotides are polymerized continuously in the 5′ to 3′ direction (Fig. 19.12). The circular viral genome is further unwound bidirectionally at the replication forks, so DNA synthesis can proceed in both

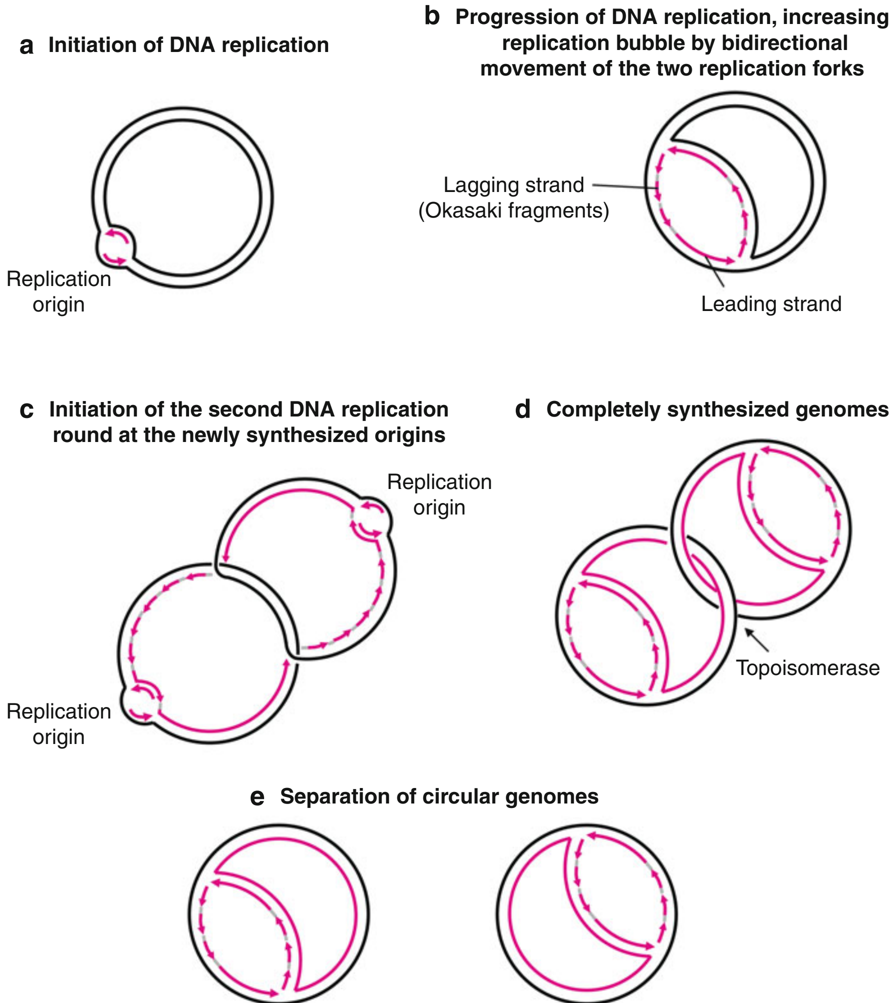


Fig. 19.12 Genome replication of polyomaviruses. The large T antigen binds to the origin of replication in the non-coding regulatory region. The DNA double strand is locally "melted" (i.e., opened, unwound and separated) at this site (**a**). Replication proceeds bidirectionally, in a semiconservative fashion, and is catalysed by cellular DNA replication enzymes (DNA polymerase-α, primase). As usual, DNA synthesis proceeds in the 5′ to 3′ direction; whereas the leading strand is synthesized continuously, the lagging strand is generated discontinuously in the form of Okazaki fragments (**b**). The newly synthesized DNA strands are shown in *red* and RNA primers on the lagging strand are shaded in *grey*. If the sequences at the origin of replication have become double-stranded, then a new round of replication will be initiated (**c**). The newly synthesized genomes are separated by the activity of a topoisomerase (**d**) and are then present as single daughter molecules (**e**)

directions. The lagging DNA strand that advances in the opposite direction is generated by discontinuous synthesis and ligation of the so-called Okazaki fragments, each of which is initiated by a short RNA primer. This replication process is largely similar to that of the cellular genome, with the difference that DNA synthesis is reinitiated in nascent daughter molecules once a new double-stranded DNA region has been formed at the origin of replication (Fig. 19.12). Replication is terminated when the two oncoming replication forks collide. Finally, a topoisomerase is required for unwinding and separation of the molecules. The use of the cellular DNA polymerase-α ensures high-fidelity DNA synthesis during replication of the viral genome because of its inherent 3′–5′ exonucleolytic proofreading activity. Hence, polyomaviruses possess high genetic stability.

The late viral genes coding for the structural proteins are transcribed only after DNA replication. For this purpose, the large T antigen binds to the enhancer sequences, and increases late mRNA synthesis. The size of these mRNAs is very heterogeneous by using different alternative splice donor and acceptor sites: there are six (in JC polyomavirus) to seven (in SV40) capped and polyadenylated transcripts that encode the different late proteins. Precursor RNAs have been found in murine polyomavirus which span the size of the viral genome multiple times. All mRNAs contain a leader sequence as a tandem repeat at their 5′ termini which is located upstream of the sequences for translation of the various structural proteins. This leader sequence contains a nucleotide sequence which has homology with the 18S ribosomal RNA of the mouse. It is believed that this might increase the translation efficiency of the different mRNA species. The region encoding the agnoprotein (LP1) is also located in the leader sequence.

The assembly of the different viral components, i.e. the self-organization of newly synthesized DNA duplexes with the cellular histones H1, H2A, H2B, H3 and H4 into minichromosomes and their association with structural proteins to form capsids, occurs in the nucleus; therefore, capsid proteins have appropriate nuclear localization signals for nuclear transport after their synthesis. In the course of this, the VP1 protein associates with the agnoprotein. It is thought that this prevents the premature oligomerization of VP1, allowing interaction only in the nucleus, so VP1 proteins can form particles with the minichromosomes. Histone H1 is removed from the complex during maturation. Progeny viruses are released by the death of the cell. In vitro, an infected cell contains up to 100,000 new virions.

Vectors Derived from SV40 Facilitate Expression of Exogenous Genes in Eukaryotic Cells

The ability of the large T antigen of SV40 to bind to the origin of replication and to initiate replication of the circular viral genome is successfully used in gene technology for the continuous expression of heterologous genes in eukaryotic cells. Expression vectors have been developed which contain the SV40-specific origin of replication, and express exogenous proteins usually

under regulation by both the late promoter and the enhancer of SV40. The vectors do not contain the genetic information for the large T antigen. However, the vector is transfected into cells that constitutively synthesize this protein. A well-known example is the cell line COS (vervet monkey cells), which contains a replication-origin-defective SV40 genome which is not able to replicate, but supplies the large T antigen for replication of the introduced vector.

19.2.6 Human Pathogenic Polyomaviruses

19.2.6.1 BK and JC Polyomaviruses

Epidemiology and Transmission

Infections with BK polyomavirus und JC polyomavirus (polyomavirus hominis 1 und 2) occur worldwide. JC polyomavirus was isolated in 1971 from the brain of a patient (with the initials J.C.) with progressive multifocal leucoencephalopathy (PML). In the same year, BK polyomavirus was isolated from the urine of a renal transplant recipient (B.K.) who was treated with immunosuppressant drugs. Subsequently, serological tests have revealed that the prevalence of both virus types is very high in the population: antibodies against BK polyomavirus can be detected in more than 80 % of adults. After infection, which predominantly affects children, both viruses persist, including in the mononuclear cells of the blood and in lymphoid tissues; BK polyomavirus has also been detected in cells of the proximal renal tubules. The viruses can be reactivated and excreted in the urine, especially in immunocompromised patients. BK polyomavirus can be found in the urine of 50 % of all bone marrow transplant recipients. Transmission probably occurs by oral infection or smear infection with the urine of infected people. BK and JC polyomaviruses are reactivated and excreted in 50 % of all pregnant women. Whether they are transmitted transplacentally to the unborn child has not yet been finally clarified.

Clinical Features

In immunocompetent children and adults, BK and JC polyomaviruses are of minor clinical significance. Severe diseases occur almost exclusively in patients with immune defects (Table 19.5). In children with an intact immune system, the primary BK polyomavirus infection rarely causes respiratory diseases and cystitis (bladder inflammation). Thereafter, the virus persists, for example, in the kidney and brain. Reactivation of the virus causes severe symptoms such as haemorrhagic cystitis and nephropathy, also known as polyomavirus-associated nephropathy, only in immunocompromised patients (e.g., bone marrow and kidney transplant patients). This leads to the loss of the donor organ in about 5 % of kidney transplant recipients. Subacute meningoencephalitis has been found in association with BK polyomavirus in some AIDS patients. The DNA of BK polyomavirus has been

Table 19.5 Diseases caused by BK and JC polyomaviruses

BK polyomavirus	JC polyomavirus
Infections of the respiratory tract, polyomavirus-associated nephropathy, haemorrhagic cystitis, ureteral stenosis (in bone marrow and kidney transplant recipients), renal graft loss, cystitis (in children), pancreatitis (very rare)	Progressive multifocal leucoencephalopathy in immunodeficient (AIDS) and immunosuppressed (transplant) patients

rarely detected in meningiomas, glioblastomas and osteosarcomas. Whether the viral infection is oncogenic has not been demonstrated so far.

JC polyomavirus does not cause a disease during primary infection, and also persists in the lymphatic tissue, mononuclear blood cells, bone marrow, brain and the kidneys. In patients with immunodeficiency, particularly AIDS patients, JC polyomavirus is responsible for the development of PML, which emerges in 2–10 % of AIDS patients. After infection (probably in childhood) and a subsequent long incubation period of several years, PML symptoms begin when the patients enter the stage of immunosuppression due to human immunodeficiency virus (HIV) infection. These include infections of the respiratory tract, polyomavirus-associated nephropathy, haemorrhagic cystitis, ureteral stenosis (in the case of bone marrow and kidney transplant recipients), loss of function of the transplanted kidney, cystitis (in children), pancreatitis (very rare) and PML in immunocompromised (AIDS patients) and immunosuppressed (transplant recipients) patients. The initial symptoms are visual and speech problems and partial paralysis, which subsequently lead to progressive dementia and death within 6 months. In some cases, brain tumours have been found in PML patients. However, whether the viral infection causes the malignancy is unclear. AIDS patients rarely die of PML since the introduction of combination therapy (highly active antiretroviral therapy) to treat HIV infections (▶ Sect. 18.1.5).

Pathogenesis

After oral transmission, BK and JC polyomaviruses most likely initially invade the tonsils, multiply there and then spread lymphohaematogenously. It is assumed that BK polyomavirus infects B lymphocytes, which transport the virus to other organs such as the kidneys, lungs, spleen, lymph nodes and liver, where the virus settles. BK polyomavirus DNA can also be detected in the brains of deceased people who did not exhibit neurological symptoms. Therefore, it is presumed that the brain is a place where BK polyomavirus persists. Furthermore, BK polyomavirus has also been detected in cells of the intestinal mucosa. However, the kidneys, through which the virus is excreted during reactivation, are the main organs for lifelong persistence. Thus, the amount of excreted BK polyomavirus can reach levels of more than 10^{10} particles per millilitre of urine in renal transplant recipients. Simultaneously, free BK polyomaviruses can also be detected in the blood. The degree of immunosuppression usually correlates with the amount of BK polyomavirus, especially in plasma.

JC polyomavirus is probably also spread in the body by infected lymphocytes. After primary infection or reactivation, infected B lymphocytes transport the viruses into the central nervous system and transmit them through the Virchow–Robin spaces to oligodendroglia and astrocytes, in which the viruses persist, or are reactivated for replication in connection with disorders of the immune system. Since DNA of JC polyomavirus and BK polyomavirus has been detected in about 20 % of examined brains of patients without relevant symptoms, it must be concluded that the viruses were able to settle there during primary infection. Persistence can also be found in lymphatic tissues, bone marrow and the kidneys; in immunodeficient patients, JC polyomaviruses are excreted in the urine. The molecular biology of latency and the mechanism of reactivation are poorly understood.

In PML patients, infectious JC polyomaviruses are produced in myelin-producing oligodendrocytes. The virus replicates lytically in these cells, and intranuclear inclusion bodies of viral proteins can be found in infected cells, which die in the further course of infection. During the progression of the disease, multifocal, patchy demyelination foci develop in the myelin sheaths of the brain. The carboxy-terminal region of the large T antigen of JC polyomavirus contains amino acids which have a certain homology with the basic myelin protein of nerve myelin sheaths. There is no histopathological evidence that the demyelination that occurs in PML is a primary autoimmune disease. Differently mutated JC polyomavirus variants (PML types) have been isolated from brains of patients. They exhibit deletions, duplications and insertions, especially in the promoter/enhancer region. Isolates from urine (archetypes) are largely uniform. Whether the mutant viruses have an altered virulence and are responsible for the development of PML symptoms is unclear; they can also be detected in B lymphocytes and in the kidneys. The frequency of PML in AIDS patients is based not only on HIV-induced immune deficiency, but also possibly on induction of JC polyomavirus expression and replication by the transactivating Tat protein of HIV (▶ Sect. 18.1.3). Induction of JC polyomavirus gene expression by human cytomegalovirus has also been shown in vitro.

KI and WU Polyomaviruses Cause Infections in Humans

In 2007, two new polyomaviruses were isolated from children with respiratory diseases in Sweden, the USA and Australia, and have been named KI and WU polyomavirus. These viruses are phylogenetically more closely related to each other than to BK and JC polyomaviruses, and belong to the group of SV40-like viruses in the family *Polyomaviridae*. Subsequent studies have shown no association of KI or WU polyomavirus infections with respiratory diseases in children or adults. These viruses have also been detected in the saliva of healthy individuals. Similar to BK and JC polyomaviruses, KI and WU polyomaviruses are widespread. The first infection probably occurs during childhood, after which the viral

DNA presumably persists for life. Initial epidemiological studies in the USA have revealed that 55–69 % of healthy adult blood donors have IgG antibodies against the structural VP1 proteins of KI polyomavirus or WU polyomavirus. Examinations of sera of children showed similar results – an indication that the infection occurs early in childhood. KI and WU polyomaviruses are reactivated and proliferate in immunocompromised patients. In lymphoid tissues of 36 % of examined AIDS patients but in only about 4 % of non-immunosuppressed control subjects, 10^5–10^6 genomes of KI and WU polyomaviruses per million cells have been detected,. Whether reactivation of the virus is associated with specific symptoms in immunocompromised individuals is still unclear.

Immune Response and Diagnosis

Primary human BK polyomavirus and JC polyomavirus infections lead to the synthesis of IgM and IgG antibodies, which are predominantly directed against viral VP1 proteins and exhibit strong cross-reactivity. They can be detected by ELISA. Usually, only the levels of IgG antibodies increase during reactivation. However, serology is clinically irrelevant. For example, the antibody concentration does not increase during the development of PML. The diagnosis of PML is made by PCR detection of JC polyomavirus DNA in the cerebrospinal fluid. Little is known about stimulation or inhibition of the immune system by these infections. It has been found that viral proteins lead to a reduced stimulation of lymphocytes. In kidney transplant recipients, the urine and blood plasma are routinely analysed for the presence of BK polyomavirus DNA by quantitative PCR to monitor viral reactivation, and to control the course of infection.

BK polyomavirus can be propagated in some cell lines, such as human embryonic kidney cells. Similar to SV40, vacuolization, inclusion bodies and cell lysis are observed after an incubation period of 1–2 weeks. JC polyomavirus can be propagated only in embryonic amnion and brain cell cultures, especially in oligodendrocytes.

Merkel Cell Polyomavirus: – A New Human Tumour Virus?

When animals such as newborn mice and hamsters are infected experimentally with human BK polyomavirus and JC polyomavirus or SV40 (Sect. 19.2.6), they develop tumours. The genome of BK polyomavirus is rarely integrated in the genome of transformed cells; however, JC polyomavirus frequently integrates its genome into the host cell DNA. No causal relationship with human tumours has been demonstrated either for these viruses or for the newly discovered KI and WU polyomaviruses. In contrast to them, Merkel cell polyomavirus seems to be causally related to a neoplastic disease in humans. This virus is relatively closely related to

B-lymphotropic polyomavirus of monkeys, and thus it does not belong to the group of SV40-like viruses. The Merkel cell polyomavirus genome has been found integrated in the cellular genome of the cells of a rare skin cancer, Merkel cell carcinoma. This neuroendocrine, very aggressive malignant tumour develops frequently in elderly and immunocompromised individuals. The Merkel cell polyomavirus genome has been detected in the tumour cells of nearly 80 % of the patients studied so far. The integration site in the chromosomal DNA seems to remain also in metastases, which develop from the primary tumour; this underlines the possible causal relationship with malign cell transformation. This new human polyomavirus is widespread; antibodies can be detected in about 40 % of healthy blood donors in the USA. There is evidence that this virus infects children as well because it can be found in the pharyngeal lavage of children with respiratory diseases, persisting in the tissue. Unlike other human polyomaviruses, no reactivation of Merkel cell polyomavirus has been demonstrated in AIDS patients.

Therapy and Prophylaxis

There are no vaccines against both viral infections. Attempts at therapy have been made with cidofovir in PML patients; however, a reliable conclusion about the success is not possible. Treatment of HIV infection (▶ Sect. 18.1.5) influences the progression of PML positively. In kidney transplant recipients, attempts are being made to control the development of polyomavirus-associated nephropathy by reducing concentration of the drug used in immunosuppressive therapy.

19.2.7 Animal Pathogenic Polyomaviruses

Polyomaviruses play a minor role in veterinary medicine. Solely budgerigar fledgling disease polyomavirus is widespread, and can a cause mortality of up to 80 % in infected herds. This virus causes subclinical infections in chicken, and can be easily cultivated in chicken cells in vitro.

Other polyomaviruses provoke clinically inapparent infections in their hosts, such as bovine polyomavirus. This virus seems to be transmissible to humans. Virus-specific antibodies have been found in human sera, but, similarly to cattle, no signs of disease have been detected.

The course and significance of SV40 infection in monkeys will briefly be discussed in the following section. SV40 is also veterinarily irrelevant. However, this virus is extremely important as a model system for studying various molecular biological processes and for historical reasons.

19.2.7.1 Simian Virus 40

Epidemiology and Transmission

The natural hosts of SV40 are Asian macaques, particularly rhesus monkeys (*Macaca mulatta*), cynomolgus macaques and African green monkeys. The virus establishes a persistent infection in the kidneys of the host; it is excreted in the urine and is transmitted within the animal population. There is only one genetically relatively stable serotype. Between 1955 and 1963, SV40 was accidentally transmitted to several million people by contaminated poliovirus vaccines (see the box entitled "The First Polio Vaccines Were Contaminated with Simian Virus 40") and vaccines against adenovirus types 3 and 7. In humans, SV40 appears to induce infections with very low virus production. It was found that children who had been inoculated orally with SV40-containing, attenuated poliovirus vaccines excreted SV40 in the stool for up to 5 weeks, but did not develop detectable antibodies. By contrast, volunteers who were infected intranasally with SV40-contaminated respiratory syncytial virus preparations exhibited a low-titre antibody response. In addition, such antibodies are also found in 2–10 % of people born after 1962. Since these individuals have not been exposed to SV40-containing vaccines, this can be assessed as an indication that the virus is capable of spreading in the human population. However, it should be considered that such results might also be based on serological cross-reactivity of antibodies against the human KI, WU and Merkel cell polyomaviruses. Moreover, about half of all zookeepers and laboratory workers who have had contact with monkeys, primary monkey cells and the virus have SV40-specific immune responses. In individual cases, SV40 genomes have been detected by PCR analysis in normal tissue and cells of the peripheral blood.

Clinical Features

Infections with SV40 are asymptomatic in healthy animals. The virus is detectable in the kidneys and the urine for life. However, the virus can be detected in mononuclear cells of peripheral blood as well as in the brain, lung, lymph nodes and spleen of simian immunodeficiency virus infected, and thus immunosuppressed monkeys (▶ Sect. 18.1.6). These monkeys frequently develop symptoms of PML (Sect. 19.2.5), and occasionally also astrocytomas.

Symptoms which may be associated with acute SV40 infections have never been observed in humans. Nonetheless, since the introduction of sensitive PCR methods for detection of viral DNA, SV40 sequences have been repeatedly found in the tissues of human tumours. These include particularly brain tumours in children and adults, ependymoma, mesothelioma and osteosarcoma. DNA sequencing has revealed that these sequences did not originate from laboratory strains, and hence they are no contaminations. A causal relationship of SV40 with tumorigenesis has not been demonstrated in these patients. However, it appears also possible that

cross-reactions with the new human polyomaviruses may have influenced the results of some older research and publications (Sect. 19.2.5).

Pathogenesis

The first transformed cells were discovered in mouse fibroblasts and baby hamster kidney cells infected with murine polyomavirus in 1959 and 1960. In 1971, the isolation of SV40 from a kidney cell line of rhesus monkeys by Benjamin Sweet and Maurice Hilleman was a great sensation, and entailed intensive research because this simian virus was found to cause cancer in newborn hamsters. Similar diseases could not be found in rhesus monkeys. Both murine polyomavirus and hamster polyomavirus were found to induce tumour formation only in other species and not in their natural hosts. No causal relationship between human cancer and polyomavirus infection has been determined so far.

Whether SV40 and murine polyomavirus cause cancer depends on whether the virus is able to undergo a productive infection cycle; i.e. whether the cells are permissive for infection. The reproductive lytic infection is associated with the generation of a large number of progeny viruses, and always ends with the death of the cell. Therefore, transformation can only occur if the cells are non-permissive for the lytic replication cycle. The viral cycle is terminated after synthesis of the T antigens, which leads to an abortive infection (► Chap. 6, Sect. 19.4.5). Hence, transformation is not only dependent on the activity of the large T antigen; cellular functions also play an important role. Cell transformation and tumour diseases are caused by interaction of the large T antigen of these viruses with cellular tumour-suppressor proteins, which become impaired in their function. These proteins normally ensure there is a controlled cell division process. If this is prevented by interaction with the T antigen, the cells enter the S phase of the cell cycle unrestrained, leading to unregulated proliferation. In addition to these protein interactions, mutations in the host cell DNA may also be involved in cancer development, as up to ten copies of the viral genome are integrated at different sites in the chromosomal DNA of SV40-transformed cells. To what extent these integration events are involved in the initiation of transformation has not yet been conclusively clarified. However, they can play an important role in tumour progression and metastasis.

The First Polio Vaccines Were Contaminated with SV40

Between 1955 and 1963, polioviruses were cultivated in primary kidney cells from rhesus monkeys. At that time it was not known that these cells were also infected with the monkey virus SV40. The first vaccine developed (“Salk vaccine”) was an inactivated vaccine against polio (Sect. 14.1), which additionally contained not only inactivated polioviruses, but also presumably killed SV40 particles. Approximately 100 million people were vaccinated

with these vaccines. By contrast, the live vaccine developed by Albert Sabin some years later consisted of attenuated polioviruses. However, since these viruses were also cultivated in monkey cells that were contaminated with SV40, the vaccine also contained active SV40 particles. Millions of Americans and Europeans were immunized with this vaccine between 1955 and 1963. Therefore, presumably, all people who were vaccinated during this period against poliovirus were also infected with SV40. It is estimated that 10–30 million people have been exposed to replication-competent SV40.

Immune Response and Diagnosis

SV40 infection is demonstrated in infected animals and humans by determination of antibodies against the structural VP1 protein by Western blot analysis and ELISA and by detection of viral genomes using PCR. Attention must be paid to possible cross-reactivities using both methods.

Therapy and Prophylaxis

There is neither a vaccine nor an antiviral therapy.

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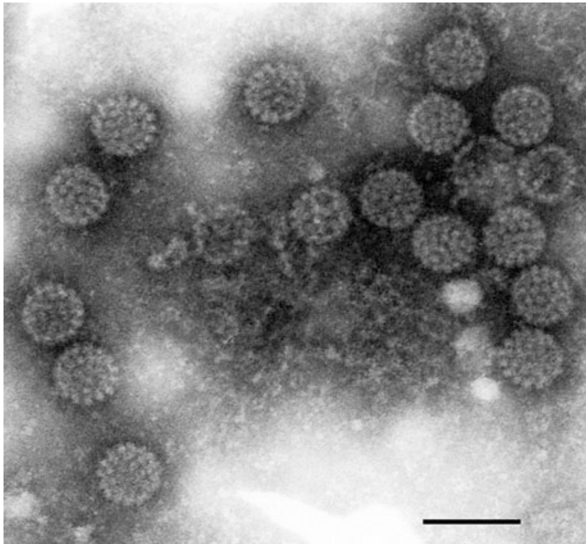
19.3 Papillomaviruses



Warts are benign neoplastic growths of the skin and have been known for many centuries. In 1894, C. Licht and G. Variot first demonstrated that warts are caused by infectious agents, as they could experimentally transmit these growths by means of raw wart tissue. Transmission by bacteria-free ultrafiltrates followed in 1907. Richard Shope isolated the first papillomavirus from a rabbit in 1932 [(Shope) cottontail rabbit papillomavirus]. He also showed that benign tumours are capable of developing into malignant cancer: the multistep hypothesis of carcinogenesis was born. As has become clear in recent years, certain human papillomaviruses cause not only benign skin diseases, but also different malignant epithelial tumours, especially cervical carcinoma. In 2008, Harald zur Hausen, the longtime head of the German Cancer Research Center (Heidelberg), was awarded the Nobel Prize in Physiology or Medicine for his research on the causal relationship between infection with human papillomaviruses and cervical carcinoma, as well as for the development of vaccines based thereon.

19.3.1 Classification and Characteristic Prototypes

Papillomaviruses are widespread. They cause warts (papillomas) in humans and many vertebrates; they have been isolated from dogs, horses, cattle, sheep, elk, deer and birds, among other animals (Table 19.6). Papillomaviruses have a very pronounced host and tissue specificity. Because papillomaviruses are strictly epitheliotropic, and can replicate only in terminally differentiated keratinocytes, it has not been possible to cultivate them in vitro. Therefore, bovine papillomavirus (BPV)

Table 19.6 Characteristic prototypes of papillomaviruses

Genus	Human virus	Animal virus
Alphapapillomavirus	HPV-2, HPV-6, HPV-7, HPV-10, HPV-16, HPV-18, HPV-26, HPV-32, HPV-34, HPV-53, HPV-54, HPV-61, HPV-71, HPV-90 (wart viruses)[a]	*Maccaca fascicularis* paplillomavirus types 3–11
		Rhesus papillomavirus type 1b
Betapapillomavirus	HPV-5, HPV-9, HPV-49, HPV-92, HPV-96[a]	*Maccaca fascicularis* paplillomavirus types 1 and 2
Gammapapillomavirus	HPV-4, HPV-48, HPV-50, HPV-60, HPV-88, HPV-101, HPV-109, HPV-112, HPV-116, HPV-121[a]	
Deltapapillomavirus		Bovine papillomavirus types 1 and 2[a]
		Ovine papillomavirus type 2[a]
		European elk papillomavirus[a]
		Reindeer papillomavirus[a]
Epsilonpapillomavirus		Bovine papillomavirus type 8
Zetapapillomavirus		*Equus caballus* papillomavirus 1
Etapapillomavirus		*Fringilla coelebs* papillomavirus
Thetapapillomavirus		*Psittacus erithacus timneh* papillomavirus
Iotapapillomavirus		*Mastomys natalensis* papillomavirus
Kappapapillomavirus		Cottontail rabbit papillomavirus (Shope papillomavirus)
		Rabbit oral papillomavirus
Lambdapapillomavirus		Canine oral papillomavirus
		Felis domesticus papillomavirus 1
		Procyon lotor papillomavirus
Mupapillomavirus	HPV-1, HPV-63	
Nupapillomavirus	HPV-41	
Xipapillomavirus		Bovine papillomavirus, types 4, 6 and 9–12[a]
Omikronpapillomavirus		*Phocoena spinipinnis* papillomavirus

(*continued*)

Table 19.6 (continued)

Genus	Human virus	Animal virus
Pipapillomavirus		Hamster oral papillomavirus
		Micromys minutus papillomavirus 1
Rhopapillomavirus		*Trichechus manatus latirostris* papillomavirus 1
Sigmapapillomavirus		*Erethizon dorsatum* papillomavirus 1
Taupapillomavirus		Canine papillomavirus type 2
Upsilonpapillomavirus		*Tursiops truncatus* papillomaviruses 1 and 2
Phipapillomavirus		*Carpa hircus* papillomavirus 1
Chipapillomavirus		Canine papillomavirus types 3 and 4
Psipapillomavirus		*Rousettus aegytiacus* papillomavirus 1
Omegapapillomavirus		*Ursus maritimus* papillomavirus 1
Dyodeltapapillomavirus		*Sus scrofa* papillomavirus 1
Dyoepsilonpapillomavirus		*Francolinus leucoscepus* papillomavirus 1
Dyozetapapillomavirus		*Caretta caretta* papillomavirus 1
Dyoetapapillomavirus		*Erinaceus europaeus* papillomavirus 1
Dyothetapapillomavirus		Feline papillomavirus type 2
Dyoiotapapillomavirus		*Equine caballus* papillomavirus 2

HPV human papillomavirus

[a]The viral species listed in the table contain different subtypes and distinguishable isolates according to the classification of the International Committee on Taxonomy of Viruses (ICTV). The table uses the names of the individual papillomavirus types that are commonly used in the literature. In addition to the individual virus types, the ICTV recently suggested the creation of papillomavirus species (e.g. alphapapillomavirus species 1–14). Similar types of papillomaviruses are classified into a species, e.g. human papillomavirus types 3–11 have been classified into alphapapillomavirus species 12. In the table, viral species are not listed and selected papillomavirus prototypes are given. As new virus types are continually being identified, interested readers should consult the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Tree&id=151340&lvl=3&lin=f&keep=1&srchmode=1&unlock) for further information

has been used as a model system for exploring the biology and pathogenesis of this viral infection. This also explains why most human papillomaviruses – more than 100 human papillomavirus (HPV) types have been identified today, and many more are expected to be identified – could be characterized only after refinement of the methods for nucleic acid isolation and sequencing. New virus types are now defined as those that match by less than 90 % with known types in terms of nucleotide sequences encoding the proteins E6, E7 and L1. Virus types whose genomes have been characterized exclusively by PCR amplification and subsequent sequence analysis are supplemented with the provisional designation "cand" (e.g., candHPV-86). This reference to the non-final assignment is omitted if the sequence is confirmed after cloning and new sequence analysis of the genome.

The subdivision of papillomaviruses into 30 genera and many species is determined on the basis of their host and organ specificity, sequence characteristics and genome organization (Table 19.6). The human papillomaviruses are assigned to the genera *Alphapapillomavirus*, *Betapapillomavirus*, *Gammapapillomavirus*, *Mupapillomavirus* and *Nupapillomavirus*. Alphapapillomaviruses preferentially infect the mucosa of the mouth and the anogenital region, causing benign (HPV-6, HPV-7, HPV-54, HPV-61, HPV-71) or malignant (HPV-16, HPV-18, HPV-32) tumours as well as benign and malignant tumours (HPV-26, HPV-34, HPV-53); some of these species (HPV-2, HPV-10) also cause cutaneous lesions. The conserved open reading frame ORF5 constitutes a characteristic genetic trait, and resides between the genes encoding the early and late proteins. ORF5 is absent in betapapillomaviruses (HPV-5, HPV-9, HPV-49), which infect especially the skin, are activated in immunocompromised patients and are causally connected with the development of epidermodysplasia verruciformis, a form of skin cornification with papulation. The absence of ORF5 also characterizes the gammapapillomaviruses (HVP-4), which infect the skin and cause benign lesions. The mupapillomavirus (HPV-1, HPV-63) also cause benign skin warts; however, inclusion bodies are found in the cytoplasm of infected cells, which are characteristic of this viral species. Furthermore, the regulatory long control region (LCR) is significantly larger. The nupapillomavirus (HPV-41) are genetically characterized by several ORFs which are located in the L1 gene region.

Members of the genera *Deltapapillomavirus*, *Epsilionpapillomavirus* and *Zetapapillomavirus* infect solidungulates and even-toed ungulates. They cause fibropapillomas in ruminants (BPV-1) and cutaneous warts in cattle and horses. BPV-3 is classified into the genus *Xipapillomavirus* because it lacks, unlike BPV-1, the gene encoding the E6 protein. The avian papilloma viruses are classified into the genera *Etapapillomavirus* and *Thetapapillomavirus* and cause cutaneous lesions in various bird species; these viruses lack the reading frame for the E6 protein, but the E7 reading frame encodes a protein that complements the E6 functions. The genera *Iotapapillomavirus*, *Kappapapillomavirus* and *Pipapillomavirus* infect rodents: unlike the other papillomavirus genera, the iotapapillomavirus possesses a significantly larger E2 protein, but ORF5 is absent. In contrast, the E6 protein of cottontail rabbit papillomavirus, a prototype of the genus *Kappapapillomavirus*, is significantly larger and has an additional open reading frame (ORF8), which is located in the region of the region encoding the early gene products. The hamster

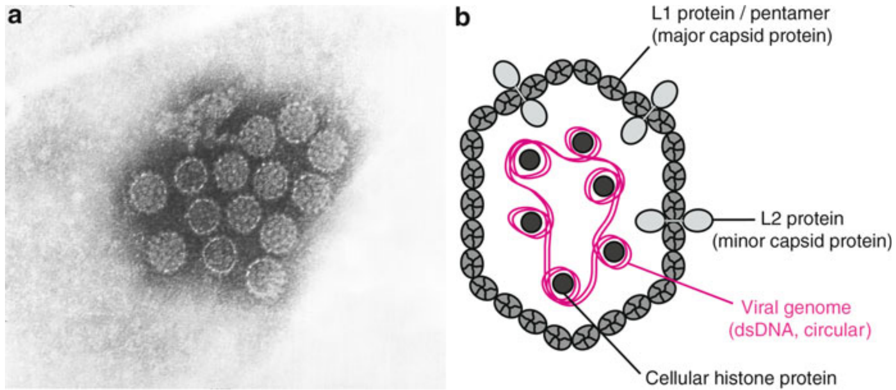


Fig. 19.13 Papillomavirus particle. (**a**) Electron micrograph of canine papillomavirus particles obtained from a biopsy of an oral papillomatosis of a dog. (**b**) Structure of a virus particle. *dsDNA* double-stranded DNA

oral papillomavirus is classified in a separate genus, as the open reading frame encoding the E2 protein partially overlaps with that of the L2 protein. The genus *Lambdapapillomavirus* contains the viral species that infect both the skin and the mucous membrane of dogs and cats. A significantly increased intergenic sequence is located between the early and late regions of the genome. *Phocoena spinipinnis* papillomavirus belongs to the genus *Omikronpapillomavirus* and infects the genital region of porpoises. Other members of this genus and also of the genus *Upsilonpapillomavirus* infect various species of cetaceans. In addition, there are a number of papillomavirus isolates which have been isolated from both humans and various animal species that are recombinants between members of distinct genera. These have been assigned to various newly created genera or have not yet been definitively classified, and thus have not yet been assigned to specific genera. These include, for example, the papillomavirus of the European hedgehog, which has some similarities with betapapillomaviruses.

19.3.2 Structure

19.3.2.1 Virus Particle

Like polyomaviruses, papillomavirus particles are small capsids without a surrounding envelope (Fig. 19.13). The diameter is 55–60 nm, i.e., slightly larger than that of polyomaviruses. The icosahedral capsids are composed of two structural proteins: the L1 protein is the principal capsid component; it forms 72 pentameric capsomers and is capable of self-assembling into particulate structures. In addition, virus particles additionally contain 12–72 molecules of the capsid protein L2, which are predominantly localized on the inner side of the particles. Inside the capsid is the viral genome.

19.3.2.2 Genome Organization and Structure

Similar to polyomaviruses, papillomaviruses also have a covalently closed, circular, double-stranded DNA genome, which is, however, considerably larger (roughly 8,000 base pairs). The supercoiled DNA is associated with cellular histone proteins into a nucleosome-like structure. All papillomaviruses have a similar genome organization (Fig. 19.14). The circular genome can be divided into two regions: a region that encodes the early proteins E1 to E7 as well as E8, and a second region, which contains the genes encoding the late synthesized structural proteins L1 and L2. The transcription of these two regions proceeds using different open reading frames of only one DNA strand. That means that, in contrast to polyomaviruses, all viral functions are encoded on a single strand (Figs. 19.9 and 19.14). The early region contains multiple, overlapping open reading frames (E1 to E7/E8), and the coding capacity is very effectively used by using the different open reading frames. The late region of the genome encodes the two structural proteins L1 and L2. Multiple splice donor and acceptor sites are located at many sites in the genome and are used for the synthesis of the various gene products. A non-coding region of approximately 1,000 base pairs is situated between the end of the late and the beginning of the early region. It is referred to as the long control region (LCR) because it accommodates most of the viral *cis*-acting control elements such as promoters, enhancers and the origin of replication.

19.3.3 Viral Proteins

19.3.3.1 Early Proteins

E1 Functions

Depending on the virus type, reading frame E1 encodes phosphoproteins with molecular masses between 68 and 85 kDa and which are required for replication of the viral genome. Their sequence is highly conserved among the different virus types, and has both structural and functional similarities to the large T antigen of SV40 (ATPase and helicase activity, DNA binding to AT-rich regions in the origin of replication; Table 19.7). Binding of the E1 protein to the sequences at the origin of replication is mediated and stabilized by the E2 protein. The E1 proteins are phosphorylated in their active form and assembly into hexamers, which in turn interact with the complex of cellular DNA polymerase-α and primase, as well as with other proteins. The phosphorylation degree of the E1 protein is probably important for the expression of the various functions which are active at different stages of the viral infection cycle: E1 regulates a restrained form of viral genome replication in undifferentiated and for lytic infection not permissive epithelial cells of the skin. In this stage, the genomes are present in a constant number of 50–400 copies (multicopy state) after the initial replication. The function of the replication-modulating activity of the E1 protein is the maintenance and segregation of the genome copies during cell division. A different function of E1 is activated when infected cells (initially undifferentiated) have developed into terminally differentiated epidermal cells. These cells in the outer layers of the skin are permissive for

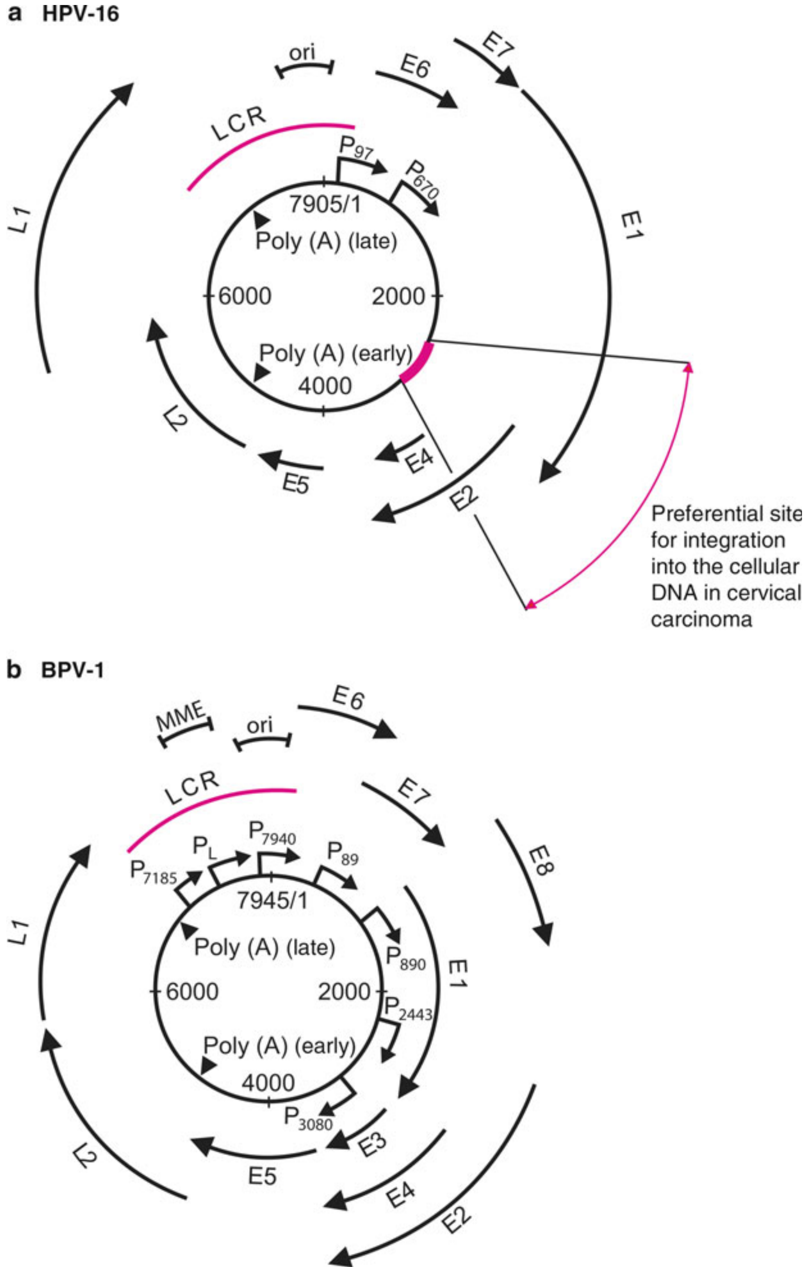


Fig. 19.14 Genome structure of papillomaviruses. (**a**) Pathogenic human papillomavirus type 16 (*HPV-16*). (**b**) Bovine papillomavirus type 1 (*BPV-1*). The genomes consist of a circular, double-stranded DNA, which is shown as the *innermost circle*. The polyadenylation signals used for the synthesis of early and late transcripts are indicated by *arrows*. Only one DNA strand is transcribed. The mRNAs synthesized during replication, their location on the genome and the translation

Table 19.7 Features and functions of papillomavirus proteins

Protein	Molecular mass	Localization	Function
Early proteins			
E1	68–85 kDa	Nucleus	Binds to the replication origin and unwinds it; interacts with cellular DNA polymerases and helicases during genome replication; interacts with E2
E2	48 kDa	Nucleus	Transactivator and transrepressor (depending on the differentiation status) of early gene expression; binds in complex with E1 at the replication origin; binds to the minichromosome maintenance element and host chromosomes and causes the transmission of viral genomes to daughter cells during cell division
E2-Tr	31 kD	Nucleus	Transrepressor in bovine papillomavirus type 1; interaction with E1 during replication
E3	Unknown	Unknown	Unknown
E5 (bovine types)	~5 kDa	Cytoplasmic membrane	Homodimer, transformation; interaction with cellular receptors for platelet-derived growth factor β and autocrine stimulation
E5 (human types)	~8 kDa	Cytoplasmic membrane	Transformation; interaction with epidermal growth factor receptors
E6	~16 kDa	Cytoplasm	Transformation; interaction with E6-associated protein (E3 ubiquitin ligase); induction of proteolytic cleavage and degradation of p53, nuclear factor X1-91 and Bak; induction of cell division; telomerase activation; apoptosis inhibition
E7	~10 kDa	Nucleus	Homodimer, transformation; phosphorylated; interaction with RB105, activation of E2F and induction of E2F-dependent cellular promoters
E8	Unknown	Unknown	Unknown; in bovine papillomavirus type 1, it provides the leader exon for synthesis of some E2 proteins
Late proteins			
L1	57 kDa	Cytoplasm/ nucleus virion	Main (major) capsid protein; pentamer; attachment, neutralizing antibodies
L2	75 kDa	Cytoplasm/ nucleus virion	Minor capsid protein; proteolytic cleavage by furin; involved in release of virus particles from endosomes and in transport of the viral genome to the nucleus
E1/E4	11 kDa	Cytoplasm	Interaction with the cytokeratin scaffold; phosphorylated

Fig. 19.14 (continued) products are shown in the *outer circles*. A region of about 1,000 base pairs (long control region, *LCR*) does not encode proteins. It contains the origin of replication (*ori*), the minichromosomal maintenance element (*MME*) and the main promoters for regulating gene expression. The integration region of malignant human papillomavirus types, which is opened during integration into the cellular genome, is marked in *red*

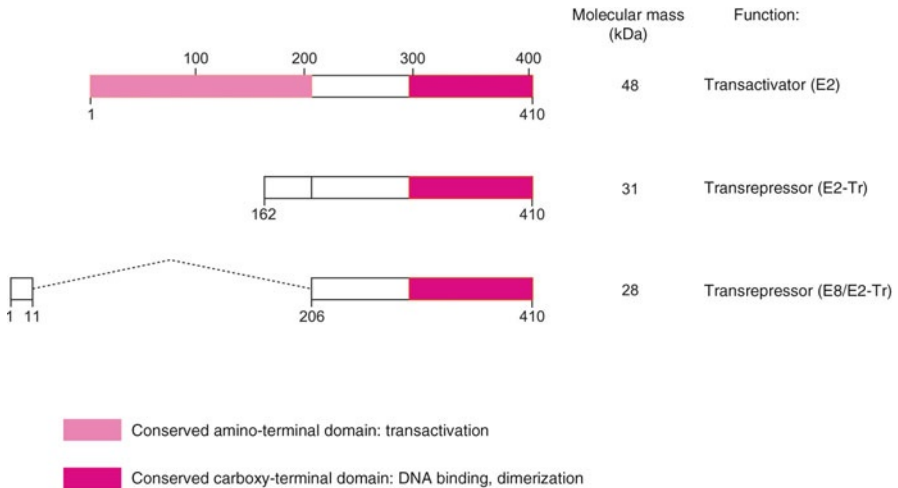


Fig. 19.15 Gene products expressed by the E2 reading frame of BPV-1 and their functional activities. The protein domains which are conserved in various types of papillomaviruses are given in *red*. The *numbers* indicate amino acid positions, starting at the first codon of the respective open reading frames. Early during viral replication, both transactivator E2 (48 kDa) and the amino terminally truncated protein E2-Tr (31 kDa) are synthesized. The transactivating domain is not part of the protein E2-Tr. Therefore, E2-Tr and also the protein E8/E2-Tr (28 kDa) have transrepressor activities

lytic infection. In these cells, a replication switch occurs from the largely stable multicopy status to productive viral replication. This results in high numbers of replicated genomes and is accompanied by the generation of infectious progeny viruses.

E2 Functions

The E2 reading frame encodes several different forms of dimeric, DNA-binding proteins, which are well studied particularly in BPV (Fig. 19.15). Since they exhibit a strong homology to the respective protein of human papillomaviruses, it is believed that the E2 proteins of both virus types have the same functions. These are important regulatory proteins for controlling transcription and replication. The E2 protein binds to 5′-ACC(N)$_6$GGT-3′sequences, which are located in different promoter elements in the LCR and near the origin of replication. In the latter case, it interacts with the E1 protein, stabilizing binding of E1 to the origin of replication. The E2 protein, which is encoded by the entire E2 open reading frame, has a molecular mass of 48 kDa. It consists of two functional domains. The carboxy-terminal region contains a DNA-binding and a dimerization domain. The DNA-binding function is activated when the E2 proteins are present as dimers. The second domain comprises a transactivating domain at the amino-terminus. Both E2 domains are connected by an amino acid linker, whose size and sequence differ among the different virus types.

E2 proteins bind conditional enhancers in the LCR sequences and regulate transcription of the E6 and E7 genes.

If a promoter which resides within the E2 reading frame is used for transcription in BPV-1, this leads to the synthesis of a truncated protein variant of E2, E2-Tr. This variant lacks 161 amino-terminal amino acid residues, and has a molecular mass of 31 kDa. The transactivation domain is absent in E2-Tr; however, the dimerization domain and the DNA-binding functions are still present. Hence, E2-Tr is still able to bind to its recognition sequence, but it cannot induce transcription; consequently, its acts as a transrepressor. Whether the E2 protein functions as a transactivator or as a transrepressor is determined by the concentration, and thus the ratio, of both protein variants. Both the homodimer $(E2\text{-}Tr)_2$ and the heterodimer (E2/E2-Tr) are not able to activate transcription. The activity of the different E2 promoters is influenced in an autoregulatory manner by these complexes and also by cellular factors which are synthesized on the basis of the degree of cell differentiation. This sophisticated regulatory mechanism has not yet been completely elucidated.

This regulatory process is–at least in BPV-1 and some HPV types – even much more complicated. In these viruses, a third version of the E2 protein is synthesized and is translated from an mRNA in which sequences of the E8 reading frame are linked with regions that code for the carboxy-terminal DNA-binding region of E2 by a splicing mechanism. The result is a protein (E8/E2-Tr, 28 kDa) which contains 11 amino acids of E8 linked to 205 amino acids of the carboxy-terminal region of E2. Like E2-Tr, it also functions as a transrepressor (Fig. 19.15). Since many HPV types do not have a reading frame coding for BPV E8, not all types are able to synthesize an E8/E2-Tr protein. It remains unknown whether they possess an analogous gene product.

The various E2 proteins collectively regulate the transcription of early genes. The principal function of the E2 protein of the highly oncogenic HPV-16 and HPV-18 is to suppress the transcription of the E6 and E7 genes in differentiated cervical cells. By contrast, it has been ascertained that E2 proteins of HPV-8, along with cellular factors, bind to the viral promoter of the late genes, and repress their expression.

E6 Functions

In highly oncogenic papillomavirus types, the E6 protein contributes to transformation, but it cannot induce this process alone. The E6 protein of HPV-16 is 151 amino acids long, contains two zinc finger domains and is complexed with Zn^{2+} ions. This is an indication that it capable of binding to DNA. The E6 protein of HPV-16 is involved in regulating the p97 promoter in the LCR. Sequence analysis of the splice donor and splice acceptor sites in the E6 reading frame suggest that in addition to the full-length form of the E6 protein two smaller versions may additionally be synthesized.

The transforming properties of the E6 protein are based on the formation of a complex with the cellular E6-associated protein (E6-AP), which has E3 ubiquitin ligase activity. Simultaneously, the E6 protein interacts with several cellular

proteins, leading to ubiquitination of the complexed cell proteins by E6-AP, and to their ubiquitin-dependent degradation in the proteasomes. These include:

1. The tumour-suppressor protein p53. Papillomavirus types which have a very high transforming potential (HPV-16 and HPV-18) can interact with the cellular protein p53. The interaction between E6-AP and p53 causes the ubiquitination of p53 and its proteolytic degradation. In these cells, there is a lack of tumour suppressor p53. The concentration of p53 is especially elevated in cells which are exposed to mutagens, and thus have increased DNA damage levels. It usually functions as a transcription factor that activates the expression of genes whose products lead to cell cycle arrest in the G_2 phase, delaying the transition into the S phase. These genes include, for example, the inhibitor of cyclin-dependent kinase $p21^{CIP}$ and various factors of DNA repair systems. The p53 deficiency gives rise to the accumulation of mutations, as the cells enter the S phase of the cell cycle before the repair systems can repair the damaged DNA. Furthermore, p53 induces the expression of genes that regulate apoptosis (▶ Chap. 6, Sects. 19.1.3, 19.2.3 and 19.4.3).
2. Nuclear factor X1-91 (NFX1-91), a transrepressor. NFX1-91 functions as a repressor by binding to the promoter of human telomerase reverse transcriptase, thus preventing the expression of this enzyme. The E6-/E6-AP-mediated proteolytic degradation of NFX1-91 activates expression of the cellular telomerase – an enzyme that is hardly present in normal cells, but is highly expressed in tumour cells. Telomerase counteracts the continuous shortening of repeat sequences at the telomeres, i.e. the chromosome ends. This shortening usually occurs in the cells of an organism during each replication of the genome, and correlates with cell ageing.
3. The proapoptotic protein Bak. Bak proteins are synthesized in large quantities in the cells of the upper, differentiated layers of the skin, in which it regulates the induction of apoptosis. E6-/E6-AP-mediated ubiquitination and proteolytic degradation of Bak proteins prevent the cell from entering apoptosis. It has been found that the E6 proteins of highly oncogenic papillomavirus types such as HPV-16 bind to Bak proteins with a significantly stronger affinity than those of HPV-11, which is only weakly oncogenic.
4. Various PDZ-domain-containing proteins. PDZ domains describe amino acid motifs that interact with other proteins. The motif X-S/T-X-V/L/I, which is responsible for protein interaction, is located in the carboxy-terminal domain of the E6 proteins of highly oncogenic papillomaviruses. Some cellular interaction partners have been identified (such as hDlg, hScribble, MUPP1 and the tyrosine phosphatase PTPN13). However, whether their degradation is related to the oncogenic effect has not yet been conclusively clarified.

E7 Functions

The E7 protein is relatively small and is 98 amino acids long. It is the main cause of the transforming potential of certain HPV types. The E7 protein is phosphorylated at serine residues 31 and 32 by casein kinase 2. A zinc finger

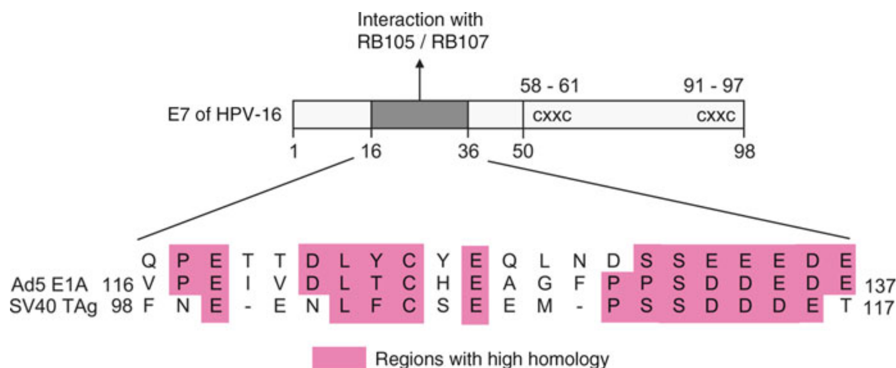


Fig. 19.16 E7 protein of HPV-16. *Numbers* refer to the amino acid positions, starting from the amino terminus. The E7 protein possesses conserved Cys-X-X-Cys motifs in the carboxy-terminal region, which characterizes it as a zinc finger protein. Between positions 16 and 36 there is a domain that contains many amino acids with homology to the large T antigen of SV40 and the E1A protein of adenoviruses. This region mediates the interaction with the cellular tumour suppressors RB105 and RB107. *Ad5* human adenovirus 5

motif located in the carboxy-terminal region, which consists of the repeated units Cys-XX-Cys, is important for correct protein folding and dimerization. The carboxy-terminal region is structurally and functionally similar to the CR3 domain of the E1A protein of adenoviruses (Sect. 19.4.3). Apart from this sequence homology in the carboxy-terminal region, there is another remarkable homology to the E1A protein of adenoviruses: the first 37 amino acids of the E7 protein are highly homologous to the CR1 and CR2 domains of the E1A protein and to the corresponding region in the large T antigen of SV40, which is responsible for binding to retinoblastoma proteins RB105 and RB107 (Fig. 19.16). Depending on the degree of phosphorylation, the tumour-suppressor proteins RB105 and RB107 are involved in the control of cellular promoters by binding and inactivating the cellular transactivator E2F. Interaction with the T antigen and E7 or E1A proteins dissolves the E2F/RB105 complex independently of the RB105 phosphorylation status, and abrogates the control function of RB105. The uncontrolled release of E2F transactivators induces the transcription of genes which are regulated by E2F-dependent promoters and promote the entry of the cell into the S phase of the cell cycle (▶ Chap. 6, Fig. 19.17). The consequence is unregulated cell division.

The E7 proteins of HPV types which like HPV-16 or HPV-18 entail a high risk for induction of malignant cell growth interact with the cellular RB105 protein by their domains that are homologous to the adenoviral E1A gene product. As a result, the E2 factors are released from the complex, and can be transported into the nucleus, where they bind to the control elements in the E2F-dependent promoters. In HPV-6 and HPV-11, which are only rarely associated with malignant tumours, there is no high affinity of E7 proteins for RB105/RB107.

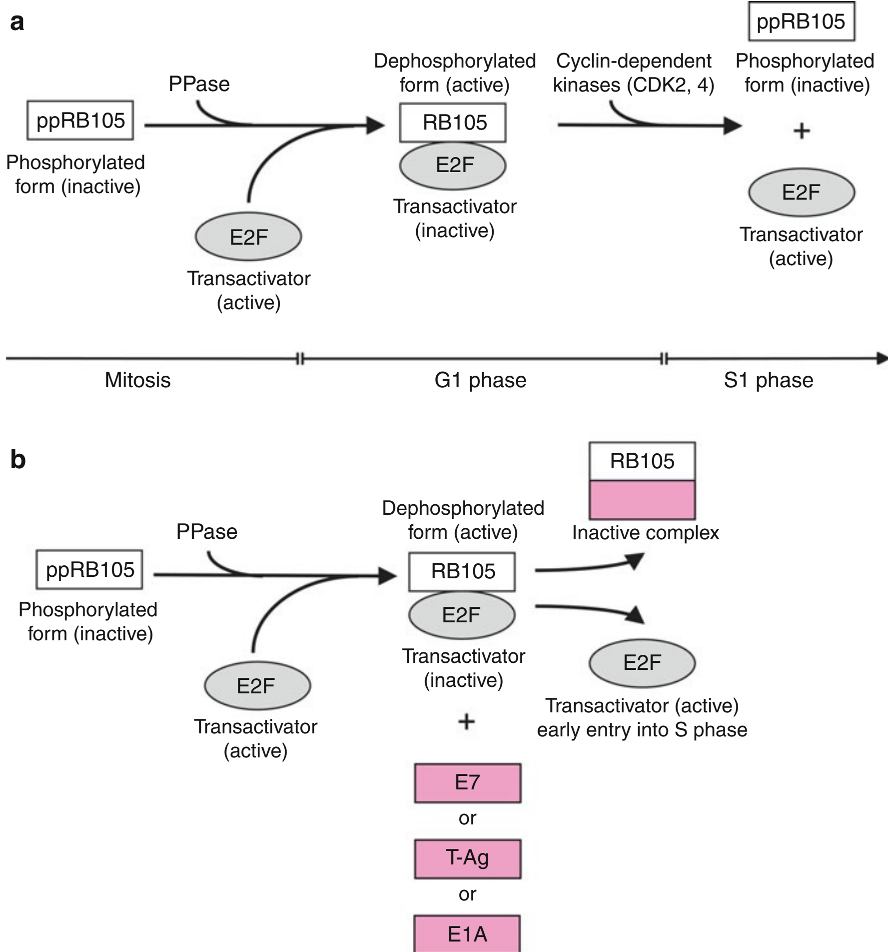


Fig. 19.17 Function of tumour-suppressor proteins RB105 and RB107. (**a**) In uninfected cells. Retinoblastoma proteins are phosphorylated and inactive during mitosis. The retinoblastoma protein is dephosphorylated and activated by a cellular phosphatase (*PPase*) during the transition from mitosis into the G_1 phase of the cell cycle. It binds in its dephosphorylated form to E2F proteins, preventing promoter binding and induction of genes whose proteins are required for entry into the S phase. When the cells enter the S phase, retinoblastoma proteins are phosphorylated by cyclin-dependent kinases (CDK2 and CDK4), whereby they are inactivated again. Phosphorylated retinoblastoma proteins release the E2F factors, which in turn can exert their DNA-binding and transactivating functions. (**b**) In infected cells, the viral protein E7 (papillomaviruses), viral protein E1A (adenoviruses) or the large T antigen (*T-Ag*; SV40) is present in the cell. These viral proteins interact with the RB105 tumour suppressor in the G_1 phase, in which they are associated with the E2F factors. Binding of viral proteins leads to the dissolution of the complex. The E2F proteins are released, activated, and can activate the relevant promoters. This effect leads to premature entry of cells into the S phase

Other Early Functions

The primary transforming potential of BPV-1 has been identified as the 44 amino acid long E5 protein. The E5 protein forms homodimeric membrane proteins which interact with the membrane-anchored platelet-derived growth factor β (PDGF-β) receptor, and activates it in a ligand-independent manner, i.e. not by exocrine binding like PDGF-β. This mechanism is also known as virocrine stimulation. The interaction of the E5 protein with the PDGF-β receptor causes its dimerization and phosphorylation of tyrosine residues in its cytoplasmic domain. This in turn gives rise to the accumulation of cellular SH2-domain-containing proteins, whereby a continuously active signal translation complex is generated.

The E5 protein of HVP-16 is 84 amino acids long and can transform fibroblasts in vitro. It induces proliferation of keratinocytes. It possibly binds to the cellular epidermal growth factor (EGF) receptor, like the binding of the E5 protein of BPV-1 to the PDGF-β receptor.

The functions of the gene products encoded by the E3 and E8 reading frames are unknown. E3 has not been found in the genome of most HPV types.

19.3.3.2 Late Proteins

The genes encoding the papillomavirus late proteins are expressed only in terminally differentiated keratinocytes. These include the two structural proteins, the major capsid protein (57 kDa) and the minor capsid protein (75 kDa), which are encoded by the genes *L1* and *L2*, respectively. The two proteins are present in a ratio of about 30:1 in infectious viruses. L1 proteins form pentamers, which are linked by intermolecular disulphide bonds. They have the inherent ability to form icosahedral particles. If the viral genome is enclosed in the particles, the degree of cross-linking between L1 capsomeres increases. However, virus particles are infectious only if they contain at least small amounts of L2 proteins; possibly, the L2 proteins additionally influence the cell tropism of the virus. A conserved sequence is located in the amino-terminal domain of the L2 protein of papillomaviruses, which is recognized and cleaved by the cellular protease furin. This proteolytic cleavage occurs at the cell surface, and results in increasing viral infectivity. In addition, after the virus particle has entered the cell through receptor-mediated endocytosis, L2 proteins are involved in the destruction of the endosomal membrane and in the transport of the viral genome to the nucleus.

Moreover, there is an E1/E4 protein (11 kDa) which is synthesized the late phase of the replication cycle, although it resides within the early gene region. It is generated by alternative splicing, and fuses the five amino-terminal amino acids of the E1 protein to the carboxy-terminal domain of the E4 protein (85 amino acids in HPV-11); the E4 reading frame does not have its own start codon. The function of this phosphoprotein that accumulates in the cytoplasm has not been definitively resolved. It induces the collapse of the cytokeratin matrix in differentiated keratinocytes. It probably interacts with a cellular RNA helicase of the family of DEAD-box proteins. An overview of the features and functions of the different papillomavirus proteins is given in Table 19.7.

19.3.4 Replication

The replication of papillomaviruses occurs in two phases, and these are dependent on the degree of cell differentiation. Papillomaviruses initially infect undifferentiated keratinocytes in the basal skin layers. In these cells, the copy number of their genomes is increased and remains constant in the undifferentiated cells. The production of progeny viruses occurs in terminally differentiated cells, and is associated with a high replication rate of the viral genome. The entire reproductive cycle of papillomavirus infection extends over a period of about 3 weeks, the time that a keratinocyte needs to complete the entire differentiation process.

The viruses reach the undifferentiated keratinocytes by penetrating into small injuries of the outer horny layer of the skin. The cellular receptor responsible for attachment of papillomavirus particles is not yet conclusively known. There are a number of experimental findings that demonstrate that papillomaviruses initially interact by their surface-exposed L1 protein domains with laminin 5, a protein of the extracellular matrix secreted by keratinocytes. Concomitantly, it has been shown that most papillomaviruses bind to heparan sulphates on the cell surface through their L1 proteins, using these molecules as the primary receptor. After this initial interaction, structural rearrangements seem to occur in the capsids. Cyclophilin B, a cell-surface-associated peptidyl-prolyl *cis*/*trans* isomerase, might also be involved as a chaperone. As a result, the L2 protein domain containing the furin cleavage site becomes accessible. These structural changes enable the viruses to bind to their secondary receptors. Heterodimeric integrin molecules containing $\alpha_6\beta1$ or $\alpha_6\beta4$ chains serve as secondary receptors in some, but not all papillomavirus types. The interaction with integrins does not seem to occur in HPV-11, HPV-33 and BPV-4. In subsequent steps, it is necessary that the cellular receptor interacts with F-actin; thereby the virus-receptor complex is retrogradely transported from the cellular surface extensions towards the cell body. As shown for HPV-16, the L2 protein is capable of interacting with still unknown cell surface components by its amino-terminal domain.

Subsequently, the virus particles penetrate into the cells by endocytosis. Functional activities of the L2 protein are necessary to ensure that the virus particles can leave the endosomes: the endosomal membrane is destroyed by the carboxy-terminal domain of L2 proteins. Thereafter, the L2 proteins complexed with the viral genome interact with dynein, a motor protein that is associated with microtubules. As a result, the complex consisting of the L2 protein and the viral genome is transported along the microtubules to the nuclear pores, and finally into the nucleus. The viral genome is located in the nucleus of undifferentiated cells within nuclear domain 10 (promyelocytic leukaemia nuclear bodies), where the early viral genes (E genes) are transcribed. This process is controlled by several viral promoters which are situated in the LCR upstream of early genes (Fig. 19.14), and are affected in their activity by various cellular factors. For instance, the cellular protein YY1 negatively regulates the activity of viral promoters. A similar negative regulatory effect is exerted by the transcription factor Oct-1, the nuclear factor for induction of IL-6 (NF-IL-6) expression, and the retinoic acid receptor. A positive regulatory

effect on these viral promoters is exerted by, among others, the enhancer-binding protein Sp1, the transcription factors activator protein 1 (AP-1), JunB and keratinocyte-specific transcription factor 1, and activated glucocorticoid receptor complexes. In addition to this complex transcriptional control, multiple splicing events are possible. As a result of this, monocistronic mRNAs are formed which code for the different versions of early proteins. Nevertheless, some polycistronic mRNAs are also synthesized: e.g., the E1 gene is encoded as third open reading frame in an mRNA whose synthesis is regulated by the p97 promoter. The open reading frames encoding the E6 and E7 proteins are located upstream of the E1 sequences. The end of the E7 gene is adjacent to the start codon AUG of the E1 gene. A ribosome entry site, as mediated by 5′-cap structures and internal ribosome entry site sequences (▶ Sects. 14.1 and ▶ 14.5), does not exist in this case. After initial binding to the 5′ end of the mRNA sequences, ribosomes may seek the start codons by a scanning mechanism, starting the synthesis of the E1 and E1/E4 proteins. All transcripts of early genes use the same polyadenylation site (Fig. 19.14).

Transcription of the genes that encode the E1 protein (DNA helicase) and the E2 protein (transcriptional activator) is important for the first processes after infection, because these proteins are essential for viral genome replication. Several copies of E1 proteins bind along with E2 proteins to the sequences at the origin of replication in the LCR, causing unwinding of the double-stranded DNA within the origin of replication sequences. This leads to the recruitment of the cellular DNA polymerase/primase-α complex to the viral origin of replication; in the subsequent polymerization steps, the helicase activity of the E1 protein unwinds the DNA double strands, and the DNA polymerase is responsible for replication of the viral genome for up to 50–400 copies per cell during the first replication cycles. This copy number remains constant, and by the involvement of E1 and E2 proteins is passed on to daughter cells during division in undifferentiated cells, multiplying the viral genomes. In the course of this process, E2 proteins interact with a stretch of sequences within the regulatory LCR of the viral genome, which is known as the minichromosome maintenance element, and comprises between four (most HPV types) and six (BPV-1) E2-binding sites. Concomitantly, E2 proteins interact with similar DNA sequences present in the chromosomal DNA of host cells. This complex formation ensures that viral genomes are equally inherited by daughter cells along with the chromosomes during cell division. The E2 transcriptional activator is required for transcription of other early genes, but its activity is modulated by the repressing variants E2-Tr and E8/E2-Tr. The latter probably exists only in BPV-infected cells or in human virus types that possess an E8 reading frame. If the repressor activities of E2 protein variants are absent, the transcription of E6 and E7 genes is induced, and the viral genome copy numbers increase tenfold to 20-fold.

The upper epithelial layers of the skin, which are non-dividing and eventually die, are continuously regenerated from the cells of the basal layer. The division of skin cells leads to the differentiation of basal epithelial cells into keratinocytes in a vertical direction, i.e. towards the skin surface (Fig. 19.18). A productive infection

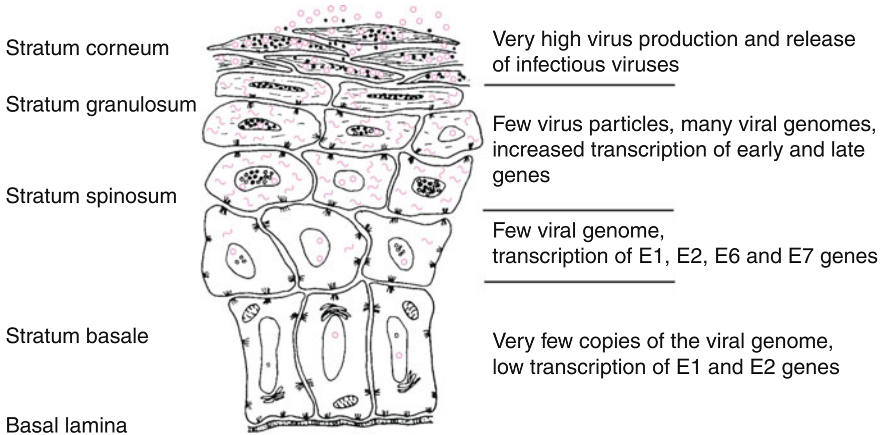


Fig. 19.18 Dependence of the papillomavirus gene expression on the degree of differentiation in epidermal cells. A cross-section of the epidermis is shown. The denominations of the different skin layers are indicated at the *left*. *Open circles within the nuclei* represent papillomavirus genomes. *Black dots* represent virus particles which are produced especially in the upper skin layers, i.e., the terminally differentiated keratinocytes of the stratum corneum, from whence they are released into the environment (Slightly modified after Cossart et al. 1995)

cycle of papillomaviruses is possible only in cells that are in the S phase of the cell cycle, during which DNA polymerases and other cellular factors are available for viral genome replication. Therefore the viruses must counteract the processes by which cells delay their entry into the S phase or inhibit cell division. This is done by the activities of the E6 and E7 proteins, which are synthesized in relatively small quantities, and induce cell division during the productive infection cycle. The E6 and E7 proteins inhibit cellular tumour suppressors such as p53 and RB105/RB107, which negatively regulate cell division. These transforming activities of both viral proteins are not manifested during the lytic replication cycle because virus-producing cells finally die.

The cellular environment changes because of the progressing differentiation. The E2-dependent, early promoter is no longer used. Instead, the E2-independent, late P_L promoter becomes active; it is located within the reading frame for the E7 protein. The increased transcription of E1 and E2 genes is induced and the polyadenylation signals at the end of the early region of the genome. The increased production of E1 and E2 proteins, in turn, gives rise to an increased replication of viral genomes, which accumulate to concentrations of more than 1,000 copies per cell. Concurrently with the further progressing cell differentiation, the cellular environment changes once more, and the promoter for the late viral genes (P_L), which is located in the LCR, becomes active. A precursor mRNA is synthesized from the P_L promoter, and spans the entire genome excluding the early polyadenylation signal. This transcript is used for the synthesis of the capsid proteins L1 and L2 as well as the E4 protein, which are encoded in the late region of the genome. This precursor mRNA uses an alternative polyadenylation signal located

at the end of the region of the late genes. The P_L promoter appears to be active even in undifferentiated cells, in which, however, transcription is prematurely terminated, and the polyadenylation signal of early mRNA species is used. This is an additional indication that many, still unknown cellular regulatory factors are involved in this process. Simultaneously with the production of the L1, L2 and E4 proteins, the replication mode switches, and E1 proteins induce, along with the cellular DNA polymerase-α and other factors, the synthesis of a large number of circular viral genomes. In terminally differentiated keratinocytes, the newly synthesized genomes form complexes with cellular histones, and assemble with capsid proteins L1 and L2 into infectious papillomavirus particles, which are released after death of the cell. However, the cell death associated with the production of infectious papillomaviruses does not limit the infection: owing to the biphasic course of the viral replication cycle, dying cells are continuously replaced by cells from the undifferentiated layers of the skin which contain multiple viral genomes. Hence, a persistent form of infection can be established which is associated with a sustained, continuous release of virus particles in spite of the death of virus-producing cells.

E6 and E7 proteins do not exert a transforming effect during the productive replication cycle. They inhibit the functions of cellular proteins such as p53 and RB105/RB107 that negatively regulate cell division. A productive infection cycle of papillomaviruses is only possible when cells are in the S phase of the cell cycle. Therefore, the virus must inhibit cellular proteins which delay the entry into the S phase. This is performed by the proteins E6 and E7, which are synthesized in relatively small quantities, and cannot exert their transforming effect during the productive infection cycle because virus-producing cells die.

Genetically Engineered Vectors Contain Papillomavirus Early Genes
In genetic engineering, the ability of papillomaviruses to induce episomal replication in eukaryotic cells is exploited for the expression of heterologous genes. Vectors which are derived from BPV-1 and contain the early region and the LCR regulatory sequences are commonly used. This corresponds to 69 % of the viral genome. Late genes are deleted and replaced by the desired heterologous gene under control of a eukaryotic promoter. After transfection into the cell of choice, the advantages of such expression systems are that the vector is amplified by the papillomavirus early functions, which also cause a high cell division rate owing to their transforming properties.

19.3.5 Human Pathogenic Papillomaviruses

19.3.5.1 Papillomaviruses (Wart Viruses)

Epidemiology and Transmission

The prevalence of papillomaviruses in the human population is very high, and is clinically manifested as warts in skin and mucosa. The most widespread virus

types are those which cause common warts on the hands and feet (verruca vulgaris and verruca plana). However, HPV-5 is also found in all population groups. It is the commonest papillomavirus type, and is associated with the disease epidermodysplasia verruciformis, which is manifested as multiple warts, and can develop into skin cancer in immunosuppressed patients. Oncogenic types such as HPV-16 and HPV-18 are associated with cervical cancer throughout the world. Approximately 500,000 new cases of cervical cancer are diagnosed worldwide every year, and about 250,000 women die. In more than 90 % of cases, cervical cancer is caused by HPV infections, of which about 70 % are attributed to HPV-16 and HPV-18. About one third of these cases are fatal. In developed countries, e.g. in the USA, the introduction of screening programmes and vaccination has led to a reduced incidence of cervical cancer: it has been estimated that there are 11,000 new cases and 3,800 deaths annually. A geographically different distribution is found only for some types. For instance, HPV-13 and HPV-32 are preferentially encountered in Central America, South America, Alaska and Greenland.

The transmission of cutaneous viruses accompanied by the formation of warts occurs predominantly from the age of 5 years by direct contact with infected skin areas, or via contaminated objects (e.g., towels) within the family. Papillomaviruses are also frequently transmitted in recreational facilities (swimming pools, sports clubs, etc.) through the collective use of such facilities by many people (wood gratings and similar objects play an important role). Moreover, the genital papillomavirus types are transmitted through sexual intercourse from the age of puberty onwards. In rare cases, genital papillomaviruses can be transmitted vertically to neonates during parturition. These perinatal infections are usually manifested as papillomas of the larynx, nose and throat in children and adolescents. In immunocompromised individuals (HIV patients, transplant patients), a significantly increased wart formation and frequent malignant transformation can be observed.

Clinical Features

Human papillomaviruses infect cells of the outer skin and epidermal mucosa, and principally cause local cell proliferations in the infected area, which are predominantly manifested as benign warts that usually regress spontaneously. Strikingly, some HPV types can be associated with specific pathohistological wart types which are localized in certain body regions (Table 19.8). There are two main centres of clinical manifestation: the cutaneous area of the skin and mucous membranes. They can be correlated with certain HPV types:

1. The cutaneous area commonly contains warts as raised skin lesions with hyperkeratosis, especially on the hands and legs, flat warts, which usually occur solitarily on the hands and face, are only slightly raised, and plantar warts, which can reach deep into the skin layers, predominantly occur on the soles. The causative agents of these wart types include members of the gammapapillomaviruses, mupapillomaviruses and nupapillomaviruses (HPV types 1, 2, 3, 4, 7, 10, 26–29, 41, 48, 50, 60, 63, 65 and 88), and also some members of

Table 19.8 Skin lesions and tumours preferentially caused by the different HPV types

Infected region	Clinical picture	Specific features	HPV types
Cutaneous skin	Plantar warts	Individual warts, preferentially in plantar areas; benign	1, 4, 63
	Common warts	Frequent formation of warts, usually on the hands; benign	1, 2, 4, 26–29, 41, 40, 43, 48, 57, 60, 63, 65
	Flat warts	Frequent formation of warts on arms, legs and face; benign	3, 10, 27
	Butcher's warts	Common warts on the hands of butchers, slaughterers, etc.; benign	7
	Epidermodysplasia verruciformis	Frequent formation of warts; malignant	5, 8, 14, 17, 20, 47
	Epidermodysplasia verruciformis (in immunosuppressed individuals)	Frequent formation of warts; usually benign	3, 9, 12, 19, 21–25, 36–38, 41, 46–50
Oral mucosa	Juvenile laryngeal papillomatosis	Commonly in children, strong growth; benign	6, 11, 13, 44, 74
	Focal, epithelial hyperplasia	Oral lesions; benign	13, 32
Anogenital mucosa	Condylomata acuminata	Exophytic lesions of the skin and mucous membrane; usually benign, rarely develop into malignant forms	6, 11, 13, 40, 42–44, 74
	Flat condylomata, cervical intraepithelial neoplasia (grades I–III)	Low risk of malignant transformation; usually benign, can turn into malignant forms	6, 11, 16, 18, 26, 30, 31, 33–35, 39, 40, 42–45, 51–53, 55–59, 61, 66–71, 74, 82
	Cervical carcinoma, cervical intraepithelial neoplasia (grade III)	High risk of malignant transformation; precancerous, invasive tumour	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 52, 58, 59, 62, 66–68, 70, 73
	Bowenoid papulosis	Pigmented skin lesions; malignant or precancerous	16, 18, 31, 33

alphapapillomaviruses, such as HPV-2 and HPV-10, which preferably infect the cutaneous epidermis and predominantly cause benign, common skin warts. Epidermodysplasia verruciformis, a rare skin disease with formation of multiple warts throughout the body, is mainly associated with betapapillomaviruses (HPV types 5, 8, 9, 12, 14, 15, 17, 19, 25, 36, 38 and 49). It occurs in immunocompromised patients with an impaired cellular immune response. Otherwise, this disease is found only in individuals carrying an autosomal recessive hereditary predisposition. Studies of affected families have shown that two independent loci are responsible for the increased susceptibility to the disease; they are located on chromosomes 2 and 17. The gene locus on chromosome 17 has been investigated in detail: mutations in the genes *EVER1* and

EVER2 are associated with the disease. Both genes encode proteins of unknown function. Most such patients exhibit an impaired cellular immune response. Approximately half of them develop malignant tumours in a period of about 20 years after the occurrence of multiple warts, which are primarily associated with the HPV-5 and HPV-8, and rarely correlate with the HPV-14, HPV-17, HPV-20 and HPV-47. Ultraviolet light is an additional factor which plays in an important role in the transition from initially benign lesions to intraepithelial neoplasias and invasive carcinomas.

2. Papillomaviruses associated with infections of the epithelial mucosa are classified into the genus *Alphapapillomavirus*, and can be divided into two groups. Slightly raised, multiple papillomas and epithelial hyperplasia are found in the oral cavity and the larynx, often even including the vocal cords. Such juvenile laryngeal papillomas occur predominantly in children, and may be life-threatening owing to their location in the respiratory tract when they constrict the airways. Malignant transformation is rarely observed. HPV-6, HPV-11, HPV-13, HPV-32, HPV-44 and HPV-74 are frequently detected in these lesions. The second group of papillomavirus-associated diseases of the mucosa are manifested in the anogenital area. Condylomata acuminata (genital warts, which are soft, clearly verrucous hyperkeratoses) and bowenoid papulosis (flat, multiple, often pigmented lesions) are primarily found in young adults on the external genitals and in the anal region. Even condylomata acuminata are usually correlated with benign HPV types (6, 11, 13, 40, 42–44, 47, 54 and 55), and rarely exhibit malignant transformation. The multiple bowenoid papulosis is also predominantly benign, notwithstanding its association with HPV-16. However, another form of Bowen's disease, which occurs with solitary skin lesions at the age of more than 50 years, frequently exhibits a propensity for intraepithelial neoplasias. Therefore, it is considered as a precancerous condition.

Cervical papillomavirus infections are manifested as flat condylomata or low-grade intraepithelial neoplasias, which can be stained with a 5 % acetic acid solution. They are frequently multicentric. Only certain HPV types are causally involved in the development of cervical carcinoma: HPV-16 is detected in more than 50 % of cases, whereas HPV-18 is found in 18–20 % of patients. Furthermore, the following HPV types are also frequently detected: 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 62, 67, 68, 70 and 73 (Table 19.8). The same distribution of types is also found in high-grade intraepithelial neoplasias in the mucosa and the cervix [squamous intraepithelial lesion or cervical intraepithelial neoplasia (CIN) I–III, increasing numerals indicate increasing severity of the neoplasm]. These intraepithelial neoplasias represent carcinoma in situ and are precursors of invasive carcinoma that have not yet penetrated the basal lamina of the epithelium. The latent period between primary infection and tumour formation is approximately 20–40 years.

The fact that HPV-16 and HPV-18, which can be detected in more than 90 % of cervical cancers, also occur in healthy women implies that not all women who have

Table 19.9 Cofactors involved in papillomavirus carcinogenesis

Virus type	Carcinoma	Cofactor
HPV-5, HPV-14, HPV-17, HPV-20, HPV-47, HPV-49, HPV-88	Squamous cell carcinoma (epidermodysplasia verruciformis)	Recessive genetic factors, MHC class I types, UV light
HPV-6, HPV-11, etc.	Laryngeal tumour	X-rays
HPV-16, HPV-18, HPV-31, etc.	Malignant anogenital tumours, cervical carcinoma	Tobacco smoke, hormones, viral infections in the mucosa (herpes simplex virus, cytomegalovirus, human herpesvirus 6, human immunodeficiency virus)
Cottontail rabbit papillomavirus (Shope rabbit papillomavirus)	Skin tumours	Tar
Bovine papillomavirus type 4	Digestive tract	Ferns (silica)

had contact with these virus types inevitably develop a persistent infection with propensity for high-grade intraepithelial neoplasias or carcinomas. However, such cases are only rarely observed. Women with detectable HPV-16 or HPV-18 infections have an about 11 times higher risk of developing a high-grade intraepithelial neoplasia within 2 years in comparison with women who are not infected with these virus types. Cofactors seem to play an important role in the development of papillomavirus-associated malignancies of the genital region. These factors could be hormones, concurrent infections with other viruses (including herpesviruses and human immunodeficiency viruses) or cigarette smoking (Table 19.9). Genetic predisposition or disorders of the cellular immune system appear to be involved as well.

Pathogenesis

HPV-Associated Skin Warts (Benign Tumours)

Most warts are benign, self-limiting ailments of the skin which frequently regress spontaneously after some time (up to several months). The viruses penetrate through minute lesions of the outer keratinocyte layer into basal epithelial cells, where the viral early proteins E1 to E7 are synthesized. The episomal genome is replicated in low copy number and inherited by daughter cells during cell division. All forms of warts have in common that they are separated from the peripheral blood and connective tissue by an intact basal epithelial cell layer, the basal lamina (Fig. 19.18). A cellular hyperplasia is found in all outer skin layers above the stratum basale. Highly vacuolated cells of the squamous epithelium with nuclear alterations (koilocytes) are histologically detectable, and the outermost stratum corneum is hyperkeratotic. The disease-associated local thickening of the skin is attributable to the induction of local cell proliferation. This requires the functions and activities of the viral proteins E6 and E7, which are produced in the undifferentiated skin layers and interact with cellular tumour-suppressor proteins. This interaction drives

infected cells into the S phase of the cell cycle. The number of differentiating cells increases because of the enhanced cell division rate, which allows the virus to perform productive replication and synthesis of the late proteins L1 and L2 as well as the generation of progeny viruses. This localized induction of cell proliferation is expressed as a wart or condyloma.

HPV-Associated Cancer (Malignant Tumours) and Cell Transformation

The molecular events that lead to transformation of normal skin keratinocytes to malignant cells occurs in three phases, which include intracellular, intercellular and immunological (MHC-dependent) processes. The emergence of cervical carcinoma can be divided into a series of histologically and cytologically differentiable stages (CIN → CIN II → CIN III → invasive carcinoma). The notion that these stages constitute a single causal chain has been questioned in recent years. Gene expression of HPV-6 and HPV-11 (low risk of malignancy) observed in stages CIN I and CIN II and in condylomata is considered as the cause of cell proliferation, but only rarely leads to development of cervical carcinoma.

In most malignant genital tumours, the viral genome does not exist in an episomal state in the nucleus, but it is integrated into the cellular DNA. The degree of integration seems to increase with the progression of malignant transformation (stages CIN II and CIN III). Malignant tumours are monoclonal, i.e. the viral DNA is integrated at the same genome site in all cells of the tumour. The integration of the viral genome occurs randomly, probably in undifferentiated epithelial layers during the persistence stage. This is frequently the result of a recombination event with the genome of the host cell. This is possibly promoted by specific sequences in the genome of "malignant virus types". The viral genome appears to be opened preferentially in the E1 and E2 gene regions (Fig. 19.14), so the nucleic acid sequences of these regions constitute the transition sites to cellular DNA. The integration event predominantly leads to a disruption of the E2 reading frame, and occasionally also to a destruction of the E1 reading frame (insertional mutagenesis). This leads to a loss of E2 protein functions, and, consequently, no infectious papillomaviruses can be generated. Therefore, viral gene expression is also blocked at an early stage. In the case of genital human papillomaviruses with high risk of malignant transformation (HPV-16 and HPV-18), the E2 protein presumably acts as a transrepressor, and represses the transcription of the E6 and E7 genes in undifferentiated skin cells. If the E2 gene is disrupted by the integration event, this will lead to a loss of E2 protein function, which, in turn, gives rise to a deregulated overexpression of E6 and E7 genes. Subsequently, E6 and E7 proteins are present in relatively high concentrations in the cells. In such cases, the abundant proteins interfere with cell cycle regulation by interacting with tumour-suppressor proteins, thus leading to high proliferation rates and cell transformation.

In addition to these cell-division-stimulating functions, E6 and E7 proteins also impinge on interferon-induced defence mechanisms, especially in highly oncogenic papillomaviruses. Particularly affected are the functions that are mediated by class I interferons (IFN-α and IFN-β). Normally, cellular interferon regulatory factor (IRF)-3 is phosphorylated as a result of a viral

infection, and is transported into the nucleus, where it induces the expression of the IFN-β gene and that of chromosome-binding protein, a cofactor of transcription activation. In particular, the E6 protein of HPV-16 binds to IRF-3, causes binding of E6-AP and the protosomal degradation of IRF-3, leading to a suppression of IFN-β production. By contrast, the E7 protein of HPV-16 binds to another member of the IRF protein family, namely IRF-9. This prevents its translocation into the nucleus, thus inhibiting its transcriptional activator function. Furthermore, the E6 proteins of HPV-16 and HPV-18 inhibit the activity of the tyrosine kinase Tyk2. Upon binding of IFN-α to IFN-α receptor, this cellular enzyme interacts with the cytoplasmic domain of the activated receptor, and induces along with Jak kinases the phosphorylation of STAT1 and STAT2 proteins. These are subsequently transported into the nucleus, where they bind to interferon-stimulated response elements (ISRE), and induce the expression of interferon-stimulated genes (▶ Chap. 8). Binding of the E6 protein to the kinase Tyk2 inhibits this process, and hence the expression of IFN-α-regulated genes. It is obvious that the versatile E6 and E7 proteins influence the function of cellular proteins in multifarious ways.

Integrated papillomavirus genomes are not detected in every cervical carcinoma. The overexpression of the E6 and E7 proteins is probably achieved in different ways. For example, the fact that episomal HPV DNA from cervical carcinomas frequently displays mutations in the YY1-binding sites within the LCR which are associated with the inability to bind the cellular factor YY1 can also lead to overexpression of the E6 and E7 genes.

The selective introduction of human chromosome 11 into tumour cells leads to an attenuation of the malignant potential of papillomaviruses. Furthermore, mutations in chromosome 11 are often observed in tumour cells. Hence, it can be inferred that a cellular interfering factor (CIF) which affects tumorigenesis must be coded in this chromosome. CIF possibly controls the activity of protein phosphatase 2A (PP2A). This enzyme regulates the finely tuned balance of phosphorylated and non-phosphorylated versions of cellular factors such as YY1, Oct-1 and NF-IL-6. These factors bind to viral promoters in the LCR and repress the transcription of early genes. Deletion or mutation of the CIF-coding region on chromosome 11 alters or abolishes the activity of the phosphatase, and thus the phosphorylation level of the transrepressing activity of the proteins. This causes the loss of their promoter-binding properties, and they are no longer able to suppress the expression of early genes. This also leads to overexpression of the E6 and E7 proteins.

Besides these intracellular events, exogenous factors also appear to influence proliferation of infected cells. HPV-infected, non-malignant cells exert a bimodal effect on activated macrophages: they induce the synthesis of monocyte chemotactic protein 1 (MCP-1, which corresponds to the chemokine CCL2), which attracts other activated macrophages in vivo that eliminate infected cells. Simultaneously, activated macrophages secrete various cytokines which repress E6/E7 expression. It is known that TNF-α, TNF-β and

interferon-γ (IFN-γ) can inhibit the expression of E6 and E7. In contrast, HPV-immortalized cell lines and HPV-positive tumour cell lines are only able to stimulate macrophages to secrete just small amounts of IL-1, IL-6 and IL-8 as well as TNF-α and granulocyte–macrophage colony-stimulating factor. The decreased production of these cytokines impairs the immunological and inflammatory response against HPV-immortalized cells, which cannot be effectively eliminated.

Besides the suppression of immunological responses by reducing secretion of cytokines, other components of the immune system also appear to be involved in the genesis of cervical carcinomas. For instance, women with the MHC class II type DQw3 have a very high risk of developing cervical cancer. Presumably, the carriers of this HLA type are not able to present HPV-specific epitopes efficiently. In addition, disorders of the cellular immune system also frequently lead to the occurrence of high-grade intraepithelial neoplasias or invasive carcinomas independently of whether they are of genetic nature (Wiskott–Aldrich syndrome) or are triggered by medical treatment with immunosuppressive drugs in organ transplant recipients or by infection with HIV. This finding and the increased incidence of certain HLA types in HPV-associated tumours are further evidence of an essential involvement of the cellular immune system in controlling infections with HPV types which constitute a high risk for malignant tumour development.

Immune Response and Diagnosis

Many exposed surface domains of L1 proteins are conserved among the various papillomavirus types, and hence are accessible to antibody binding. Nevertheless, the papillomavirus infection does not induce a protection against re-infection with the same or a similar virus type. The inability of the organism to establish a protective humoral immune response against papillomaviruses is explained by the location and the course of infection in the outermost skin layers. Virus particles or viral proteins do not come into contact with immunologically active cells in the peripheral blood. Therefore, there is just an irregular antibody production against the L1, L2, E2, E4 and E7 proteins. In women with cervical carcinomas, there is evidence that the amount of antibodies against early and late proteins differs in comparison with control subjects. Antibodies against E7, E4 and L1 are frequently found, but in only 50 % of cases.

Warts, condylomata and malignant tumours are frequently observed in patients with defects in the cellular immune response, e.g. in AIDS patients. This suggests that the T-cell response plays a central role in HPV infection. Spontaneous regression of skin warts, which is normal, probably occurs because of infiltration of cytotoxic T cells into the skin layers which recognize virus-infected cells as foreign. Frequently, such spontaneous regression occurs after injuries or traumatic events, which facilitate the migration of T lymphocytes. Furthermore, it is also observed in small tumour cell clusters. In such cases, cytokines such as TNF-α and transforming growth factor β (TGF-β) seem to trigger Fas-induced apoptosis.

Since virus-specific antibodies are not a reliable criterion for ascertaining the infection, diagnosis is performed solely by detecting the viral DNA in biopsied material and smear samples using the PCR technique or similar methods (such as the hybrid capture assay). Today, the detection of viral DNA by PCR is complemented by the cytological Papanicolaou test, which detects malignant cells. The Papanicolaou test allows the rough distinction into low-pathogenicity and high-pathogenicity papillomavirus types, it is considered to be the state of the art. However, some of the commercially available hybridization tests in strip format provide a far more accurate typing and allow, along with sequencing, the detection of mixed infections. As far as we know, the viral DNA is present as an episome in the "prevalent infection". The progressive severity of the disease and the number of cellular atypias (from CIN I to CIN III and metastatic carcinomas) are correlated with the integration frequency of the viral DNA into the genome of tumour cells. Therefore, to assess the risk of the development of a tumour and its time course, a distinction between the two forms of viral DNA can be made in addition to the determination of the virus type. However, this method has not yet been used in routine diagnostics.

Therapy and Prophylaxis

Most warts are harmless; therefore, a specific therapy is not necessary. If they are located at troublesome sites, or if their removal is advisable for cosmetic reasons, then etching with silver nitrate ($AgNO_3$) or cryotherapy is recommended. A keratolysis can be induced by treatment with salicylic acid or 5-fluorouracil. Most warts will disappear after a few days. Plantar warts are sometimes removed surgically. Laryngeal papilloma can be induced to regress by injection of IFN-α and IFN-β. Laser therapy, electroexcision or electrocoagulation also shows good results in this context. Systemic application of IFN-α and INF-β can be successfully used to treat cellular atypias. Even immune modulators such as imiquimod can be used to treat cutaneous warts. They stimulate particularly the non-adaptive immune response by activation of Toll-like receptors, leading to the evanescence of the lesions (▶ Chaps. 8 and ▶ 9).

Surgical removal is the only method to eliminate malignant forms of the disease in the genital area. Early diagnosis plays a very important role in the prognosis. In advanced stages, there is a danger that growing malignant cells break through the basal membrane and invade the bloodstream after neovascularization of the in situ carcinoma, and so metastases can develop in other organs.

A vaccine against high-risk HPV types is currently available. The confinement of infection to the outer layers of the epidermis has proven to be an aggravating factor in the development of a vaccine. Protection by virus-neutralizing antibodies has seemed questionable because they do not reach the infection site. The first vaccine protecting against infection with genital papillomaviruses – Gardasil (Sanofi Pasteur MSD) – was approved in the European Union in 2006, followed by the approval of another vaccine, Cervarix (GlaxoSmithKline), in 2007. Both vaccines rely on genetically engineered virus-like particles that are formed by recombinant expression of the viral L1 gene. The L1 proteins have the capacity

to assemble into particulate structures that resemble infectious virions but do not contain a viral genome. Owing to their particulate structure, these virus-like particles induce the production of neutralizing antibodies. Cervarix contains virus-like particles of HPV-16 and HPV-18, whereas the quadrivalent vaccine Gardasil additionally contains particles of HPV-6 and HPV-11. Both vaccines protect against infection with the respective viruses and – to a lesser extent – against related HPV types, preventing the development of cancers and anogenital warts which are caused by them. The use of the vaccine is currently recommended for girls before puberty, i.e., girls between 12 and 17 years. In this way, infection with these virus types should be fundamentally prevented. However, since the effect on the incidence of cervical carcinoma will be perceptible only after a few decades, the implementation of regular cancer screening must continue. In addition, new therapeutic vaccines are being developed for application in patients with cervical carcinoma. These vaccines are directed against the E6 and E7 proteins. The cellular immune system of vaccinated people should be enabled to recognize malignant cells, and to prevent or restrain tumour development.

19.3.6 Animal Pathogenic Papillomaviruses

19.3.6.1 Animal Papillomaviruses (Wart Viruses)

Epidemiology and Transmission

Numerous papillomaviruses are known which cause severe and economically important infectious diseases in animals. All have in common the induction of skin tumours, which are usually histopathologically classified as fibropapillomas, and rarely as fibrosarcomas (Table 19.10). These viruses are transmitted directly or by contaminated objects such as harnesses or halters. Small skin injuries are a prerequisite for infection. Papillomaviruses are normally very host-specific. An exception seems to be the infection of horses with some BPV types (BPV-1 and BPV-2) which cause equine sarcoid.

Clinical Features

After an incubation period of several weeks, the formation of warts becomes apparent. The respective papillomaviruses cause the skin lesions listed in Table 19.10. The warts are painless, but may be mechanically troublesome, such as warts on the teats during milking, or oral warts as an obstacle during eating. The infiltrating growing equine sarcoid can become very large, leading to a propensity for bleeding owing to secondary infections.

Pathogenesis

The pathogenesis of animal papillomavirus infections is very similar to that of human papillomaviruses. Through skin lesions the virus enters the cells of the stratum basale of the epithelium, in which the viral DNA is replicated and the early genes are expressed (Fig. 19.18). Thus, animal pathogenic viruses can also lead to a long-term malignant cell transformation and tumorigenesis

Table 19.10 Selected animal pathogenic papillomaviruses and their diseases

Genus	Virus	Symptoms
Deltapapillomavirus	Bovine papillomavirus types 1 and 2	Cutaneous fibropapilloma
		Equine sarcoid (fibrosarcoma)
	Reindeer papillomavirus	Fibroma
	European elk papillomavirus	Fibroma
	Ovine papillomavirus types 1 and 2	Cutaneous papilloma
Epsilonpapillomavirus	Bovine papillomavirus type 5	Teat papilloma
Zetapapillomavirus	*Equus caballus* papillomavirus 1	Cutaneous papilloma
Kappapapillomavirus	Cottontail rabbit papillomavirus	Cutaneous papilloma
	Rabbit oral papillomavirus	Cutaneous papilloma
Lambdapapillomavirus	Canine papillomavirus	Oral papilloma
	Feline papillomavirus	Cutaneous papilloma
Xipapillomavirus	Bovine papillomavirus types 3, 4 and 6	Cutaneous and intestinal papilloma
Thetapapillomavirus	*Psittacus erithacus timneh* papillomavirus	Papilloma

(warts, sarcoid). Late genes are expressed in the epithelial cells of the stratum granulosum, and exhibit an advanced degree of differentiation. Synthesis of the structural proteins and assembly of the various components into progeny viruses eventually occur in dying cells (stratum corneum) and are shed from the skin surface. The emergence of papillomas is predominantly monocausal. However, in addition to BPV-4, bracken fern also plays a role as a cofactor in the formation of tumours of the digestive tract and urinary bladder in cattle (Table 19.9).

Immune Response and Diagnosis

The papillomas of domestic animals usually heal spontaneously, with the exception of equine sarcoid and intestinal papillomatosis of cattle. This is the result of a slowly developing, but effective immunity. Virus-specific antibodies are detectable and correlate with the protective effect. The diagnosis is performed by the characteristic histological picture or by detection of the virus in the warts by electron microscopy.

Control and Prophylaxis

Stall-specific vaccines based on wart material extracted from the affected animal can be produced and can be inactivated with formaldehyde. They have been used with some success in cattle and dogs.

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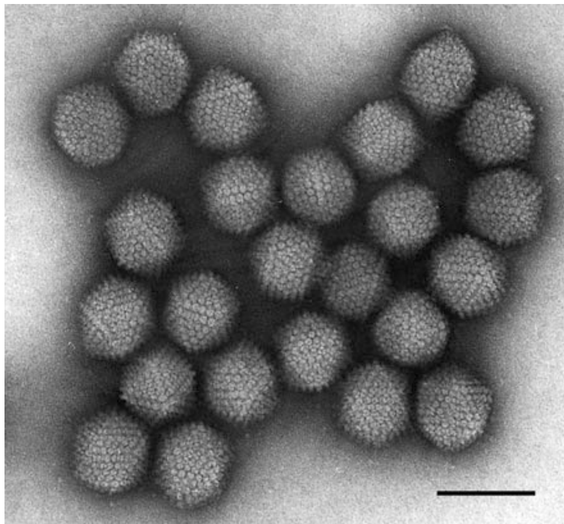
19.4 Adenoviruses



In 1953, Wallace P. Rowe and co-workers isolated adenoviruses from tonsils and adenoid tissue which could be multiplied in cell culture. Today, more than 52 different human adenoviruses are known. They predominantly cause respiratory illnesses, but also ailments in the gastrointestinal tract and the conjunctiva of the eye. In 1962, John J. Trentin and colleagues showed that human adenovirus 12 (HAdV-12; other types are denominated similarly) is capable of causing malignant tumours in rodents. This was the first indication that there are also human pathogenic viruses with carcinogenic potential. However, a causal relationship with human tumours has not been ascertained so far. In addition to the human adenoviruses, a large number of other adenovirus species have been discovered in mammals and birds.

19.4.1 Classification and Characteristic Prototypes

The family *Adenoviridae* is divided into five genera (Table 19.11). The genus *Mastadenovirus* contains the different adenovirus species of mammals (humans, monkeys, cattle, sheep, swine, dogs) and the genus *Aviadenovirus* contains those of birds. Because of molecular and structural differences, the genera *Siadenovirus*, *Atadenovirus* and *Ichtadenovirus* have been created and other adenoviruses of mammals, birds, reptiles, and fishes have been assigned to them.

Human adenoviruses of the genus *Mastadenovirus* are classified into seven viral species (HAdV-A to HAdV-G), which in turn are divided into 67 serotypes. The assignment of serotypes to the viral species is based on several criteria, including similarities in genome organization and DNA sequences, the host tropism, the carcinogenic potential in rodents and the growth properties in cell cultures. Serotypes are assigned to different viral species when antibodies directed against their surface structures are not cross-neutralizing, and when a phylogenetic distance of more than 10 % can be calculated on the basis of a genetic distance matrix of nucleotide sequences coding for the viral protease, the protein pVIII, the hexon protein and the DNA polymerase. If the phylogenetic distance of individual serotypes is less than 5 %, additional similarities can result in adenoviruses which were isolated from different hosts being assigned to a common species. This is the case for the chimpanzee isolates simian adenoviruses 22, 23, 24 and 25 and bovine adenovirus 2, which, on the basis of their sequence homologies, have been assigned to HAdV-E and ovine adenovirus A, respectively.

RNA Splicing Was Discovered in Adenoviruses

An important molecular process that occurs in eukaryotic cells was first observed in adenoviruses: RNA splicing, a regulated molecular process during which smaller RNA species are cleaved from a frequently very large precursor RNA molecule in the nucleus. These "mature", translatable mRNA molecules may contain sequences from distant regions of the genome.

Table 19.11 Characteristic prototypes of adenoviruses

Genus	Human virus	Animal virus
Mastadenovirus	Human adenovirus A (human adenoviruses 12, 18, 31 and 61) Human adenovirus B (human adenoviruses 11, 50) Human adenovirus B1 (human adenoviruses 3, 7, 16, 21) Human adenovirus B2 (human adenoviruses 11, 14, 34, 35 and 55, and simian adenovirus 21) Human adenovirus C (human adenoviruses 1, 2, 5, 6 and 57, and bovine adenovirus 9) Human adenovirus D (human adenoviruses 8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51, 53, 54, 58, 60, 62–67) Human adenovirus E (human adenoviruses 4 and simian adenoviruses 22–26, 30, 36–39) Human adenovirus F (human adenoviruses 40 and 41 and simian adenovirus 19) Human adenovirus G (human adenovirus 52 and simian adenovirus 1)	Simian adenovirus A (simian adenoviruses 3, 4, 6, 9, 10, 14, and 48) Bovine adenovirus A (bovine adenovirus 1) Bovine adenovirus B (bovine adenovirus 3) Bovine adenovirus C (bovine adenovirus 10) Canine adenovirus (canine adenoviruses 1 and 2) Equine adenovirus A (equine adenovirus 1) Equine adenovirus B (equine adenovirus 2) Murine adenovirus A (murine adenovirus 1) Ovine adenovirus A (ovine adenoviruses 2–4, bovine adenovirus 2) Porcine adenovirus A (porcine adenoviruses 1–3) Porcine adenovirus B (porcine adenovirus 4) Porcine adenovirus C (porcine adenovirus 5) Bat adenovirus A (bat adenovirus TJM) Bat adenovirus B (bat adenovirus 2)
Aviadenovirus		Fowl adenovirus A (fowl adenovirus 1; CELO virus) Fowl adenovirus B (fowl adenovirus 5) Fowl adenovirus C (fowl adenoviruses 4 and 10) Fowl adenovirus D (fowl adenoviruses 2, 3, 9, and 11) Fowl adenovirus E (Fowl adenoviruses 6–8) Goose adenovirus (Goose adenoviruses 1–3) Falcon adenovirus A Turkey adenovirus B (turkey adenovirus 1)
Siadenovirus		Turkey adenovirus A (turkey adenovirus 3; turkey haemorrhagic enteritis virus, marble spleen disease virus of pheasants) Frog adenovirus A (frog adenovirus 1)
Atadenovirus		Ovine adenovirus D (ovine adenovirus 7 and goat adenovirus 1) Duck adenovirus A (duck adenovirus 1; egg drop syndrome virus) Bovine adenovirus D (Bovine adenoviruses 4, 5, and 8 and bovine adenovirus strain Rus) Snake adenovirus A
Ichtadenovirus		Sturgeon adenovirus A

CELO chicken embryo lethal orphan

Only after splicing are they transported to the cytoplasm, where they are translated into proteins. RNA splicing was discovered simultaneously by Philip A. Sharp and Louise T. Chow in 1977. This discovery revealed that mRNA is not always collinear with the respective gene sequences in eukaryotes. The old "one gene–one enzyme" hypothesis was refuted.

19.4.2 Structure

19.4.2.1 Virus Particle

Adenoviruses have a diameter of 80–110 nm. They have non-enveloped capsids, which have an icosahedral structure exhibiting 20 faces and 12 vertices. Each of the vertices has a spike-like protein protrusion (Fig. 19.19). Adenovirus particles consist of 252 capsomers, namely 249 hexons and 12 pentons. The vertices are made up of pentons;

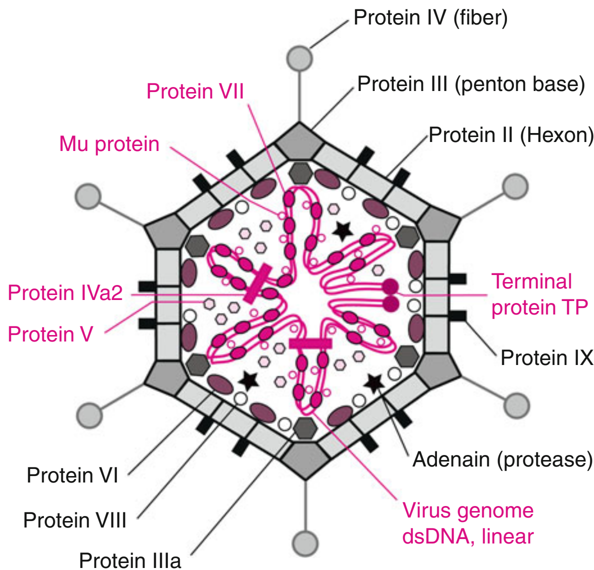


Fig. 19.19 Structure of an adenovirus particle. Fibre proteins (IV, spikes) are located at the 12 vertices of the icosahedral particle, and project from the shell surface. They are associated with penton base proteins (III), which are situated at the vertices of the icosahedron, and are complexed with penton-associated IIIa proteins at the inner side of the particles. The faces are made up of hexon proteins (II), which are associated with IX proteins outside the particles and with the proteins VI and VII inside the protein shell. The linear double-stranded DNA genome is associated with the proteins V, VII and Mu in a core structure in the interior of the particle. The 5′ genome ends are covalently linked to the terminal protein (*TP*). Furthermore, the particles also contain a few copies of both the IVa2 protein and the protease adenain. The Roman numerals are the internationally used designations for the structural proteins (see the text). *dsDNA* double-stranded DNA

the name refers to their location at the fivefold symmetry axes of the icosahedral capsid and the five neighbouring capsomers at the faces with which they are associated. Pentons are composed of a penton base and a fibre protein. The penton base is a pentamer of the viral structural protein III (80 kDa). Each of the 12 penton bases is associated with a fibre protein, which is a homotrimeric complex that protrudes 9–30 nm from the vertices. The adenoviral fibre protein has a knob at the end of the shaft. The length of the shaft differs among the various mastadenovirus species. The length and flexibility of the fibre protein are determined by the number of homologous protein domains (pseudo-repeats), which can range from six in viruses of the HAdV-B species to 23 among the members of the HAdV-A species. Each repeat unit consists of a consensus sequence that forms two antiparallel β-sheet domains which are connected with a β-turn and a loop region of variable sequence and size (Table 19.12). HAdV-F viruses of serotypes 40 and 41 possess two genes encoding fibre proteins. Each of the two fibre proteins is associated with a penton base in these virus types. Since the two fibre proteins have different shaft lengths, they protrude different distances from the capsid surface as well. The animal pathogenic aviadenoviruses have also two different fibre proteins. In this case, both fibre proteins are anchored to each vertex. Another viral polypeptide is also connected with penton base proteins, the penton-associated protein (IIIa). The capsomers that form the faces of the icosahedron are called hexons; the designation refers to the six neighbouring capsomers that are adjacent to them. Each capsid face is composed of 12 hexons, each of which is a trimer of the viral hexon protein (II, 120 kDa). The hexons are also linked with hexon-associated proteins: the IX proteins are found on the particle surface at the contact sites between the hexons, whereas polypeptides VI and VIII are located inside the capsid. The interior of the particle contains a nucleoprotein complex, consisting of the viral DNA genome and about 160 and 800 units of proteins V and VII, respectively. The V protein is absent among members of atadenoviruses. A terminal protein (TP) is covalently linked with each 5′ end. Furthermore, the viral genome is associated with a few units of the multimeric protein IVa2 and with about 100 copies of the Mu protein. The protein Mu is a small, basic protein of 36 amino acids which is formed by proteolytic cleavage of the precursor protein pX, and resembles cellular protamines. The viral protease adenain, which is responsible for the cleavage, is also part of the virion. The Roman numerals II to IX that are used to denominate the structural proteins of adenoviruses correlate with the migration behaviour which is observed after their electrophoretic separation in polyacrylamide gels. Protein I has been omitted because this is a complex of two proteins.

19.4.2.2 Genome Organization and Structure

The genome of adenoviruses is double-stranded, linear DNA with a length of 36–38 kb (depending on the virus type, 35,937 base pairs in HAdV-2). The two 5′ ends of the genome are covalently linked to a serine residue of a terminal protein (TP; 55-kDa). The two TP molecules are able to interact with each other by non-covalent interactions, thus keeping the genome in a quasi-circular state (Fig. 19.20a). The DNA is complexed with two arginine-rich viral proteins, proteins V and VII, which thus have a pronounced basic character. Protein VII

Table 19.12 Association of human adenoviruses A to G with specific diseases, their oncogenic potential in the rodent system, their receptor preference and characteristics of their fibre proteins

Adenovirus species	Target organs and diseases	Incidence in human infections	Oncogenic potential in rodents	Cellular receptors		Number of repeats in the shaft of fibre proteins/ fibre length
				Penton base	Fibre proteins[a]	
A	Intestine–gastroenteritis	Rare	High	Integrins $\alpha v\beta 3$/ $\alpha v\beta 5$	**CAR**	23 repeats/28–31 nm
B1	Respiratory tract, conjunctiva/eye – acute respiratory illness, pneumonia, conjunctivitis	Frequent	Medium	Integrins $\alpha v\beta 3$/ $\alpha v\beta 5$	**CD46**, CD80, CD86	6 repeats/9–11 nm
B2	Respiratory tract, conjunctiva/eye, kidney/urinary tract – acute disease of the respiratory tract, pneumonia, conjunctivitis, acute haemorrhagic cystitis, severe progression in immunosuppressed patients	Frequent	Medium	Integrins $\alpha v\beta 3$/ $\alpha v\beta 5$	**CD46**, CD80, CD86	6 repeats/9–11 nm
C	Respiratory tract, conjunctiva/eye, lymphocytes – mild respiratory disease	Frequent	Very low	Integrins $\alpha v\beta 3$/ $\alpha v\beta 5$	**CAR**, MHC class I, VCAM-1	22 repeats/23–31 nm
D	Conjunctiva/eye – severe epidemic keratoconjunctivitis with HAdV-8, HAdV-19a and HAdV-37	Rare	Very low	Integrins $\alpha v\beta 3$/ $\alpha v\beta 5$	**Sialic acid**, CD46, CAR	8 repeats/12–13 nm
E	Respiratory tract, conjunctiva/eye – acute respiratory disease, conjunctivitis (also severe cases)	Rare	Very low	Integrins $\alpha v\beta 3$/ $\alpha v\beta 5$	Unclear, CAR?	12 repeats/17 nm
F	Intestine – gastroenteritis	Rare	Very low	Unknown	Unclear, CAR?	Short fibre protein, 12 repeats; long fibre protein, 21/22 repeats/about 29 nm
G	Intestine – gastroenteritis	Rare	Unknown	Unknown	Unknown	9 or 17 repeats/unknown

CAR coxsackievirus and adenovirus receptor, *VCAM-1* vascular cell adhesion molecule 1

[a]The principal receptors for attachment are indicated in *bold*

is composed of approximately 23 % arginine and has a molecular mass of 18.5 kDa. It is associated with the genome throughout its entire length, and interacts with amino acid sequences at the inner side of the capsids. Hence, it is responsible for the tightly condensed, ordered packaging of the DNA genome within the virion. Protein V is also associated with the DNA and has a molecular mass of 48.5 kDa. It is also responsible for proper folding of the

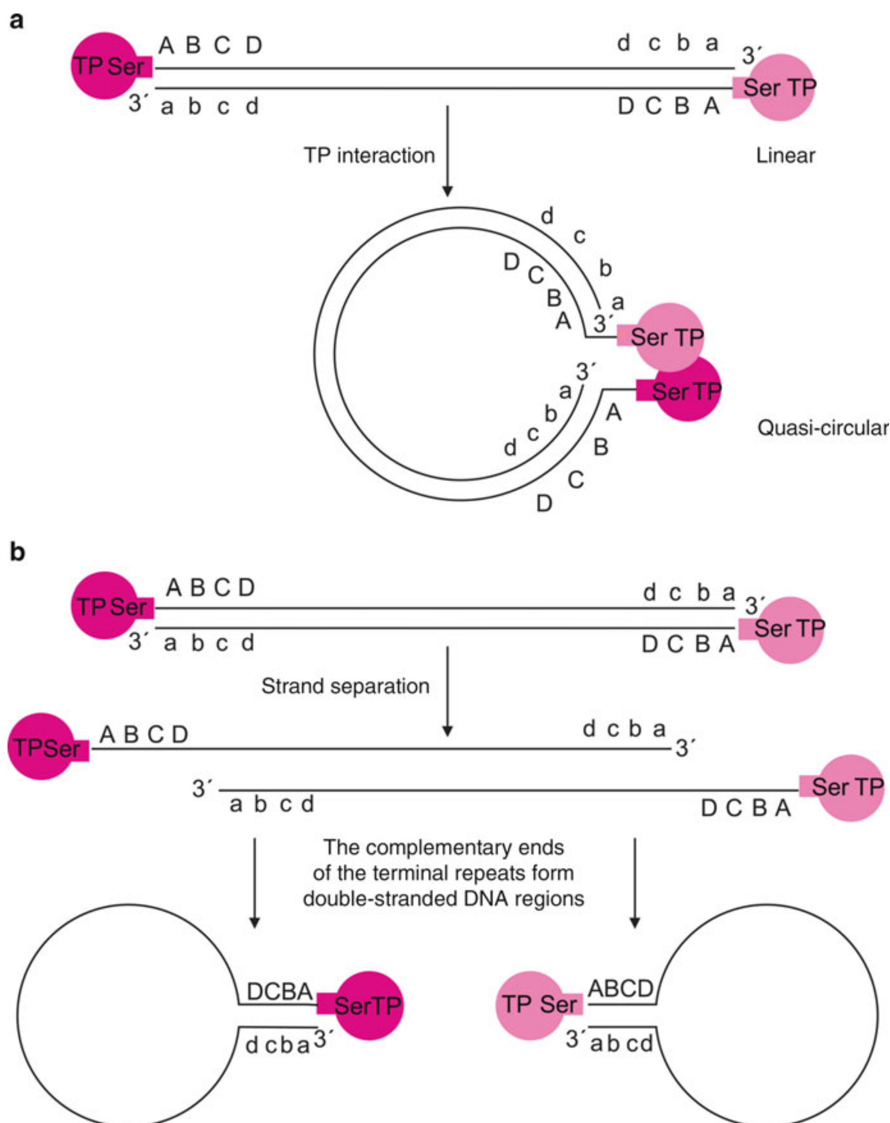


Fig. 19.20 (continued)

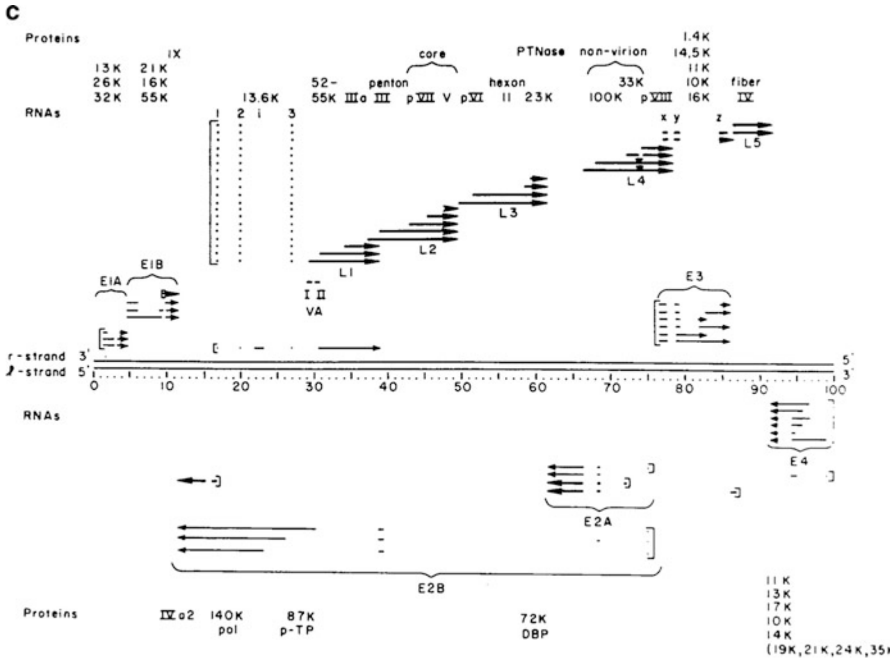


Fig. 19.20 Genome structure of adenoviruses. (**a**) The adenovirus genome consists of a linear, double-stranded DNA. Each 5′ end is covalently linked to a serine residue of the terminal protein (*TP*). The terminal proteins interact with each other, maintaining the genome in a quasi-circular state. (**b**) The adenovirus genome contains inverted repeats at the ends. This is indicated by *upper-case letters* and *lower-case letters*. If the double-stranded genome is converted into single-stranded DNA, then the ends can form double-stranded regions. They are called "panhandle" because of their appearance. (**c**) Transcription map of human adenovirus 2 (HAdV-2) and its mRNA families. The double-stranded DNA genome of the virus (*r* and *l* strands) is represented by the *double lines in the middle*, and is divided into genome units. *Arrows* indicate the different, partially spliced mRNAs and the direction of transcription. *Thick arrows* represent late mRNAs; *thin arrows* represent early transcripts. Most late mRNAs start at genome unit 16.3 and contain a tripartite leader sequence of non-coding exons, which are indicated by the numbers *1*, *2* and *3*. Some late mRNAs contain a fourth leader segment (*i*); it encodes a protein of 14 kDa. *Thick arrows* also represent some transcripts derived from early active transcription units, which, however, are detectable in increased quantities only late during the infection cycle. In addition, proteins are indicated by abbreviations or the corresponding molecular weights. The *Roman numerals* denote the corresponding viral structural proteins (From Shenk 1996)

genome within the virion, as it non-covalently interacts with the DNA in addition to its interaction with the inner side of pentons.

Inverted nucleic acid sequence repeats are located at the ends of the genome and are 54–166 base pairs long, depending on the virus type (103 base pairs in HAdV-2 and HAdV-5, and 164 base pairs in HAdV-12). Purified genomes that are separated into single strands by heat denaturation followed by hybridization during annealing exhibit a "panhandle-like" structure in the electron microscope because the inverted

repeats at the ends can form double-stranded regions (Fig. 19.20b). These termini are important for initiation of DNA replication.

Both DNA strands encode proteins whose genes are clustered into groups which are located in close physical proximity according to their biological functions in the same phase of the infection cycle (Fig. 19.20c). The genome consists of five coding regions, of which four (E1 to E4) are expressed early during infection. The group of late expressed genes (L) encodes the viral structural proteins.

19.4.3 Viral Proteins

19.4.3.1 Early Proteins

E1 Proteins

The E1 genes are transcribed first during the viral infection cycle. Therefore, they constitute the group of adenoviral immediate early genes because their expression does not require the previous synthesis of other viral factors. According to the current international alignment, their loci are located at the left end of the genome and contain approximately 4,000 base pairs, constituting 11 % of the genome. The E1 region contains two active transcription units: one encodes the group of E1A proteins and the other encodes the group of E1B proteins. Both groups of proteins possess oncogenic potential, and thus are capable of inducing malignant transformation of rodent cells in vitro.

Two mRNA species are transcribed from the E1A genes. These transcripts have identical ends, and are 900 and 1,000 nucleotides long; however, they differ by the excision of different intron sequences from the precursor RNAs (Fig. 19.21). The two early forms of E1A proteins are translated from these transcripts. These proteins have a length of 289 and 243 amino acid residues in HAdV-5. They have identical amino and carboxy termini, and are phosphorylated in differing degrees by the PPA2, whose activity is regulated by the viral protein E4 (14-kDa protein). The differing phosphorylation pattern confers on them a heterogeneously appearing molecular mass. A nuclear localization sequence is situated at the carboxy terminus. Comparison of the amino acid sequences of E1A proteins from different adenoviruses has revealed that they contain three highly conserved regions (CR1 to CR3). The CR3 domain is absent in the short E1A variant because this coding region was removed from the mRNA by alternative splicing. Various E1A protein functions are associated with the CR domains. In the viral infection cycle, the E1A proteins act primarily as transcriptional activators, and induce transcription of the E2, E3 and E4 genes. E1A does not bind directly to regulatory DNA regions, but exerts its transactivating function by interacting with host proteins that binds to the promoter regions of the E2, E3 and E4 genes. The consensus sequence of a zinc finger motif resides in the CR3 domain. Using this zinc finger motif, the E1A protein interacts with the cellular transcription factor TFIID that binds to TATA boxes which are located upstream of the transcription start. In addition to the viral E2, E3, and E4 genes, some cellular genes are also activated, e.g. the gene encoding the cellular chaperone hsp70, a growth factor for epithelial cells, and the tubulin gene.

In addition to the activation of viral and cellular promoters, E1A proteins have a variety of other functions. They act not only as transactivators, but also as

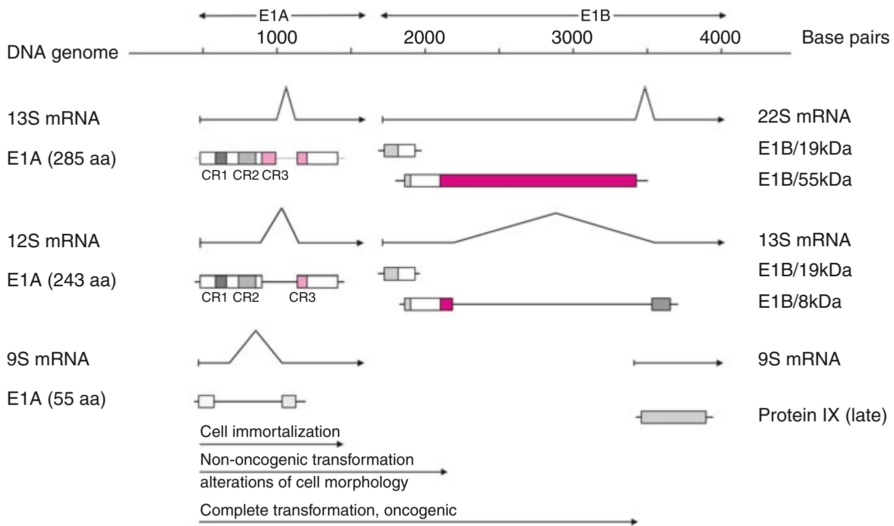


Fig. 19.21 Principal products of the E1 region of HAdV-2. The E1 region of the double-stranded adenoviral DNA genome encoding the E1 proteins is shown in the *upper part*. With use of different splice donor and acceptor sites, three mRNAs are formed from the E1A region (*left half*), which encode two different forms of E1A proteins, as well as an additional small protein. The location of the CR1 and CR3 domains, which are conserved within E1A proteins of different adenovirus types, is indicated in *light red*. Three transcripts are synthesized from the E1B region. Both the 19-kDa E1B protein and the 55-kDa E1B protein are translated from the largest mRNA, whereas the smaller transcript is used for the synthesis of the 19-kDa E1B protein and a shortened version of the 55-kDa protein. The third mRNA uses a different start codon, and is necessary for translating the late structural protein IX. The *arrows in the lower part* indicate the sequences of the E1 region which are responsible for cell transformation and immortalization. *aa* amino acids

transrepressors. This activity has been particularly associated with the 243 amino acid long E1A variant. The E1A protein is similar to the E7 gene product of papillomaviruses and to the large T antigen of SV40 with respect to another function: E1A proteins are capable of inducing DNA synthesis in resting cells which are arrested in the G_1 or G_0 phase of the cell cycle, thus enabling them to enter the S phase. This property correlates with the transforming activity of E1A proteins. It is based on the interaction with the cellular tumour-suppressor protein RB105, as well as the proteins RB107 and p130. All these proteins are members of the pocket protein family and exert similar functions. They are nuclear phosphoproteins that interact with other protein factors and control their function via "pocket"-like domains. The postulated mechanism is described in ▶ Chap. 6 and Sect. 19.3.3 (Figs. 19.16 and 19.17). In the case of E1A proteins, this activity resides in the CR1 and CR2 domains, through which the E1A protein interacts with the cellular retinoblastoma tumour suppressors, thus dissociating the complex between dephosphorylated RB105/RB107 proteins and cellular E2F transactivators, which keep E2F proteins inactive. The E2F factors are activated, enter the nucleus, bind to promoter regions and induce the transcription of cellular genes that determine entry into

the S phase. Furthermore, E1A proteins also bind to cellular histone acetyltransferases (p300, p400), affecting their function. The histone–DNA complexes are altered by changing the acetylation pattern of the basic lysine and arginine residues of histones. Moreover, E1A proteins also interact with CREB-binding protein. In this way, the activity of cellular promoters of the group of cyclic AMP response elements is altered.

In addition to the two major E1A proteins, other versions are also synthesized from the E1A gene in the late infection phase (Fig. 19.21a). These E1A variants have the same amino terminus, but are translated from mRNA molecules which are generated by differential splicing. These minor variants are probably involved in the regulation of the manifold functional activities of E1A proteins.

An mRNA of 2,200 nucleotides in length is transcribed from the E1B genes. Two different start codons initiate the translation of the two E1B proteins (19 kDa and 55 kDa), which differ in their sequences and are encoded by two overlapping reading frames (Fig. 19.21). The small E1B protein (19 kDa) inhibits apoptosis and exerts a similar effect as the antiapoptotic protein Bcl2 and related polypeptides. Virus mutants with defective 19-kDa E1B protein show an accelerated cytopathic effect in infected cells. The larger E1B protein (55 kDa) plays an important role in cell transformation: its co-expression with E1A proteins is a prerequisite for the development of the complete transformed state in rodent cells. This function is explained by the interaction with p53, another cellular tumour-suppressor gene (▶ Chap. 6, Sects. 19.1 and 19.3).

The molecular effects of p53 differ from those of retinoblastoma proteins. However, p53 also prevents entry into the S phase of the cell cycle. The tumour-suppressor protein p53 is induced and present in high concentrations in cells that contain mutations and DNA damage, and causes cell cycle arrest at the transition between the G_1 and the S phase. This delay allows the cells to eliminate DNA damage by using their DNA repair mechanisms before replication starts, and the mutated sequences are passed on to daughter cells. If DNA repair is not successful, damaged cells are eliminated from the organism by the induction of apoptosis. The 55-kDa E1B protein binds to the amino-terminal transcriptional activation domain of p53 and inhibits its activity. This leads to a premature entry into the S phase. This activity of the 55-kDa E1B protein is supported by another viral protein (E4-ORF6, 34 kDa), which also interacts with p53. The interaction with the E1B protein and its cooperation with E4-ORF6 leads to p53 ubiquitination and SUMOylation, which – like ubiquitination – also induces the proteolytic degradation of p53. The 55-kDa E1B protein is functionally similar to the large T antigen of SV40 and the HBx protein of hepatitis B viruses, which also bind to the p53 protein and inhibit its activity. Like the adenoviral E1B protein, the E6 protein of papillomaviruses not only binds to p53, but it additionally induces its ubiquitination and proteasomal degradation. All these processes emphasize that proliferating cells are necessary for viral replication. Polyomaviruses, papillomaviruses, adenoviruses and probably also hepatitis B viruses and herpesviruses have evolved similar mechanisms to reverse the G_0/G_1 phase, and to induce cell cycle progression towards the S phase of the cell cycle.

The large E1B protein has another important function that ensures the controlled progression of viral infection: it promotes, also in cooperation with E4-ORF6, the nuclear export of late mRNA species that encode structural proteins, whereas cell-specific mRNAs are retained in the nucleus.

E2 Proteins

The expression of E2 proteins depends on the transactivating function of E1A proteins; therefore, they are not considered to be immediate early genes. The E2 gene region encompasses more than 20 kb in the central region of the genome, and is divided into two sections, E2A and E2B (Fig. 19.20c). The different mRNA products are formed by using different splicing signals from large RNA precursor molecules. The E2 gene product is required for replication of the viral DNA genome. The E2A gene encodes a protein (72 kDa) which is multiply phosphorylated at the amino-terminal domain and capable of binding to single-stranded DNA. It binds to the single strands that arise during replication of the viral DNA, thus preventing duplex structure formation and protecting them from degradation by nucleases (Fig. 19.22b). The E2B region encodes two proteins: the first protein is the precursor polypeptide pTP (80 kDa) of the mature TP (55 kDa), which is formed by a viral protease during morphogenesis; the second protein is the viral DNA polymerase (140 kDa).

E3 Proteins

The E3 region contains the genetic information for a number of relatively small proteins which are not essential for the viral replication cycle, and are not conserved among adenoviruses. However, they can influence the course of infection, and are also associated with the establishment of persistent adenovirus infections. E3 proteins are also translated from mRNA molecules which arise from a larger precursor mRNA by using alternative splicing. The various E3 gene products have the following functions: A glycosylated 19-kDa protein (E3-gp19K) reduces the concentration of MHC class I proteins on the surface of infected cells by preventing their correct glycosylation in the ER and in the Golgi apparatus. Consequently, these membrane proteins cannot be transported to the cell surface, and the cells are no longer able to present peptides from viral proteins in complex with MHC class I antigens. Thus, the deficiency of MHC class I proteins on the cell surface hinders the recognition and elimination of infected cells by cytotoxic T lymphocytes. It is controversial whether this process is involved in the development of the oncogenic potential of adenoviruses because particularly members of highly oncogenic species HAdV-A such as HAdV-12 and HAdV-31 do not have a gene encoding an E3-gp19K protein. Probably, the mentioned activity of the E3-gp19K proteins is closely related to the ability of adenoviruses to establish persistent infections.

Another protein of the E3 cluster (E3-14.7 K) renders infected cells insensitive to TNF-α-mediated induction of apoptosis. Hence, it counteracts an activity of the E1A protein that increases the sensitivity to cytolysis. The receptor internalization and degradation (RID) protein complex composed of proteins of the E3 region (E3-10.4 K or RID-α and E3-14.5 K or RID-β) also antagonizes the TNF-α-mediated cytotoxicity. It causes the internalization and degradation of Fas proteins (CD95) which are bound to their receptors on the cell surface.

Furthermore, the RID protein complex induces internalization of EGF receptors on the cell surface. In addition, an E3 gene encoding the E3-12.5 K protein is

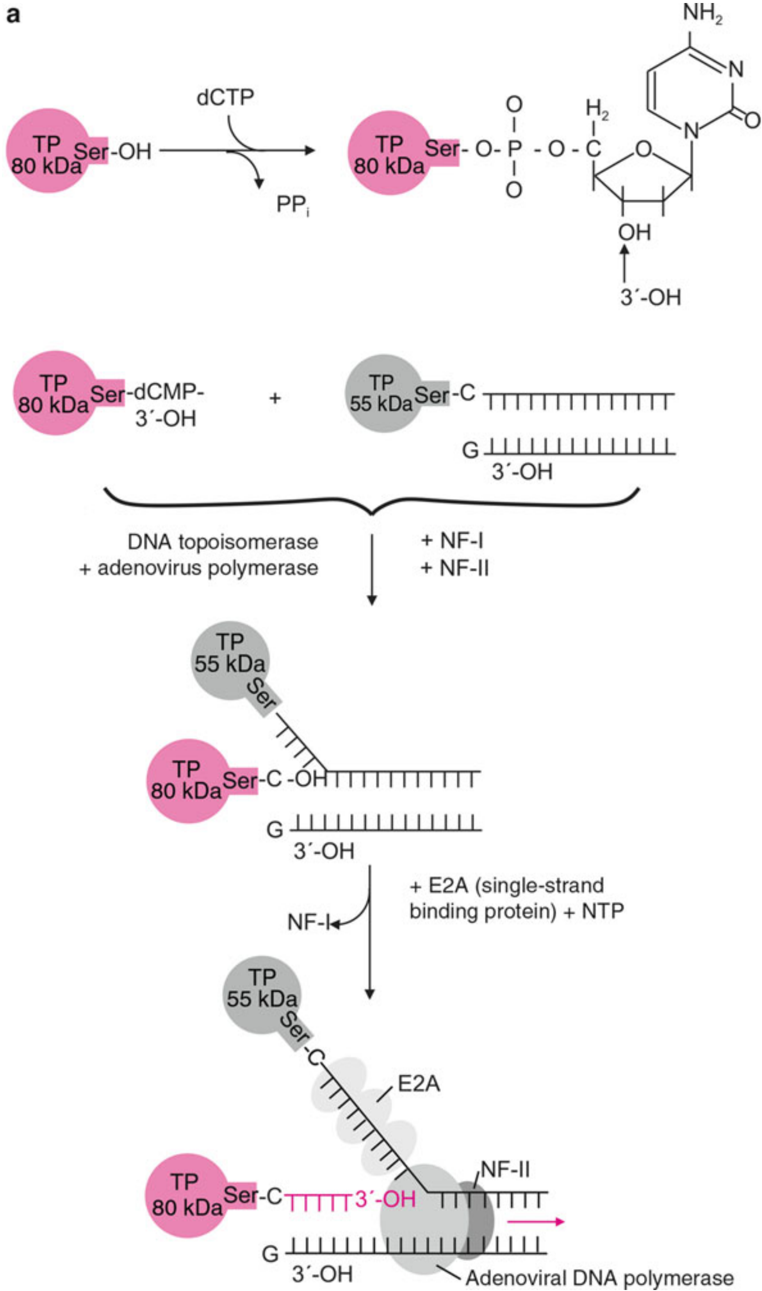


Fig. 19.22 (continued)

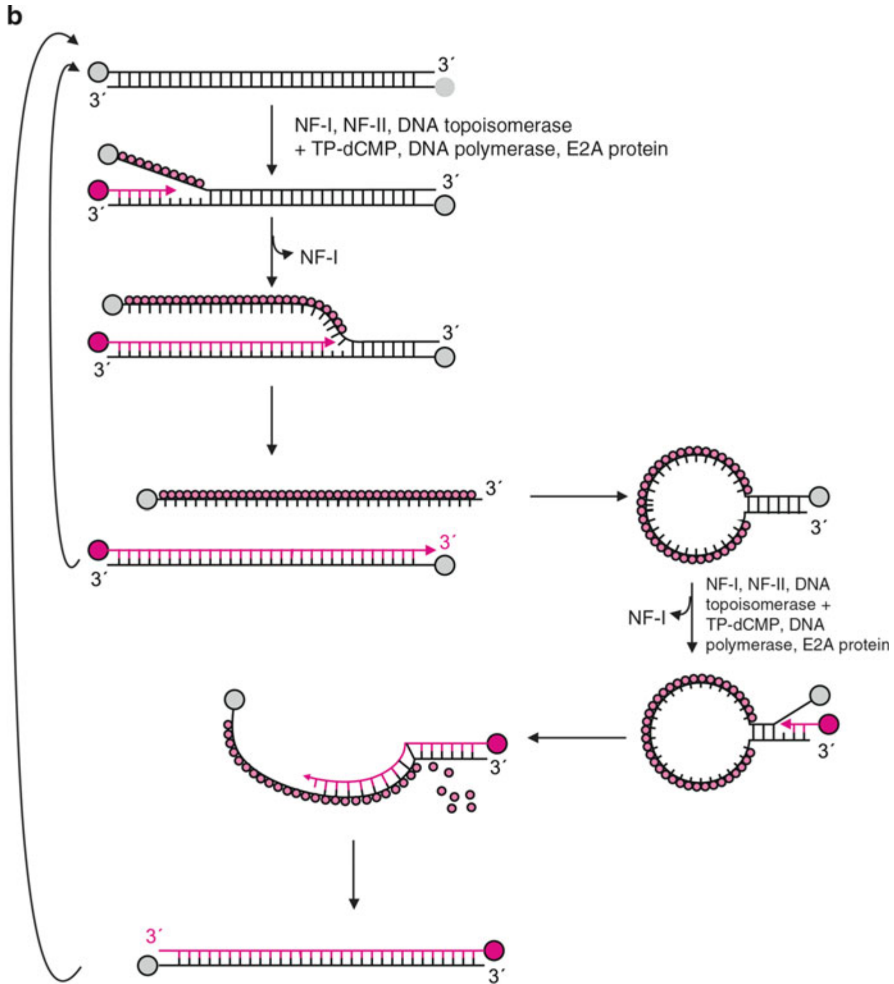


Fig. 19.22 Adenovirus genome replication. (**a**) Initiation of DNA polymerization. The terminal protein (*TP*) is initially synthesized as a product with a molecular mass of 80 kDa. A dCMP residue is covalently linked to the hydroxyl group of a serine in the terminal protein by an esterification reaction under cleavage of pyrophosphate. The 80-kDa terminal protein interacts with the 55-kDa terminal proteins, which are covalently linked to the 5′ ends of the genome. An initiation complex is formed under the participation of the cellular DNA topoisomerase, nuclear factors NF-I and NF-II and the adenoviral DNA polymerase. The cytosine, which is bound to the 80-kDa terminal protein, hybridizes with the guanosine residue at the 3′ end of the genome and thereby a parent strand (that with the covalently bound 55-kDa terminal protein at the 5′ end) is progressively displaced from the DNA duplex, and complexes with the single-strand binding protein E2A. The cytosine provides the necessary primer 3′-OH end, to which the complementary nucleotides are consecutively added during DNA synthesis. Nuclear factor NF-1 is no longer required for the following steps. (**b**) Elongation and double-strand synthesis. The DNA polymerization is initiated by binding of the complex 80-kDa terminal protein (*large red circles*) and dCMP. The displaced parental strand associates with E2A proteins during polymerization (*small red circles*), and the

present in all adenoviruses, with the exception of HAdV-F (HAdV-40 and HAdV-41). Its function is unknown.

The E3-11.6 K protein, or adenovirus death protein, is synthesized early in large concentrations, but is produced particularly during the late phase of the infection cycle of HAdV-C. It is then involved in the lysis of infected cells, and in the release of progeny viruses. Again, only HAdV-C viruses produce an E3-6.7 K protein. It is a glycosylated protein which is embedded in the membranes of the ER, and is retained in this compartment. Its function has yet to be revealed.

E4 Proteins

The open reading frames localized in the E4 region at the right end of the viral genome are labelled according to their arrangement as E4-ORF1 to E4-ORF7 (Fig. 19.23). Their transcription direction is opposite to that of the E1, E3, and late genes (Figs. 19.20c and 19.23). E4 genes are regulated by a common promoter, and the different mRNAs are generated from a common precursor transcript by using alternative splicing.

The gene for the protein E4-ORF1 is present in the genome of all human adenovirus types, with the exception of HAdV-40 and HAdV-41 (HAdV-F). However, E4-ORF1 gene expression has only been demonstrated in cells infected with HAdV-9 und HAdV-26 (HAdV-D). The E4-ORF1 protein of HAdV-9 has 125 amino acid residues, and induces the formation of breast tumours in rodents. The E1A and E1B proteins do not play a role in this process. Nevertheless, the actual function of the E4-ORF1 protein remains largely unknown. It has sequence homology to cellular dUTP pyrophosphatases (UTPases) and binds to a group of proteins that are characterized by the presence of PDZ domains. These are scaffolding proteins which mediate contacts between membrane-anchored and cytoplasmic proteins, and are important for the development of cell-to-cell contacts, and for signal transduction. Whether and how these potential functions of the E4-ORF1 protein play a role in tumorigenesis in rodents is not well understood.

The functions of the E4-ORF2 protein are unknown. The HAdV-5 E4-ORF2 protein contains 136 amino acids, and is present in the cytoplasm during the early infection phase.

The protein of the E4-ORF3 reading frame (11 kDa) stabilizes late viral transcripts in cooperation with the E4-ORF6 gene product. In addition, it interacts with

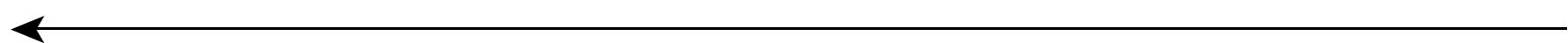


Fig. 19.22 (continued) insertion of complementary nucleotides into the other parental strand continues until the end of the genome. Then, two viral genomic molecules coexist: a single-stranded parental DNA molecule associated with E2A proteins and a newly synthesized double-stranded DNA genome (semiconservative replication mode). Whereas the double-stranded DNA can be replicated again, the ends of the single strand form a "panhandle"-like structure by their complementary inverted repeats (Fig. 19.20a, b). The double-stranded ends of the genome ("panhandle") induce binding of another initiation complex. During this polymerization reaction, the E2A proteins are continuously displaced from the single-stranded DNA template. The result of this process is a new double-stranded DNA genome which also consists of a parent and a newly synthesized DNA strand

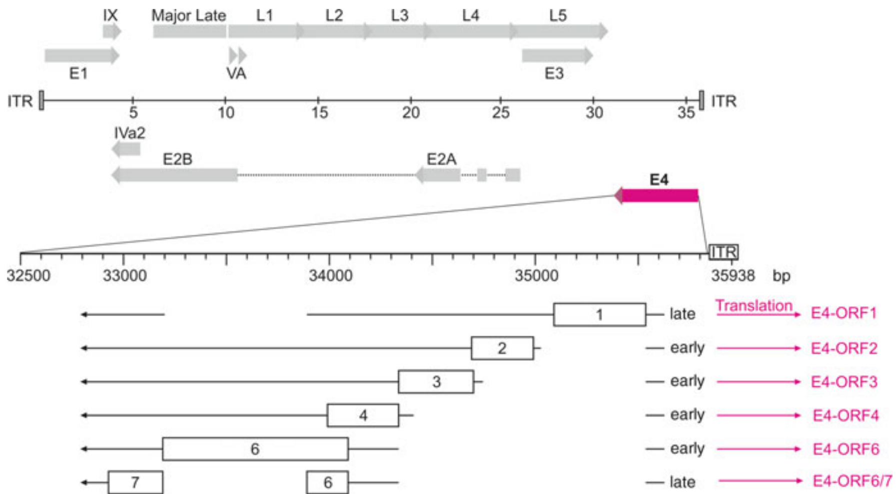


Fig. 19.23 Main transcripts and gene products of the E4 region of human adenovirus 5. *Upper part*: overview of the localization of the E4 region in the HAdV-5 genome. *Lower part*: main transcripts of the E4 region, time of their synthesis, location of the open reading frames, introns and exons, and the translated proteins E4-ORF1 to E4-ORF6/7. *ITR* inverted terminal repeat

the DNA-dependent protein kinase, a component of the DNA repair system for double-strand breaks. Presumably, this prevents the formation of concatemers during genome replication, and promotes the synthesis of monomeric adenovirus genomes. Furthermore, there is an interaction of E4-ORF3 with the 55-kDa E1B protein, whereby it regulates, like E4-ORF6, the transport of viral transcripts from the nucleus to the cytoplasm. Moreover, it is also responsible for the rearrangement of nuclear bodies which are found early after cell infection. This reorganization is a prerequisite for efficient viral replication.

The E4-ORF4 protein (14 kDa) binds to various forms of the regulatory subunit of protein phosphatase 2A (PP2A). This enzyme complex influences the phosphorylation level of some cellular proteins (e.g. c-Fos and AP-1) and that of the viral E1A proteins. It also inhibits transcription of the E2 gene and the E4 gene. The interaction with PP2A is also essential for inducing the apoptotic function of the E4-ORF4 protein. This is performed by a p53-independent mechanism, possibly by activation of cellular caspases. The main function of the adenoviral E4-ORF4 protein, in complex with PP2A, is to dephosphorylate members of the SR protein family. SR proteins are essential splicing factors that regulate alternative splicing, among other things. They are required for splicing transcripts from the L1 region. Although initially only the mRNA for the 52/55-kDa L1 protein is formed from the precursor transcript, later alternative splicing processes lead to the generation of the mRNA species from which the penton-associated protein IIIa is translated. Phosphorylated SR proteins act as repressors, preventing the splicing events that generate the IIIa mRNA. The E4-ORF4/PP2A-induced dephosphorylation of SR splicing factors abolishes their repressor effect.

One of the viral proteins that are encoded in the E4 region was briefly mentioned in the explanation of the functions of the 55-kDa E1B protein. In addition to the E1A and 55-kDa E1B proteins, the E4-ORF6 protein (34 kDa) is considered as the third oncogene of adenoviruses. It is a Zn^{2+}-ion-binding early protein that interacts with the cellular tumour-suppressor protein p53 in a way similar to that of the E1B protein. However, the interaction of both viral proteins occurs independently of each other with different domains of p53: whereas the E1B protein interacts with the amino-terminal domain of p53, E4-ORF6 binds to the carboxy-terminal region. This interaction targets p53 for proteasomal degradation by a still unknown mechanism. This leads to a loss of p53 functions, rendering the cells unable to induce the expression of the different specific genes that regulate the cell cycle and induce apoptosis. In this way, the E4-ORF6 protein enhances E1A- and E1B-mediated malignant cell transformation. In addition, it promotes, interacting with the E1B protein, the transport of viral late mRNA species from the nucleus to the cytoplasm, concurrently inhibiting that of cellular transcripts. This progressively reduces the metabolic activity of the cell during viral replication.

E4-ORF6/7 is translated from an alternatively spliced mRNA of the E4 region in which ORF6 is fused to ORF7. In HAdV-5, this process leads to the generation of a 17-kDa fusion protein whose amino-terminal region is equivalent to the first 58 amino acids of the E6 protein, and which are fused to 92 residues of the E7 reading frame. The E4-ORF6/7 protein complements the E1A transactivator protein, and thus regulates the expression of viral and cellular genes that are controlled by E2F factors. It acts to stabilize the complex formation between E2F transcription factors and promoter sequences.

19.4.3.2 Late Proteins

Late viral proteins are predominantly structural proteins. Nevertheless, non-structural proteins are also found to be encoded in the late gene region. The latter include several chaperones which are active during viral morphogenesis and promote packaging of the genomes into the precursor particles (Table 19.13). Some of the early proteins are even synthesized in the late phase of infection, especially the E2A protein, which binds to single-stranded DNA. Except for hexon-associated protein IX, late proteins are coded by DNA sequences that span 80 % of the genome. Only the gene encoding protein IX is located in the E1B gene region; however, it is translated using another reading frame (Fig. 19.21).

The structural proteins are translated from mRNA molecules which are spliced from a large precursor transcript that contains more than 30,000 nucleotides. The synthesis of the precursor mRNA is controlled by the major late promoter. Every mature mRNA species begins with an identical, non-coding leader sequence, which consists of three short RNA fragments that are assembled by splicing (Fig. 19.20c). The leader region is followed by the different sequences encoding the various viral structural proteins. Some structural proteins (IV, VI, VII, VIII, Mu) are synthesized as large precursor proteins, and their mature forms arise only by proteolytic processing by the viral protease adenain (23 kDa) during the assembly of the individual components into precursor capsids. Adenain is a papain-like cysteine

Table 19.13 Properties and functions of selected mastadenovirus proteins

Protein/coding region	Molecular mass	Modification	Function
E region			
E1A	40 kDa/289 aa	Phosphorylated	Immediate early protein: transactivator; zinc finger motif; binding to TFIID; binding to histone acetyltransferases; binding to tumour-suppressor proteins RB105, p107, p130; immortalization; transformation with E1B promotes entry into the S phase of the cell cycle
	26 kDa/243 aa	Phosphorylated	Immediate early protein; transrepressor; binding to RB105, immortalization; transformation with E1B promotes entry into the S phase of the cell cycle
E1B	55 kDa/496 aa	Phosphorylated	Immediate early protein, together with E4-ORF6 causes SUMOylation and degradation of p53, together with E1A causes transformation of the cell; regulation of RNA transport
	19 kDa/176 aa	Phosphorylated	Immediate early protein; inhibitor of apoptosis; active in DNA synthesis; reduces the occurrence of cytopathic effects; partially membrane-associated
E2A	72 kDa	Phosphorylated	Binds to single-stranded DNA, active in DNA replication; protein precursor (pTP) of the terminal protein (55 kDa); the terminal protein is covalently linked to the 5′ ends of the genome; acts as a primer in initiation of DNA synthesis
E2B	80 kDa		
	40 kDa		DNA polymerase
E3-gp19K	19 kDa	Glycosylated	Reduction of MHC class I proteins on the cell surface
E3-14.7K	14.7 kDa		Reduction of the susceptibility to TNF-α-mediated apoptosis
E3-10.4K (RID-α)	10.4 kDa		Proteins RID-α and RID-β bind together to epidermal growth factor receptors and to Fas/TNF receptor complexes causing their internalization and degradation
E3-14.5K (RID-β)	14.5 kDa		
E3-12.5K	12.5 kDa		Function unknown, not present in HAdV-40 and HAdV-41
E3-11.6K (adenovirus death protein)	11.6 kDa		Adenovirus death protein; causes cell lysis
E3-6.7K	6.7 kDa	Glycosylated	Function unknown; embedded in the ER membrane
E4-ORF1	125 aa		Induction of mammary tumours in HAdV-9-infected rats
E4-ORF2	17 kDa/136 aa		Cytoplasmic protein; function unknown
E4-ORF3	11 kDa		Acts along with E4-ORF6 and 55-kDa E1B in regulating mRNA transport

(*continued*)

Table 19.13 (continued)

Protein/coding region	Molecular mass	Modification	Function
E4-ORF4	14 kDa		Binds to protein phosphatase 2A; affects phosphorylation of cellular and viral proteins, thus it regulates the transcription of cellular and viral proteins
E4-ORF6	34 kDa		Interaction with 55-kDa E1B and degradation of p53; regulation of mRNA transport from the nucleus to the cytoplasm; Zn^{2+} binding
E4-ORF6/7	17 kDa		Stabilizes binding of E2F factors to the promoter region; functionally complements E1A proteins
L region/structural proteins			
L3, protein II	120 kDa		Hexon protein, in the capsomer as a trimer; induces binding of species-specific antibodies
L2, protein III	80 kDa		Penton base, homopentamer, penton component; interaction with integrins αv β3/α vβ5; induces binding of species-specific, neutralizing antibodies
L1, protein IIIa	66 kDa	Phosphorylated	Associated with penton base, hexon and protein VI
L5, protein IV	62 kDa	O-glycosylated	Fibre protein, homotrimer, penton component; interaction with CAR, CD46 or sialic acid; induces formation of serotype-specific, neutralizing antibodies
Protein IVa2	50 kDa		Multimeric structural protein associated with the viral genome involved in packaging of genomes into newly formed capsid precursors; chaperone?
L2, protein V	48.5 kDa	Phosphorylated	Nucleocapsid component; interacts with both the genome and the inner side of capsids
L3, protein VI	55 kDa		Associated with hexon and protein V; forms dimers that interact in turn to form trimers; localized on the inside of the capsids; promotes the release of virus particles from endosomes after penetration of virus particles into the cell; carboxy-terminal peptide is cleaved by the protease adenain; protease activation
L3, protease (adenain)	23 kDa		Papain-like cysteine protease; cleavage of the precursor proteins pTP, pX, pIIIa, pVI, pVII and pVIII into terminal protein, Mu, IIIa VI, VII and VIII, respectively; necessary for the infectivity of virions
L2, protein VII	18.5 kDa		Nucleocapsid component; arginine-rich; associated with the DNA genome; involved in transport of the viral genome to the nuclear pores and into the nucleus

(*continued*)

Table 19.13 (continued)

Protein/coding region	Molecular mass	Modification	Function
L4, protein VIII	13 kDa		Hexon-associated structural protein; localized on the inner side of capsids; stabilizes hexon interaction
E1B, protein IX	11.5 kDa		Hexon-associated structural protein; localized at the outside of capsids, at the contact sites of hexon capsomers; stabilizes hexons
L2, protein X	7 kDa		Proteolytic cleavage of pX by adenain generates the Mu protein, 36 amino acid, alkaline, protamin-like protein
L region/non-structural proteins			
Protein L1, 52/55K	46 kDa		Involved in packaging of genomes into newly formed precursor capsids
Protein L1, 16.6K	16.6 kDa		Function unclear
Protein L4, 100K	100 kDa	Methylated	Late non-structural protein; chaperone; active in hexon assembly; later active in regulation of mRNA translation
Protein L4, 33K	33 kDa		Late non-structural protein; chaperone; active in morphogenesis and packaging of the genome: in complex with protein IVa2 involved in regulating the activity of the major late promoter
Protein L4, 22K	22 kDa		Involved in packaging of viral genomes into newly formed precursor capsids

ER endoplasmic reticulum, *mRNA* messenger RNA, *RID* receptor internalization and degradation

protease comprising 201–214 amino acids; it contains many alkaline amino acids and is associated with the inner side of capsids. The protease is activated by non-covalent interaction with the viral DNA, and with an 11 amino acid peptide (VIc), which is cleaved from the carboxy terminus of protein VI by adenain. Adenain is essential for both maturation and infectivity of progeny viruses. An overview of the properties and functions of the different adenoviral proteins is provided in Table 19.13.

19.4.4 Adenovirus-Associated RNA

Apart from encoding the various early and late genes, the adenovirus genome also contains the information for small, non-coding RNA molecules: the virus-associated RNA species RNAI and RNAII. They comprise approximately 160 nucleotides, are transcribed by the cellular RNA polymerase III, have a high GC content and exhibit a pronounced secondary structure. The virus-associated RNA genes are located in the late region of the genome.

Virus-associated RNAI is synthesized in quantities of more than 10^8 copies per cell; and it inhibits the activity of the double-stranded-RNA-activated protein

kinase R. Protein kinase R inactivates translation initiation factor eIF2α by phosphorylation. Inactivation of eIF2α leads to translational arrest by inhibiting the initiation of translation. This mechanism for inhibition of protein synthesis is induced by double-stranded RNA and class I interferons (IFN-α, IFN-β), which activate protein kinase R (▶ Chap. 8). Double-stranded RNA molecules are formed during the viral replication cycle because extensive regions of the genome are transcribed from both DNA strands, giving rise to large mRNA precursors. These long double-stranded RNA molecules activate protein kinase R, leading to phosphorylation of eIF2α. The large amounts of virus-associated RNAI counteract this mechanism by binding competitively to protein kinase R, and inhibiting its kinase activity. This mechanism facilitates the translation of viral proteins.

Furthermore, it has been found that virus-associated RNAI and virus-associated RNAII block the regulatory processes triggered by RNA interference during the late phase of the infection cycle. Both virus-associated RNAI and virus-associated RNAII are cleaved into small RNA molecules of 21–23 nucleotides by the double-stranded RNase Dicer. These so-called virus-associated-RNA-derived microRNAs (mivaRNA) associate with the RNA-induced silencing complex (RISC), and inhibit its function. Usually, microRNAs bind to RISC. They are short, non-coding double-stranded RNA molecules which are generated by a multistage process involving the nuclear RNase Drosha. The nuclear export receptor exportin 5 gives rise to the export of microRNAs into the cytoplasm, where they are finally processed to mature microRNAs by the Dicer endoribonuclease. RISC-associated microRNAs bind to complementary sequences in mRNA molecules. If there is entire complementarity, the degradation of the mRNA is induced; if the recognition sequence in the mRNA is not exactly complementary to the microRNA, then the translation is inhibited. In virus-infected cells, the large amounts of mivaRNAs compete with cellular microRNAs to interact with RISC, and thus inhibit the regulatory steps triggered by RNA interference.

19.4.5 Replication

Adenoviruses infect a broad spectrum of cells. They can easily be propagated in human tumour cell lines in high concentrations in vitro (HeLa, KB, etc.). In vivo, adenoviruses preferentially infect epithelial cells of the throat, nose and pharynx, as well as lungs and digestive tract. HAdV-A, HAdV-C, HAdV-E and HAdV-F and the chicken embryo lethal orphan virus from the group of animal pathogenic aviadenoviruses attach to the coxsackievirus and adenovirus receptor (CAR) (Table 19.12) by the knob structure at the end of their fibre proteins. The CAR protein is a member of the immunoglobulin superfamily, and is involved in the formation of tight junctions as a surface protein of polarized epithelial cells. It is also used as a receptor by Coxsackie B viruses (▶ Sect. 14.1.4). Neutralizing antibodies are directed against the protein regions which are involved in CAR binding. HAdV-B viruses use another cellular protein for attachment, namely

CD46, a surface protein that regulates the activation of the complement system. It protects healthy cells from the detrimental effect of the complement system by cleavage of cell-surface-bound complement components. CD46 is also used as a cellular receptor by attenuated measles vaccine viruses and by human herpesvirus 6 (▶ Sects. 15.3 and 19.5). In addition, HAdV-B viruses can also use CD80 and CD86 as cellular receptors; they are related to CD46. HAdV-D viruses bind to sialic acid residues by the knobs of their fibre proteins, which have a high density of positively charged amino acid residues. The sialic acids are terminal *N*-acetylneuraminic acid residues which are present as a modification of cell surface components. Apart from these interactions, some adenoviruses have been found to interact with other cell surface components, such as MHC class I proteins and vascular cell adhesion molecule 1.

Adenovirus Binding to the CAR – An Artefact in Cell Culture?
The use of the cell surface protein CAR as a receptor for attachment of many serotypes of human and animal adenoviruses was first described in studies of the viral replication cycle which were done in cell culture systems. In this case, CAR is equally distributed all over the cell surface, and the data that demonstrate an interaction of fibre proteins with this cell surface protein are not controversial. However, in polarized epithelial cells, which are used by adenoviruses as target cells in vivo, the CAR protein is not on the accessible side, but is part of the tight junctions at the cell–cell contacts. Therefore, an increasing line of evidence suggests that adenoviruses do not use the CAR protein as a receptor in vivo, but use other cell surface structures.

In addition to these direct interactions between the viral fibre proteins and cell surface components, adenoviruses can bind to the cell surface by the aid of some soluble proteins present in the blood, in the lacrimal fluid and in the mucosa of the lung epithelium. These components functions as “bridge molecules”, which indirectly enable the viruses to interact with certain cell types. For example, blood coagulation factors VII and X and the complement C4 binding proteins bind to the fibre proteins of HAdV-A, HAdV-B, HAdV-C and HAdV-D. In the liver, these complexes interact with heparan sulphate proteoglycan structures or with the low density lipoprotein receptors on the surface of hepatocytes, leading to penetration of adenoviruses. The same applies to dipalmitoylphosphatidylcholine, a major component of phospholipids in the lung. Dipalmitoylphosphatidylcholine binds to the hexon protein on the surface of HAdV-C viruses, mediating the interaction with the type II alveolar epithelial cells. In this way, adenoviruses can gain access to the cells of the lung epithelium during infections of the respiratory system. Analogously, lactoferrin, a molecule of the non-specific immune response that is present in the lacrimal fluid and in the lung mucosa, is capable of interacting with the surface of HAdV-C viruses, thus mediating the infection of the conjunctiva and lung epithelial cells by binding to the lactoferrin receptor.

Subsequently, cell-surface-attached adenoviruses are internalized by interaction of the penton base proteins with the heterodimeric integrins $\alpha_v\beta3$ and $\alpha_v\beta5$, which are used as co-receptors. This process is dependent on an RGD motif in the penton base protein, which is present in all adenoviruses with the exception of HAdV-40 and HAdV-41 (HAdV-F species). The latter are probably internalized considerably more slowly owing to the lack of binding to integrins co-receptors. For the simultaneous interaction of fibre knobs and the penton base with different cellular receptors, steric constraints have to be overcome especially in adenovirus types containing long fibre shafts, which must have sufficient flexibility. Interaction of the penton base protein with integrins leads to activation of phosphatidylinositol 3-hydroxykinase. This enzyme produces phospholipids that function as a second messenger, and impinge on a number of biological processes, including activation of the small GTPases Rho and Ras, and consequently the reorganization of actin filaments in the cell. Adenovirus endocytosis is regulated by GTPases of the Rho family such as Rab5, Rac1, Cdc42 and RhoA.

After the initial contact with the cell surface, the receptors move with the attached viruses to clathrin-rich regions in the cytoplasmic membrane to form endocytic vesicles, which are internalized into the cytoplasm along with the enclosed viruses. An acidification occurs in the endosomes. This changes the conformation of proteins VI, which are associated with the penton base and proteins IIIa. As a consequence, proteins VI dissociate from the particles, and induce the destruction of the endosomal membrane. In this process, an amphipathic α-helix at the amino terminus of protein VI is functionally active, which probably intercalates into the endosomal membrane, mediating its lysis. The virus particles are released into the cytoplasm, losing the penton base proteins as well as the adjacent hexons, fibre proteins and proteins IIIa. Thereafter, the residual particles bind to dyneins of microtubules by their remaining hexons, and are transported to the nuclear pores by the action of the nuclear transport signals of proteins VII. The viral DNA-protein complex enters the nucleus, and the empty residual capsids remain in the cytoplasm.

Transcription of viral genes and replication of the viral genome occur in the nucleus. This process can be roughly divided into four steps, whereby the regulated and correct execution of each step is essential for the following:

1. Transcription of early genes, translation of early proteins
2. DNA replication
3. Transcription of late genes, translation of late proteins
4. Morphogenesis

First, the cellular RNA polymerase II transcribes the E1A and EIB genes (Fig. 19.21). Modification of the 5′ ends with cap structures and polyadenylation is done by cellular enzymes as well. The E1A and E1B gene products are essential for the further course of the infection cycle, since the transactivator effect of the E1A protein is required for induction of transcription of the E2, E3 and E4 genes. The E1A protein activates cellular, *trans*-active, DNA-binding proteins, the so-called E2F and E4F factors. Their name is derived from the early adenoviral gene groups E2 and E4, whose transcription the factors influence. Since these cellular factors normally regulate the transcription of various host genes, they are also activated during

the early phase of infection. Subsequently, the viral E2 gene products are required for the replication of the viral genome. The actual replication of the viral genome occurs semiconservatively without synthesis of Okazaki fragments. This process begins with the esterification of a dCMP molecule with the β-hydroxyl group of a serine residue of the terminal precursor protein pTP (80 kDa) (Fig. 19.22a). The pTP-dCMP complex has high affinity for the viral DNA polymerase, interacting with the TP components that are covalently bound to the 5′ termini of the genome. The resulting complex acts as a primer for the initiation of DNA synthesis by the 3′-OH group of the deoxyribose moiety of dCMP. Additionally, the activities of the cellular nuclear factors NF-I and NF-II are required. NF-II is a DNA topoisomerase I.

Subsequently, the parental DNA strand containing the 5′-linked TP is progressively displaced, and the new strand is elongated by continuous insertion of nucleotides into the initiation complex (Fig. 19.22b). The topoisomerase I activity of NF-II is required for this process. The displaced DNA single strand is continuously coated with E2A proteins, which prevent its degradation by cellular nucleases. Presumably, the inverted repeats hybridize with each other at the ends of the displaced strand and form a double-stranded region, which is very similar to the ends of an adenoviral genome. This triggers the recruitment of a new initiation complex of dCMP-pTP/adenoviral DNA polymerase/NF-I/NF-II, leading to a new start of polymerization. During elongation, the double-stranded "panhandle" structure is resolved, the single-strand binding proteins are displaced by the elongating DNA polymerase, and the single strand serves as a template for the continuous synthesis of a new double-stranded DNA genome (Fig. 19.22b). The cleavage of the terminal precursor pTP into the mature 55-kDa TP, which is associated with the genome of infectious adenoviruses, is done by the proteolytic activity of adenain (23 kDa) only after completion of DNA replication.

The viral transcription pattern changes after genome replication: most of the early genes are no longer transcribed. Instead, the mRNA species coding for the viral structural proteins are synthesized from the late regions of the genome. The switching process is performed by the protein complex composed of 55-kDa E1B and 34-kDa E4-ORF6, which promotes the export of late viral mRNA species into the cytoplasm. The cellular protein synthesis is impaired. This is probably caused by a late viral protein that inhibits the phosphorylation of protein p20 of the cap-binding complex. This prevents binding of the complex to cellular mRNAs, but the late viral transcripts are not affected owing to the presence of specific sequences in their leader region. The first steps of morphogenesis occur in the cytoplasm: hexon and penton bases as well as fibre proteins assemble into capsomers. For this purpose, chaperones (protein folding catalysts) are required. They are partially encoded by the virus, and in part originate from the cell. For example, three late viral proteins with molecular masses of 100 kDa, 50 kDa (pIVa2) and 33 kDa and which are encoded in overlapping reading frames within the L4 region are necessary for trimerization of hexon proteins. They interact with hexon proteins and catalyse the formation of hexon capsomers. Chaperones are not contained in the released particles. How capsomers are then transported into the nucleus is not known. Nine hexons

shape the precursors of the faces of the icosahedron; they associate with pentons, forming a precursor form of the capsid. Viral nucleoproteins V and VII are introduced into the precursor capsid, and presumably interact with the viral genome only in the developing particles. The viral double-stranded DNA genome passes through one of the vertices into the interior of the precursor capsid. It is complexed only with pTP, which is proteolytically cleaved into TP only after genome packaging. The induction of apoptosis in the late phase of infection leads to cell death, and thus to the release of infectious virions.

19.4.6 Human Pathogenic Adenoviruses

19.4.6.1 Classification, Epidemiology and Transmission

The various human adenoviruses are characterized by different types of infection, organotropisms and symptoms (Table 19.12). Owing to these characteristics, they are classified into different species (HAdV-A to HAdV-G). Infections with serotypes of HAdV-A (HAdV-12, HAdV-18, HAdV-31) are rare and have mainly been observed in children with infections of the gastrointestinal tract. HAdV-3 and HAdV-7 belong to subspecies B1. They principally occur as an epidemic during the winter months, and are very frequent in patients with colds worldwide. Outbreaks of HAdV-7 infections have been more frequently diagnosed among members of the US military. Serotypes HAdV-11, HAdV-34 and HAdV-35 of subspecies B2 additionally cause persistent infections of the kidneys, especially in immunocompromised individuals, and can be isolated from the urine of such patients. Serotypes HAdV-1, HAdV-2, HAdV-5 and HAdV-6 of HAdV-C and serotype HAdV-3 of HAdV-B are spread endemically. They cause infections of the respiratory tract, especially in young children. HAdV-D encompasses many serotypes. They are the causative agent of keratoconjunctivitis, which is known as swimming pool conjunctivitis, and occurs particularly during the summer months. HAdV-42 and HAdV-51 have recently been isolated only from AIDS patients who had developed pneumonia as a result of immune suppression. HAdV-4, a prototype of subgenus E, has been isolated especially from recruits to the US Army who had epidemic diseases of the respiratory tract. HAdV-40 and HAdV-41 are the only known members of HAdV-F. They are found worldwide, and cause gastrointestinal infections with diarrhoea, especially in infants and young children. There is very little information about HAdV-52; it is being discussed to classify it into the new species HAdV-G. Besides these classic adenovirus species, novel virus recombinants have recently been repeatedly found, and their genomes are assembled from regions of different species. These human adenovirus recombinants frequently exhibit a high degree of virulence.

The viruses are usually transmitted by aerosol or contaminated objects and liquids. During the summer months, the frequently occurring infectious inflammation of the conjunctiva and cornea (keratoconjunctivitis) is transmitted in swimming pools by adenovirus-contaminated water. Adenoviruses can also be

transmitted in connection with faecal–oral infections of the gastrointestinal tract in regions with poor sanitary conditions, but also in paediatric clinics and within families. After infection, adenoviruses often persist for years in the tonsils, which intermittently excrete them in the saliva. Excretion of the virus is increased in immunosuppressed patients.

19.4.6.2 Clinical Features

Approximately half of all adenovirus infections are asymptomatic. The incubation period of respiratory tract infections is about 6 days. The first symptom is conjunctivitis, followed by fever and sore throat. The flu-like symptoms are often accompanied by vomiting and diarrhoea. The complaints generally subside after 1 week. Epidemic infections with HAdV-3, HAdV-4, HAdV-7 and HAdV-21 can develop into bronchitis/bronchiolitis and lung inflammation. In children under the age of 5 years, infections are usually severer than in adults. Approximately 10 % of pneumonias are causally associated with adenovirus infections in this age group.

Adenovirus infections of the gastrointestinal tract may be manifested as nausea, vomiting and diarrhoea. Here too, the incubation period is about 1 week, and the symptoms can last for 9–12 days. Keratoconjunctivitis appears along with flu-like symptoms. The incubation period is usually 6–9 days. The symptoms are watery, painful eyes, photophobia and corneal erosions, which can last up to 6 weeks. The keratoconjunctivitis caused by adenoviruses is mostly without lasting damage.

In rare cases, adenoviruses have also been associated with acute cystitis, meningoencephalitis or necrotizing enterocolitis. In immunocompromised patients (especially recipients of solid organs and bone marrow transplants), in which the immune system is not completely functional owing to immunosuppression, adenoviruses can cause persistent infections which manifest themselves as diarrhoea, kidney inflammation, hepatitis, severe, life-threatening pneumonia or meningoencephalitis. Severe infections, which frequently have a fatal outcome, are observed especially in immunocompromised children. This also applies to children treated with cytostatic agents, who show disseminated infections. Very high amounts of adenovirus particles (more than 10^9 particles per millilitre) are detectable in the blood of such patients.

19.4.6.3 Pathogenesis

Symptomatic Infection in Humans

Adenoviruses generally enter the organism through the respiratory tract. They preferentially infect epithelial cells of the pharynx, small intestine and cells of the conjuntiva, where they replicate. Viraemic spread within the organism is rarely observed, usually only in immunosuppressed patients. Protein synthesis and processing of cellular RNA species are almost completely inhibited during infection, leading to the death of infected cells. Basophilic inclusion bodies are detectable in the nuclei. The inclusion bodies are probably regions in which the various components are assembled into virus particles because paracrystalline aggregations of capsids and capsid proteins can be observed by electron microscopy in these structures. Cell fusion or multinucleated cells do not occur.

Mononuclear cells and lymphocytes migrate into the infected organ regions. The latter are largely cytotoxic T cells that recognize infected cells by peptides which are presented by MHC class I proteins. They are responsible for the elimination of the virus from the organism. Some adenoviruses evade this recognition by reducing the amount of MHC class I antigens on the cell surface by the activity of the E3-gp19K protein. In addition, the complex of RID-α and RID-β and the E3-14.7 K protein protect infected cells from the cytotoxic and proapoptotic effect of TNF-α. E1A proteins also repress the expression of STAT activators (▶ Chap. 8), leading, along with virus-associated RNAs, to a block of both interferon-dependent immune responses and RNA interference. In these ways, adenoviruses probably cause persistent infections. Especially infections with serotypes 1, 2 and 5 of HAdV-C seem to persist for longer periods in the tonsils. Recurrent outbreaks of symptomatic infections in immunocompromised patients also suggest persistence of some virus types. The establishment of chronic infections is probably counteracted by the apoptosis-promoting functions of the E4 region, which are primarily associated with the E4-ORF4 protein and are required for the release of infectious progeny viruses. Hence, it can be inferred that the establishment of persistent infections depends on the balance and regulation of antiapoptotic and proapoptotic viral functions.

Adenoviral DNA sequences have been found in some human tumours (stomach, bowl and neuronal tumours). However, a causal relationship between viral infection and tumour has not been conclusively demonstrated. In contrast to transformed rodent cells, neither a partial nor a complete integration of the adenoviral genome has been detected in human cells.

Tumorigenesis and Cell Transformation in Rodents

Some human adenoviruses (HAdV-12, HAdV-18, HAdV-31) which belong to the highly oncogenic HAdV-A (Table 19.12) cause mesenchymal tumours (sarcomas) in hamsters and other rodents in 100 % of cases after an incubation period of 2–3 months. Species B (HAdV-3, HAdV-7, HAdV-11) is considered as weakly oncogenic. In such cases, the incubation period until tumour formation is to 2 years. However, most virus types are not able to cause tumours. Nevertheless, all human adenoviruses (HAdV-A to HAdV-F) are able to transform rodent cells in vitro. The region of the genome encoding the very early adenoviral E1A and E1B proteins is sufficient for cell transformation. The functional activities of the proteins encoded in this region were described in Sect. 19.4.3. If only the E1A region is introduced into cells, these cells are indeed immortalized, but the E1A proteins alone are not able to induce the fully transformed state. The products of the two early genes E1A and E1B are required for transformation. Furthermore, there are data suggesting that the activity of a third adenoviral oncogene is also needed, the E4-ORF6 protein.

From other experiments, it is also known that the effect of E1A and E1B proteins is not sufficient for tumour development: only in vitro transformed cells which were immortalized either with the highly oncogenic HAdV-A or with the E1A and E1B genes are capable of producing tumours in syngeneic

animals (rat or hamster) after grafting. When cells are transformed with HAdV-B viruses in cell culture, they do not cause tumours in syngeneic, immunocompetent hosts, but cause them in nude mice in which the cellular immune system is genetically defective. In addition to the cell-transforming ability of all adenoviruses, the highly oncogenic group has developed additional mechanisms to evade the immune system of the host.

Presumably, different mechanisms become operative after transformation with the E1A/E1B region of oncogenic adenoviruses:

1. The E1A and E1B proteins of non-oncogenic adenoviruses contain epitopes that are presented by MHC class I proteins of the mouse. These as "foreign" labelled cells can be eliminated by cytotoxic T cells in immunocompetent animals. Such an epitope has been identified in HAdV-5. It comprises amino acids 232–247 of the E1A protein, which are coded in the second exon. It is presented by H-2Db, it acts as an immunodominant epitope and ensures that the presenting cells are recognized and killed. A corresponding T-cell epitope has not been found in the E1A proteins of HAdV-12 and other highly oncogenic adenoviruses. Therefore, they cannot be recognized by cytotoxic T lymphocytes in mice.
2. The E1A proteins of oncogenic adenoviruses are per se able to reduce the levels of MHC class I antigens in primary rodent cells, i.e. without the activity of the 19-kDa E3 protein. This effect is caused by the CR3 region of the HAdV-12 E1A protein, whereby the E1A protein acts as a transrepressor (Sect. 19.4.3) that reduces the expression of all three loci of the murine H-2 gene complex, leading to a strongly decreased (four to 20 times) MHC class I gene expression in comparison with non-transformed cells, or those transformed with the HAdV-5 E1 region. Cells containing fewer MHC antigens on their surfaces are not detected and eliminated by cytotoxic T lymphocytes.
3. A number of additional mechanisms which are considered to contribute to cell transformation and tumorigenesis have been proposed to explain the development of adenovirus-associated tumours. In transformed cells, HAdV-12 DNA is integrated into the genome of the host cell. This may result in an altered gene expression in the affected regions. The integrated sequences are methylated de novo. In this case, adjacent regions of the cellular genome may also be modified, affecting gene expression. Furthermore, extensive sequence amplifications and rearrangements are observed within the integrated viral DNA and in adjacent regions of the genome. These processes alter the expression of cellular proto-oncogenes: the expression of c-*myc* and c-*jun* is significantly increased in HAdV-12-transformed cells, whereas HAdV-5-transformed cells upregulate the expression of c-*jun*. The processes that lead to transformation are best studied in hamster cells, in which HAdV-12 is not able to perform a productive infection cycle. Although the cells are infected and the viral DNA is transported into the nucleus, DNA replication, the synthesis of late viral proteins and the production of infectious particles do not occur.

In these cells, the viral genome is integrated into the genome about 10–16 h after infection, thereby leading the changes in methylation patterns and cellular gene expression described above. Abortive infections without production of infectious virus progeny have not been observed in HAdV-2- or HAdV-5-infected hamster cells. These virus types can functionally complement HAdV-12: cells in which the left half of the HAdV-2 or HAdV-5 genome is integrated into the DNA, and thus constitutively express the pertinent gene products, facilitate the production of infectious HAdV-12 particles when infected with HAdV-12.

Hence, many different factors, of which only a few are known, seem to be involved in adenovirus-induced cell transformation and tumorigenesis.

The Shipyard Eye Was a Severe Epidemic Keratoconjuntivitis Caused by Adenoviruses

Infections of the conjunctiva and cornea (shipyard eye) were repeatedly observed in shipyards from 1920, and often had very severe symptoms. In these cases, the adenoviruses were probably transmitted by inadequately sterilized instruments by removing foreign bodies from the eyes. These infections frequently caused a long lasting opacity of the eye and were associated with lymph node swelling.

19.4.6.4 Immune Response and Diagnosis

In the context of adenovirus infection, both the non-specific and the specific immune response are effectively induced. In the early stages of the infection cycle, the production of class I interferons and proinflammatory cytokines is induced by the signal cascade, which is mediated by activation of Toll-like receptor 9 in the endosomes. The trigger is presumably non-methylated or incorrectly methylated CpG motifs in the viral DNA. Adenovirus infections also cause a long-lasting, virus-type-specific immunity, probably because of their intensive replication in the lymph nodes of the throat and intestine. Recognition of virus-infected cells by cytotoxic T lymphocytes is essential for the elimination of the virus from the organism. T-cell epitopes have been identified in a number of viral proteins, even in immediate early proteins. Antibodies are preferentially raised against capsid surface structures.

The serological diagnosis of acute adenovirus infection is based on the detection of IgM antibodies directed against the viral structural proteins. Because of the high prevalence of different adenoviruses in the human population, the great similarity of amino acid sequences in the hexon, penton and fibre proteins, and the associated cross-reactivity of antibodies, is not sufficient to determine adenovirus-specific IgG in the serum of patients. If there are serum samples from a patient before illness, a significant titre increase of adenovirus-specific IgG antibodies can be evidence of a recent infection.

Virus-type-specific neutralizing antibodies are primarily directed against the knob structures of the fibre proteins, which contain the most type-specific epitopes. The immunoglobulins against the fibre protein are neutralizing, and confer a lifelong protection against re-infections by the same adenovirus type, but not by others.

Diagnosis is performed by isolation of the virus from respiratory materials or by detecting viral DNA from throat and eye swabs as well as from faeces, urine and blood. With the exception of HAdV-D and HAdV-F, adenoviruses can be cultivated relatively well in permanent, diploid human cell lines such as HeLa cells or human embryonic kidney cells. The lytic infection of cells causes a cytopathic effect. The in vitro enriched viruses can be further characterized by haemagglutination tests, using type-specific antisera, or by PCR and subsequent sequencing of the amplified product.

19.4.6.5 Therapy and Prophylaxis

There is no approved antiviral therapy against adenovirus infection. Nonetheless, there is some evidence that the adenoviral DNA polymerase can be inhibited by cidofovir. Therefore, cidofovir therapy and the simultaneous reduction of immunosuppression is habitually applied with some success in immunosuppressed patients with life-threatening, disseminated infections. The application of IFN-β seems to have a positive effect in severe cases of keratoconjunctivitis. A vaccine against HAdV-4 and HAdV-7 has been developed, as these virus types have repeatedly caused severe epidemics, frequently concomitant with pneumonia, among US military personnel. The vaccine is approved only for use in members of the US armed forces. It is based on replication-competent HAdV-4 and HAdV-7 viruses, which are administered in enteric-coated gelatine capsules that release the viruses only in the intestine. These adenoviruses cause a disease only when they infect epithelial cells of the respiratory tract. Admittedly, they can cause a limited infection in the intestinal epithelium, but it is asymptomatic. A specific immune response is triggered during infection, and confers protection against re-infections with these virus types. However, the general application of such live vaccines is a very controversial subject because of the endemic dissemination of the various types in the population, and the associated risk of the emergence of new recombinants. The primary oncogenic potential of adenoviruses also argues against the indiscriminate use of such a vaccine, although a causal relationship between adenovirus infections and tumorigenesis has not been demonstrated in humans so far.

19.4.7 Animal Pathogenic Adenoviruses

Adenoviruses play merely a minor role in veterinary medicine. Although adenoviruses have been isolated from different animal species such as cattle, horses, pigs, dogs, rabbits and poultry, only the canine and avian adenoviruses are pathogenic.

19.4.7.1 Canine Adenoviruses

Epidemiology and Transmission

Two canine adenovirus types are distinguished: canine adenovirus 1 (CAdV-1) as the causative agent of infectious canine hepatitis, and canine adenovirus 2 (CAdV-2) as the pathogen of infectious laryngotracheitis.

Infectious canine hepatitis has been described throughout the world, but its incidence is very low. Besides dogs, foxes can also be infected with CAdV-1. They frequently develop encephalitis, also known as Rubarth's disease. The virus is excreted in and disseminated by the faeces, urine and saliva of infected dogs. The month-long excretion of the virus in the urine is of major epidemiological significance. For many years, only sporadic cases of canine adenovirus infections have been described in western Europe. The reason is that almost all combination vaccines for dogs contain a canine adenovirus component, usually a CAdV-2 vaccine virus. In comparison with CAdV-1, this virus does not cause a systemic infection, and its replication is restricted to the cells of the respiratory tract. CAdV-2 is rather a vaccine virus than a pathogen. It induces a reliable, protective cross-immunity against CAdV-1.

Clinical Features

Infections with CAdV-1 are characterized by acute gastroenteritis and hepatitis. The dogs develop fever, diarrhoea and colic. Occasionally, encephalitis and inflammation of the cornea are found as special forms.

Pathogenesis

CAdV-1 is transmitted orally to the dogs; it subsequently reaches the lymphocytes in the tonsils, and after dissemination of infected cells via the blood system, it also reaches the Peyer patches in the small intestine, from where it infects endothelial cells in various organs, especially in the liver and kidneys. The infection is associated with massive necrosis of parenchymal cells. The virus enters the brain also via haematogenous dissemination, and can cause encephalitis. Another manifestation is an opacity of the cornea (also known as hepatitis blue eye), which is caused by immune complexes. Its development is associated with the onset of antibody formation after replication of CAdV-1 in the corneal epithelium a few weeks after infection.

Immune Response and Diagnosis

The diagnosis of CAdV-1 infections can be performed by examination of the urine, from which the virus can be isolated and cultivated in cell culture, visualized by electron microscopy and detected by PCR. The virus can especially be detected in the renal tissue of dogs which died of the viral infection.

Control and Prophylaxis

Live vaccines based on an attenuated CAdV-1 are no longer in use owing to the risks of cornea opacity (hepatitis blue eye) and induction of immunosuppression. By contrast, only such vaccines are used today which contain either inactivated CAdV-1 particles or an attenuated CAdV-2 strain.

19.4.7.2 Avian Adenoviruses

Epidemiology and Transmission

Numerous adenoviruses cause diseases in poultry. They can be divided into three genera by virtue of the different immune responses against the viral hexon proteins, the group-specific antigen. Aviadenoviruses (conventional group I adenoviruses) include virus isolates from chickens, ducks and geese, including chicken embryo lethal orphan virus. The genus *Siadenovirus* comprises the haemorrhagic enteritis virus of turkeys and the marble spleen disease virus of pheasants. Atadenoviruses include, inter alia, the egg drop syndrome virus of chickens. The most important way in which avian adenoviruses are transmitted is vertical transmission through the eggs. Infected birds excrete the virus in their faeces. The horizontal transmission of these pathogens is epidemiologically important.

Clinical Features

Whereas the association of aviadenoviruses with illnesses of birds has not been determined with certainty, viruses of the genera *Siadenovirus* and *Atadenovirus* cause distinct, clearly defined diseases. Haemorrhagic enteritis of turkeys and pheasant marble spleen disease of pheasants are associated with a mortality of up to 60 %. The birds develop a systemic infection that is associated with haemorrhagic enteritis or with petechial haemorrhages in various organs. Egg drop syndrome virus causes a systemic disease in chickens that is characterized in laying hens by the production of a high percentage of shell-less eggs.

Pathogenesis

Little is known about the pathogenesis of aviadenovirus infections. In chicken, egg drop syndrome virus infects all lymphoid tissues and the pouch shell glands of the uterus, which are responsible for the formation of the eggshell. This pathogenetically important step leads to virus-induced necroses of the pouch shell glands, and thus to an impairment of eggshell formation. Thus, the hens produce almost shell-less eggs, which are also known as wind eggs.

Immune Response and Diagnosis

Adenovirus infections probably induce a lifelong protective immunity in surviving chickens. Maternal antibodies protect the chicks during the first few weeks of life. The diagnosis of avian adenovirus infections can be performed by detecting the pathogens in the affected organs, by isolation of the virus or by immunofluorescence. Typing of virus isolates is performed by immunodiffusion using type-specific sera. In addition to these methods, PCR is increasingly used for the detection of pathogens. The subsequent typing is performed by sequencing the amplified DNA fragments.

Control and Prophylaxis

An effective live vaccine based on an attenuated virus is available for haemorrhagic enteritis of turkeys. Pheasants can be immunized against marble spleen disease using an inactivated vaccine. Moreover, an effective inactivated vaccine against infection with egg drop syndrome virus is also available.

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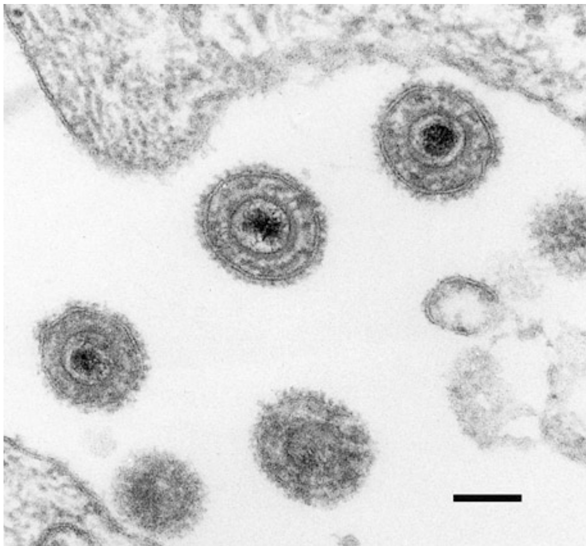
19.5 Herpesviruses



Herpesvirus infections occur in humans and many vertebrates – from monkeys and cats to frogs and fish. Although the symptoms differ widely, both the particle morphology and the molecular biological characteristics are similar in all members of this virus family. The double-stranded DNA genome is replicated in the nucleus, where the first steps of morphogenesis occur. As described by Dietrich Falke and colleagues in

1959, the capsid is primarily surrounded by a shell originating from the inner nuclear membrane, which is subsequently removed and finally replaced by the membrane of *trans*-Golgi vesicles by a sophisticated enveloping process. All herpesviruses encode several enzymes which are involved in nucleic acid metabolism and genome replication. The host cells in which herpesviruses replicate lytically perish during the generation of progeny viruses. Eight types of human herpesvirus are known. Their acute infections are manifested as diseases of various organs, predominantly the skin (herpes simplexvirus types 1 and 2 as well as varicella-zoster virus) or the lymphatic system (Epstein-Barr virus, human herpesviruses 6, 7 and 8). The name "herpesvirus" is derived from the Greek word *herpein* meaning "to creep", alluding to the creeping spread of the skin rash caused by herpes simplex virus. In pregnant women, acute cytomegalovirus infections can result in severe damage to the embryo.

A characteristic feature of all herpesviruses is their ability to remain latent in the organism for life after an initial infection. In this state, the production of infectious particles is usually blocked, and the host cells survive. The virus can be repeatedly reactivated from latency into the lytic cycle – a process which is characterized by the recurrence of the same primary or similar symptoms. Epstein-Barr virus is involved in the development of human tumours, including Burkitt's and Hodgkin's lymphoma and nasopharyngeal carcinoma. Cytomegaloviruses cause life-threatening, generalized diseases, especially pneumonia, in immunocompromised individuals. The genome of human herpesvirus 8 has been detected in the cells of Kaposi's sarcoma, primary effusion (body-cavity-based) lymphoma and multicentric Castleman's disease. This virus is causally involved in the genesis of these tumours.

Even domestic animals have a variety of herpesviruses that cause to some extent severe and economically significant infectious diseases. Apart from a few exceptions, the host range of herpesviruses is very narrow, so transmission to other species is an exception; hence, zoonoses caused by herpesviruses are unknown. Diseases caused by animal pathogenic herpesviruses have a wide spectrum, ranging from unspecific general infections to abortion and reproductive and central nervous disorders.

19.5.1 Classification and Characteristic Prototypes

Recently, the taxonomy of herpesviruses has been updated: The new order *Herpesvirales* has been created and the former family *Herpesviridae* has been split into three families, namely families *Herpesviridae*, *Alloherpesviridae* and *Malacoherpesviridae*. The revised family *Herpesviridae* retains the mammal, bird and reptile viruses, the new family *Alloherpesviridae* incorporates the viruses from fish (channel catfish virus, koi herpesvirus) and frogs (Lucké tumour herpesvirus) and the new family *Malacoherpesviridae* contains a bivalve virus (oyster herpesvirus). In terms of DNA sequence and genome organization, members of the families *Alloherpesviridae* and *Malacoherpesviridae* differ significantly from viruses of the family *Herpesviridae*. The family *Herpesviridae* can be further classified into three subfamilies on the basis of their pathogenicity and their replication properties (Table 19.14). Alphaherpesviruses have a relatively broad

Table 19.14 Characteristic prototypes of herpesviruses (order *Herpesvirales*)

Family	Subfamily	Genus	Human virus	Animal virus
Herpesviridae	*Alphaherpesvirus*	*Simplexvirus*	Human herpesvirus 1 (herpes simplex virus type 1) Human herpesvirus 2 (herpes simplex virus type 2)	Bovine herpesvirus 2 (bovine mammilitis virus)
				Cercopithecine herpesvirus 1 (herpes B virus; herpesvirus simiae)
				Cercopithecine herpesvirus 2 (simian agent 8)
				Cercopithecine herpesvirus 16 (baboon herpesvirus 2, herpesvirus papio 2)
				Atelino herpesvirus 1
		Varicellovirus	Human herpesvirus 3 (varicella–zoster virus)	Saimirine herpesvirus 1 Bovine herpesvirus 1 (infectious bovine rhinotracheitis virus)
				Bovine herpesvirus 5 (bovine encephalitis virus)
				Equine herpesvirus 1 (equine abortion virus)
				Equine herpesvirus 3 (equine coital exanthema virus)
				Equine herpesvirus 4 (equine rhinopneumonitis virus)
				Equine herpesvirus 8 (asinine herpesvirus 3)
				Equine herpesvirus 9 (gazelle herpesvirus)
				Porcine (suid) herpesvirus 1 (pseudorabies virus)
				Feline herpesvirus 1 (feline rhinotracheitis virus)
				Canine herpesvirus 1
		Mardivirus		Gallid herpesvirus 2 (Marek's disease virus type 1)
				Gallid herpesvirus 3 (Marek's disease virus type 2)
				Meleagrid herpesvirus 1 (turkey herpesvirus)
		Iltovirus		Gallid herpesvirus 1 (avian infectious laryngotracheitis virus)
				Psittacid herpesvirus 1 (Pacheco's disease virus)
		Scutavirus		Chelonid herpesvirus 5 (chelonid fibropapilloma-associated virus)

Betaherpesvirus	*Cytomegalovirus*	Human herpesvirus 5 (human cytomegalovirus)	Cercopithecid herpesvirus 5 (African green monkey cytomegalovirus)
			Cercopithecine herpesvirus 8 (rhesus cytomegalovirus, macacine cytomegalovirus)
			Pongine herpesvirus 4 (panine herpesvirus 2, chimpanzee cytomegalovirus)
	Muromegalovirus		Murine herpesvirus 1 (murine cytomegalovirus)
			Murine herpesvirus 2 (rat cytomegalovirus)
	Roseolovirus	Human herpesvirus 6	
		Human herpesvirus 7	
	Proboscivirus		Elephantid herpesvirus 1 (elephant endotheliotropic herpesvirus)
Gammaherpesvirus	*Lymphocryptovirus*	Human herpesvirus 4 (Epstein–Barr virus)	Pongine herpesvirus 1 (chimpanzee herpesvirus, herpesvirus pan)
			Pongine herpesvirus 2 (orang-utan herpesvirus)
			Pongine herpesvirus 3 (gorilla herpesvirus)
			Callitrichine herpesvirus 3 (marmoset lymphocryptovirus)
			Cercopithecines herpesvirus 12 (papiine herpesvirus 1, herpesvirus papio)
			Cercopithecines herpesvirus 14 (African green monkey Epstein–Barr-virus-like virus)
			Cercopithecines herpesvirus 15 (macacine herpesvirus 4, rhesus lymphocryptvirus)
	Rhadinovirus	Human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus)	Ateline herpesvirus 2 (herpesvirus ateles, strain 810)
			Ateline herpesvirus 3 (herpesvirus ateles, strain 73)
			Saimirine herpesvirus 2 (herpesvirus saimiri)
			Bovine herpesvirus 4 (Movar virus)
			Murid herpesvirus 4 (mouse herpesvirus, strain 68)
			Cercopithecines herpesvirus 17 (macacine herpesvirus 5, rhesus rhadinovirus)

(*continued*)

Table 19.14 (continued)

Family	Subfamily	Genus	Human virus	Animal virus
		Macavirus		Alcelaphine herpesvirus 1 (bovine malignant catarrhal fever virus)
				Alcelaphine herpesvirus 2 (hartebeest malignant catarrhal fever virus)
				Bovine herpesvirus 6 (bovine lymphotropic herpesvirus)
				Caprine herpesvirus 2
				Ovine herpesvirus 2 (sheep-associated malignant catarrhal fever virus)
				Suid herepsviruses 3–5 (porcine herpesviruses 1–3)
		Percavirus		Equid herpesviruses 2 and 5 (equine herpesvirus 2 and 5)
				Mustelid herpesvirus 1 (badger herpesvirus)
Alloherpesviridae		*Ictalurivirus*		Ictalurid herpesvirus 1 (channel catfish virus)
		Cyprinivirus		Cyprinid herpesvirus 1 (carp pox herpesvirus)
				Cyprinid herpesvirus 2 (haematopoietic necrosis virus)
				Cyprinid herpesvirus 3 (koi herpesvirus)
		Batrachovirus		Ranid herpesvirus 1 (Lucké tumour herpesvirus)
		Salmonivirus		Salmonid herpesvirus 1 (herpesvirus salmonis)
				Salmonid herpesvirus 2 (*Oncorhynchus masou* herpesvirus)
Malacoherpesviridae		*Ostreavirus*		Ostreid herpesvirus 1 (oyster herpesvirus)

The specification of the different herpesvirus types includes the systematic name and in *parentheses* the commonly used names

cell spectrum. In vitro, they multiply in short replication cycles, leading to a quick spread in cell culture. The herpes simplex and varicella-zoster viruses, which cause a vesicular rash in humans, are prototypes of this subfamily and are subdivided into the genera *Simplexvirus* and *Varicellovirus*, respectively. Betaherpesviruses, e.g. cytomegaloviruses, have a narrow cell tropism. They have a relatively long replication cycle, and thus spread slowly in vitro. Infected cells appear clearly enlarged. Members of the third subfamily, *Gammaherpesvirus*, have a very narrow host spectrum, but the duration of the replication cycle is different among the various virus types. The gammaherpesviruses infect either B lymphocytes (Epstein-Barr virus) or T lymphocytes (saimiriine herpesvirus), and induce in them a state of virus latency. Some of these viruses are also able to infect epithelial, endothelial or fibroblast cells lytically. On the basis of viral DNA sequence homology, the structure of the genome and the immunological relationship of viral proteins, the subfamilies are further divided into different genera, to which the serologically distinct virus types are assigned.. In addition to the usually used virus names, herpesvirus types are denominated according to their host and numbered chronologically in order of discovery, such as human herpesviruses 1–8 (Table 19.14).

19.5.2 Structure

19.5.2.1 Virus Particle

Herpes virions have a diameter of 150–200 nm, and are constituted of three main structural elements: the icosahedral nucleocapsid (capsid) has a diameter of 90–110 nm and contains the linear, double-stranded DNA genome; the envelope, in which several glycosylated viral surface proteins are incorporated; and the tegument, which fills the space between the nucleocapsid and the envelope and is composed of various proteins. The number of structural proteins differs among the different virus types, and is not precisely known in all cases (Fig. 19.24, Table 19.15). Human cytomegalovirus particles consist of more than 70 different viral proteins and a similar number of cellular proteins, including – apart from structural components – also enzymes and chaperones. Some herpesviruses also contain mRNA molecules in their particles. For example, nine viral mRNAs have been discovered in herpes simplex virions, whereas even some cell-specific transcripts have been found in human cytomegalovirus. Their functions remain to be elucidated. The viral core is a fibrillar protein matrix inside the nucleocapsid with which the double-stranded linear DNA genome is associated. In electron microscope images, it occasionally resembles a coil around which the DNA is wrapped as a sewing thread or copper wire. The core is enclosed by the icosahedral capsid, which is composed of 162 capsomers. The main capsid protein of herpes simplex virus has a molecular mass of 155 kDa, with six of the capsid protein molecules forming a capsomer. They are linked by disulphide bonds to other viral proteins (VP19C), which are located on the inner side of the capsids, and are associated with the capsid proteins VP23 and the DNA genome. The capsomers (hexons) form tubular structures with a hole on the surface-exposed side which do not reach into

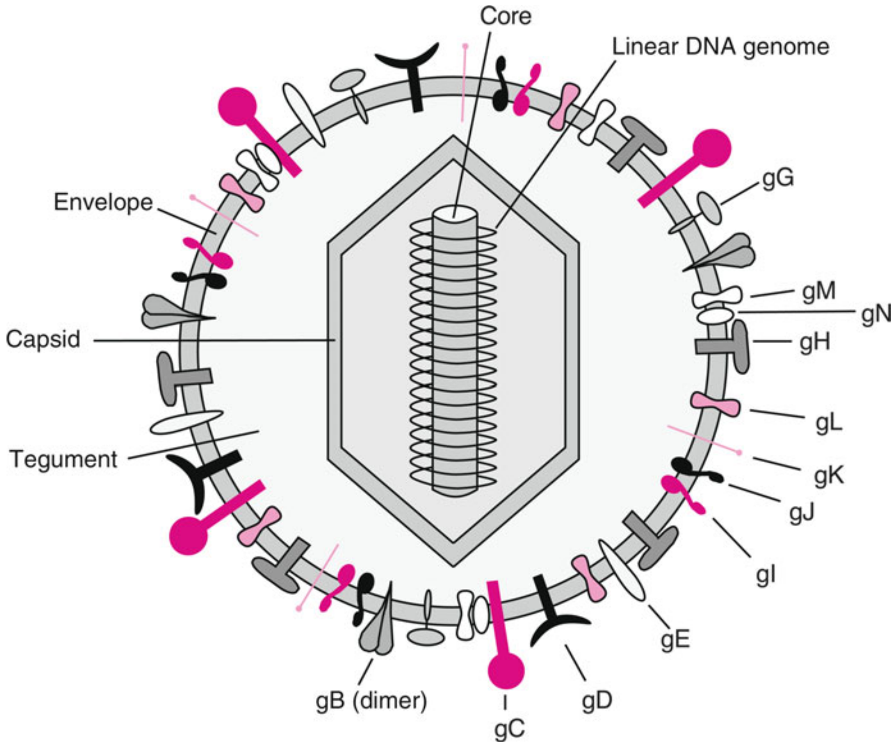


Fig. 19.24 Structure of a herpes simplex virus particle. A protein core is complexed with the linear, double-stranded DNA genome, and resides inside the virion. The core is surrounded by an icosahedral capsid that is composed of several structural proteins. The tegument, which contains several regulatory proteins, is situated between the capsid and the envelope, and is derived from the inner nuclear membrane. Twelve different proteins are embedded in the envelope of herpes simplex virus; they are indicated by different *symbols*

the capsid interior. The proteins p155 (pentons) are associated with the vertex proteins (VP26) at the 12 vertices of the icosahedrons. The phosphorylated vertex proteins have a molecular mass of 11 kDa, and assemble on the hexon units, forming a ring-like structure. In addition to infectious virions, infected cells also produce so-called L particles, which are not infectious, and consist only of an envelope and the tegument.

Intracellular capsids isolated before they are enveloped by the membrane contain a number of additional proteins that are only partially detectable in infectious particles. The VP21 proteins localized inside the capsid and the VP22a proteins encoded by reading frames U_L26 and $U_L26.5$ play a special role during morphogenesis as scaffolding proteins. They interact reciprocally and with VP5 proteins, forming a scaffold for the formation of the capsid structure. The product of the U_L26 gene is an autocatalytic protease (VP21, Pra) that cleaves itself and other members of this gene family (VP22/$U_L26.5$), generating, inter alia, a shortened

Table 19.15 Molecular properties and functions of structural proteins identified in herpesvirus particles

Molecular mass (kDa)/denomination/gene locus					
Human simplex virus	**Human cytomegalovirus**	**Epstein–Barr virus**	**Human herpesvirus 8**	**Modification**	**Function**
Capsid					
155/VP5/U_L19	153/-/UL86	154/-/BcLF1	153/-/ORF25		Major capsid protein; capsomer
50/VP19C/U_L38	34–37/-/UL46	39/-/BORF1	36/-/ORF62		Capsid protein, inside
34/VP23/U_L18	28/-/-	28/-/BOLF1	34/-/ORF62		VP23 on the capsid surface; fibrillar network between capsomers
11/VP26/U_L35	11/-/-	20/-/BFRF3	29/-/ORF65		Capsid protein; binds to dynein (Tctex1) of microtubuli
40/VP21/U_L26	-/-/UL80				Protease (Pra); assemblin inside the capsids; autocatalytically cleaved, generation of the cleavage product UL26 (also known as VP24 or Prn); the smallest active form of the protease of herpes simplex virus
38/VP22a/U_L26.5	37/-/UL80a	32/-/BdRF1	29/-/ORF175		Scaffolding protein; absent in infectious particles; substrate for UL26
74/portal/U_L6	73/portal/ UL104	68/portal/ BBRF2	68/portal/ORF43		Portal protein, 12 units per capsid; forms pores through which the viral genome is introduced into the precursor capsids
Tegument					
54/α-TIF, ICP25, VP16/U_L48	71/pp71/UL82			Phosphorylated	Transactive protein; induces the transcription of immediate early genes; causes SUMOylation of Daxx in human cytomegalovirus
74/VP13-14/U_L47				Phosphorylated	Modulates the functional activity of α-TIF
58/Vhs protein/U_L41					Virus-host shutoff; causes destabilization and degradation of host cell mRNA species
300/VP1-2/U_L36	212/-/UL48	350/-/BPLF1			Release of viral DNA, DNA cleavage

(*continued*)

Table 19.15 (continued)

Molecular mass (kDa)/denomination/gene locus					
Human simplex virus	**Human cytomegalovirus**	**Epstein–Barr virus**	**Human herpesvirus 8**	**Modification**	**Function**
18/-/U_S11	18/-/UL26	18/-/BFRF3		Myristoylated	dsRNA binding protein; post-transcriptional regulator of gene expression; inhibits activation of protein kinase R, OAS; antiapoptotic
64/-/U_L17		58/-/BGLF1			Cleavage and packaging of DNA genomes and capsids
	-/-/UL25				Interaction with pp65
	-/-/UL69				Interaction with pp65
	150/-/UL32			Phosphorylated, *N*-acetyl-glucosamine	Acceptor of phosphate groups by the virion-associated protein kinase; induces cross-reacting antibodies
57/VP18.8/U_L13	65/pp65/UL83	47/-/BGLF4		Phosphorylated	Protein kinase und acceptor of phosphate groups (in human cytomegalovirus)
		152/-/BNLF1	gp35-37/-/ORF8		Main tegument component
Envelope proteins					
100/gB/U_L27	150/gB/UL55	110/-/BALF4	-/-/ORFK8.1	Glycosylated	Penetration and particle internalization; trimer; proteolytic cleavage by furin into two parts (gp55 and gp93 in human cytomegalovirus, gp78 and gp58 in Epstein–Barr virus): cell fusion; in Epstein–Barr virus, it is not located in the envelope, but is detectable in the nuclear and ER membranes
55/gC/U_L44				Glycosylated	Attachment; binds to different components of the complement complex
44/gD/U_S6				Glycosylated	Penetration and particle internalization; influences virulence of human simplex virus in mice; absent in varicella–zoster virus

60/gE/U_S8				Glycosylated	Receptor for the Fc region of immunglobulins, forms heterodimers with gI
25/gG/U_S4					Cell-to-cell spread, coating of nucleocapsids
90/gH/U_L22	86/gH/UL75	85/-/BXLF2	-/-/ORF22	Glycosylated	Penetration and particle internalization
42/gI/U_S7				Glycosylated	Receptor for the Fc moiety of immunglobulins
10/gJ/U_S5					
38/gK/U_L53				Glycosylated	Mutants induce cell fusion
25/gL/U_L1	32/gL/UL115	25/-/BKRF2		Glycosylated	Cell fusion during transport to the cytoplasmic membrane; complex formation with gH; glycoprotein, not membrane-associated
51/gM/U_L10	45/gM/UL100	45/-/BBRF3	-/-/ORF39	Glycosylated	Integral envelope protein, highly hydrophobic
12/gN/U_L49.5	-/gN/UL73	11/-/BLRF1		Glycosylated	Complexed with gM by disulphide bridges
	47–52/gCII/US6			Glycosylated	MHC class I homologue protein
	48/gp48/UL4			Glycosylated	?
	-/gO/UL74			Glycosylated	Forms the gCIII complex along with gH and gL
	-/gp34/TRL11/IRL11				Acts as a viral gFc receptor
	-/gp68/UL119/UL118				Acts as a viral gFc receptor
	gp220/350/-/BLLF1			Glycosylated	Binding to CD21 (complement receptor CR2), neutralizing antibodies
	gp150/-/BDLF2			Glycosylated	?
	55–78/-/BILF2			Glycosylated	?
	gp42/-/BZLF2			Glycosylated	Associated with gp85/BXLF2 and gp25/BKRF2; binds to MHC class II proteins; viral penetration?
	39/-/BMRF2			Glycosylated	Binding to integrin
	30/-/U_L24				Mutants induce cell fusion; syn plaque phenotype
	25/-/U_L20				Intracellular particle transport during viral morphogenesis; cell fusion

(*continued*)

Table 19.15 (continued)

Molecular mass (kDa)/denomination/gene locus					
Human simplex virus	**Human cytomegalovirus**	**Epstein–Barr virus**	**Human herpesvirus 8**	**Modification**	**Function**
	-/-/U_L11			Myristoylated	?
	-/-/U_L43				?
	-/-/U_L45				Associated with the ER

The corresponding proteins of the different virus which possess either a significant amount of similar sequences or have similar functional properties types are listed comparatively. The specification for each protein refers to the molecular mass (kDa). The names of the proteins are given according to the rules which are explained in Sect. 19.5.2, and the denomination of the loci corresponding to them by which they are encoded in the genome of the virus. In many cases, it is not possible to provide all information for each protein because either the molecular mass or the gene localization is not known *dsRNA* double-stranded RNA, *OAS* 2′-5′-oligoadenylate synthetase, α-*TIF* α-*trans*-inducing factor

form of the protease (VP24; Prn) and the infected cell protein (ICP) 35 family, to which the scaffolding protein VP22a belongs. This protein forms the scaffold for the developing capsids during morphogenesis, and is no longer detectable in infectious virions. Proteins with similar properties have also been detected in the capsids of human cytomegaloviruses: the UL80 protein is a protease that is also referred to as assemblin. The UL80a proteinarises by autocatalytic cleavage. It is functionally analogous to the VP22 of herpes simplex viruses. A corresponding protease is encoded in ORF17.5 of human herpesvirus 8.

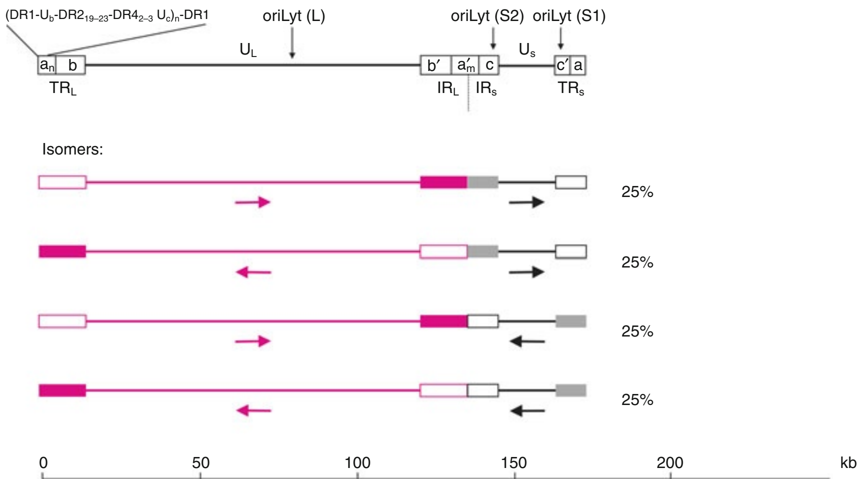
The herpes simplex virus capsids are surrounded by an envelope, into which up to 12 viral glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, gN) and two to four non-glycosylated polypeptides are embedded. They form, to some extent, protein protrusions on the surface of the virus, and perform important functions in binding to the cell receptors, in penetration and cell entry, and in the induction of a protective, neutralizing antibody response. The region localized between the capsids and the envelope, the tegument, can differ in size, and its thickness determines the slightly variable diameter of the particles. The tegument is an unstructured protein mixture that comprises up to 20 viral components. These proteins also enter the cell during infection. Some of the tegument proteins of herpes simplex virus have important regulatory functions during the early phase of the replication cycle. They include, among others, the α-*trans*-inducing factor (α-TIF), the virus-host shutoff (Vhs) protein, a protein kinase and the U_S11 gene product. The virion proteins of other herpesviruses, which are morphologically very similar to herpes simplex virus, have similar molecular masses and analogous functions (Table 19.15).

19.5.2.2 Genome Organization and Structure

The herpesvirus genome is constituted of linear, double-stranded DNA with a length between 120 and 230 kb. All virus types contain unique and repeated sequence segments that are arranged in different patterns in the respective genera (Fig. 19.25). Presumably, the two ends of the genome are close to each other within the virion. The acidic phosphate groups are saturated with basic, cellular spermine and spermidine molecules. Upon infection, the linear DNA is converted into a circular molecule, which is then present as an episome within the nucleoplasm. Both DNA strands encode more than 100 gene products (cytomegaloviruses even more than 200), some of which are expressed using different reading frames and overlapping open reading frames. Apart from a few exceptions, the expression of each gene is controlled by a specific upstream promoter which has the characteristic recognition sites for the eukaryotic RNA polymerase II. The promoter regions contain additional binding sites for cellular and viral transactivators, which facilitate a spatial and temporal gene expression during the infection cycle. Many proteins are translated from spliced mRNA molecules. The required splice donor and acceptor sites are scattered throughout the genome. In particular, Epstein-Barr virus proteins which are synthesized during latency are translated from mRNA molecules which are subject to multiple splicing processes involving extensive regions of the genome.

Herpes Simplex Viruses (Human Herpesviruses 1 and 2)

The genome has a length of about 152 kb, and is divided into a long and a short segment (long segment 126 kb, short segment 26 kb) (Fig. 19.25a). Each segment contains both a long (U_L) and a short (U_S) unique sequence region, which are flanked by inverted repeats. They are referred to as a terminal repeat and an internal repeat according to their position either at the ends of the genome or at the centre, where the two segments are joined. The repeats flanking the U_L region at the end of the genome consist of multiple repeated a regions (each comprising 465–624 base



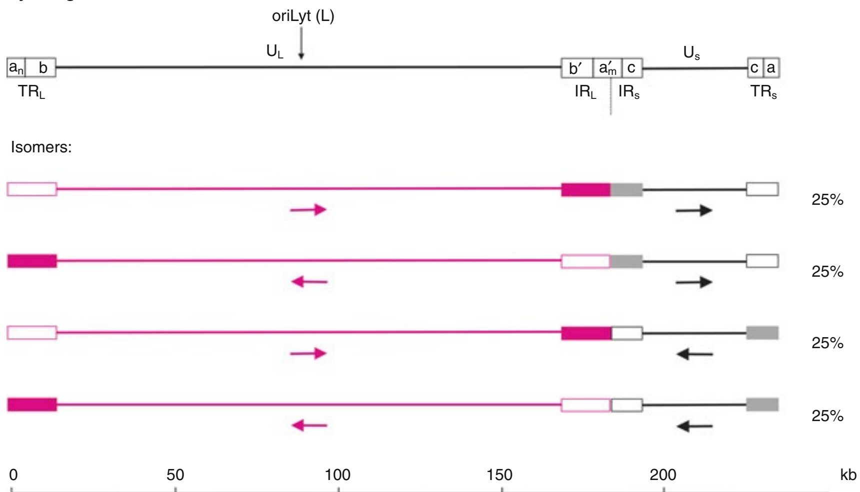
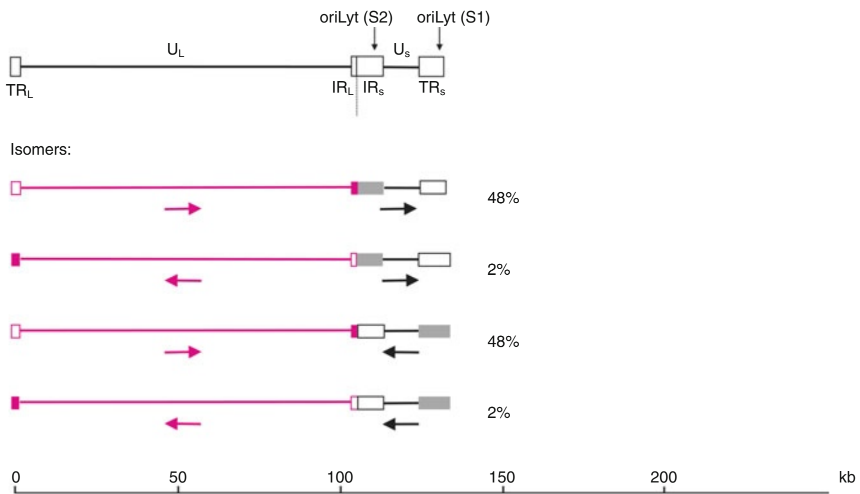
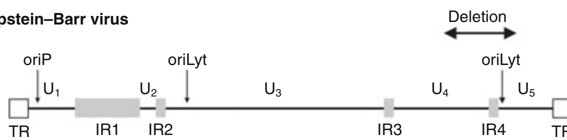


Fig. 19.25 (continued)

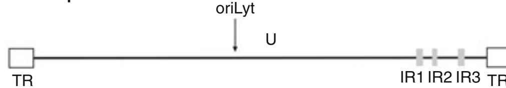
c Varicella–zoster virus



d Epstein–Barr virus



e Human herpesvirus 6



f Human herpesvirus 8

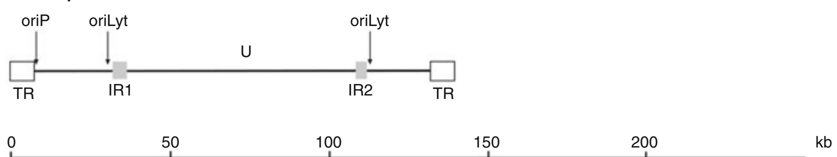


Fig. 19.25 Genome structure of herpesviruses: (**a**) Herpes simplex virus. (**b**) Human cytomegalovirus. (**c**) Varicella-zoster virus. (**d**) Epstein-Barr virus. (**e**) Human herpesvirus 6. (**f**) Human herpesvirus 8. The regions of the genome that are composed of repeat sequences are depicted as *bars*. They are located at the ends of the genome (terminal repeat, *TR*) and within the genome (internal repeat, *IR*). The unique genome segments are represented by *lines*. In addition, the replication origins which are used for the rolling-circle replication mechanism during the lytic replication cycle are indicated: oriL, oriS1 and oriS2 in herpes simplex virus, oriLyt in human cytomegalovirus, oriLyt, (S1) and oriLyt (S2) in varicella-zoster virus, and oriLyt in Epstein-Barr virus, human herpesvirus 6 and human herpesvirus 8. In the case of Epstein-Barr virus, also the replication origin which is used for replication of the episomal viral DNA (oriP) during latency is known. The unique genome segments of herpes simplex virus, cytomegalovirus and varicella-zoster virus can be arranged in different orientations to each other. This is determined by the location of the terminal repeat and the internal repeat and their orientation to one another

pairs), which are followed by a b element (8,800 base pairs). This segment is termed the TR_L (for "terminal repeat long") region. It is followed by the U_L region (108,000 base pairs), which is followed by a b′ element in reverse orientation and a differing number of a repeats. They constitute the transition between the segments and are referred to along with the subsequent c element (6,600 base pairs) as the internal repeat region (IR_L/IR_S for "internal repeat long" and "internal repeat short"). This is followed by the U_S region, which is followed by an inverted c′ unit and a sequences (TR_S for "terminal repeat short") (Fig. 19.25a). Therefore, the genome of herpes simplex virus consists of an array of repeated and unique sequence elements arranged in the pattern indicated in following scheme:

$$a_n\text{-b} - U_L - \text{b}'\text{-a}'_m\text{-c} - U_S - \text{c}'\text{-a}$$
$$TR_L - U_L - IR_L/IR_S - U_S - TR_S$$

Herpesvirus Proteins are Designated with Different Abbreviations

The nomenclature of herpesvirus proteins is very complex. Herpes simplex virus structural proteins have the initials VP (for "virion protein") and a number, which depends on the migration behaviour of the respective protein in the sodium dodecyl sulphate polyacrylamide gel electrophoresis. The most slowly migrating protein carries the number 1 (VP1). The intracellular proteins (ICP) which are synthesized in infected cells must be distinguished from them. They are numbered in an analogous manner. Since some virion proteins can also be detected intracellularly, the nomenclature of both groups overlaps. In addition, glycoproteins are abbreviated as gX, whereby upper-case letters refer to the order in which the polypeptides were discovered. The proteins gA and gF do not exist because they were subsequently proven to be precursor proteins or cleavage products of other glycoproteins. Furthermore, proteins are also denominated after their encoding open reading frames. These are designated after the genome segments, and are numbered from left to right regardless of their orientation, e.g. U_S1 and U_L19. These two proteins are coded in the first reading frame of the short unique region and in the first reading frame of long unique region, respectively. Another principle of classification is based on the time at which the relevant proteins are synthesized in the cell: α proteins are the first proteins, and are translated independently of the synthesis of other viral gene products. Therefore, they are referred to as very early, α, or immediate early proteins. β or delayed early proteins chronologically follow the α proteins and are dependent on their

Fig. 19.25 (continued) (indicated by the *lower-case letters*, *a* and *a*′ representing an example of inverted repeats). This leads to the generation of four different genome isomers. The probabilities for the occurrence of the different genome isomers in the virions are indicated. Furthermore, the location of the sequences deleted in the genome of Epstein-Barr virus isolate B95-8 is also shown. For further explanation, see the text

presence in the cell. The γ or late proteins are synthesized at the end of the cycle. Most of them are structural proteins. All terms are used concomitantly in the literature. Thus, for example, the product of reading frame U_S1, an α protein, is also termed ICP22, and the γ protein U_L19 is the capsid protein VP5 or p155. A similar redundant system has been used for the nomenclature of the cytomegalovirus gene products.

The nomenclature of the gene products of Epstein-Barr virus is not as confusing. It is based on the DNA fragments that are generated by digestion of the genomic DNA with the restriction enzyme *Bam*HI. The largest is the A fragment, the second largest the B fragment, and so on. As there are more DNA fragments than letters, the denomination of all fragments after Z is continued using lower-case letters. The different reading frames are assigned to the fragments and numbered in their order. This takes into account whether they are oriented in the right or left transcription direction. This provides an indication from which DNA strand the mRNA of the corresponding gene is read. Thus, one of the immediate early or α proteins of Epstein-Barr virus is assigned to the reading frame BZLF1 (*Bam*HI/Z fragment/left frame 1). It is the first, left-directed reading frame of the Z fragment generated by *Bam*HI digestion. In addition, the proteins are abbreviated by indicating their molecular weights: e.g., gp220/350 stands for glycoproteins of the respective size.

The genes of human herpesvirus 6 which are located in the unique region of the viral genome are designated with the abbreviation U for unique and a number: U1 is the first reading frame located adjacent to the terminal repeat TR_L at the left end of the genome, U100 is at the other end adjacent to TR_R; this numbering does not take into account the genomic orientation of the open reading frames. In the sequences of direct terminal repeats TR_L and TR_R there are seven open reading frames (TR1 to TR7). The A and B strains of human herpesvirus 6 differ by nine additional reading frames in strain B, which are named B1 to B9.

The nomenclature of the human herpesvirus 8 genes has been established following that of saimiriine herpesvirus. This virus has large sequence homology to human herpesvirus 8. The homologous genes are denominated as "ORF" with the respective numeral according to the numbering of the open reading frames (e.g. ORF2 for the dihydrofolate reductase genes which are present in the genomes of both viruses). The reading frames of regions of the genome without homology to those of saimiriine herpesvirus are also numbered in order of their arrangement, preceded by the letter "K" (for "Kaposi's sarcoma-associated virus").

Bovine herpesvirus 2, which has a genomic structure similar to that of herpes simplex virus, contains more than 133,000 base pairs.

During infection and replication of these herpesvirus types, four isomeric genome forms can arise by intramolecular recombination of identical inverted

repeats, in which the U_L and U_S regions are oriented differently to each other (Fig. 19.25a). Every isomeric genome is present in the virions, all are infectious and they do not differ in their information content, since they are present in the cell as circular episomes (Fig. 19.27). Three replication start sites have been identified in the herpes simplex virus genome: one origin is located in the centre of the U_L region (oriL), the other two (oriS) reside in the c sequences of the repeat units flanking the U_S region. They comprise about 800–1,000 base pairs, and have short, symmetrically arranged sequence elements and palindromes at their centres. At least one oriS element must be present in the genome for the correct execution of DNA synthesis; an oriL element is not absolutely essential.

The number of a units in the terminal repeat and internal repeat elements differs depending on the virus strain. They are also composed of successive short direct repeats and a unique sequence, which are conserved in all herpes simplex viruses (Fig. 19.25a): a 20-base-pair direct repeat (DR1) is located at the end, followed by a single U_b region (65 base pairs), a 19-fold- to 23-fold-occurring repeat DR2 (22 base pairs), a DR4 repeat (37 base pairs) in twofold to threefold repetition and a U_C region (58 base pairs). The DR1 element at the end of the TR_L region is only 18 base pairs long. An overhanging nucleotide is situated at the 3′ terminus. At the other end of the genome there is only one base pair of the DR1 repeats and also a 3′ overhanging nucleotide. The sequences complement a complete DR1 element during genome circularization. The DNA sequences of herpes simplex virus types 1 and 2 are very similar (85 % homology), and the arrangement of the various elements is identical.

Cytomegalovirus (Human Herpesvirus 5)

Cytomegaloviruses belong to the human pathogenic viruses with the highest coding capacity. The genome of human cytomegalovirus (strain AD169) comprises 229,354 base pairs, and contains the genetic information for approximately 200 gene products. About 30 proteins exhibit significant homology with the corresponding gene products of other herpesviruses. It is striking that the genetic information of cytomegaloviruses contains nine groups of closely related genes that occupy about 26 % of the genome. These include the RL11 and US6 genes that encode the glycoprotein families gp48 and gcII/gp47 to gcII/gp52. Like with other herpesviruses, the cytomegalovirus genome is divided into a long unique region and a short unique region, which are flanked by repeats (Fig. 19.25b) Intramolecular recombination events also generate four isomeric genomes. The origin of replication during the lytic replication cycle (oriLyt) is located in the centre of the U_L region.

Varicella-Zoster Virus (Human Herpesvirus 3)

Varicella-zoster virus belongs to the alphaherpesviruses, and its genome is the smallest of all human herpesviruses. It has only 72 open reading frames, and it lacks many of the genes that are found in herpes simplex virus. In addition to the strain Dumas (124,884 base pairs), the vaccine virus strain Oka and a few other isolates have been completely sequenced. The genome organization resembles that of herpes simplex virus, with the exception that the repeat units TR_L and IR_L, which flank the long unique region U_L (104,800 base pairs), are very short (only 88 base

pairs). By contrast, the TR_S and IR_S units are long (7,319 base pairs), and flank the U_S region, which comprises 5,232 nucleotides. The numerical values refer to the strain Dumas. Intramolecular recombination rarely occurs in varicella–zoster virus, probably because of the shortness of the TR_L and IR_L sequences, so only two isomeric genomes are usually formed, in which the S elements have different orientations (Fig. 19.25c). Animal pathogenic varicella viruses have a similar genome organization. The DNA genomes of bovine herpesvirus 1 and porcine herpesvirus have a size of approximately 140 kb. The DNA genome of feline herpesvirus is 134 kb long, whereas the genome of equine herpes viruses is about 148 kb long.

Epstein-Barr Virus (Human Herpesvirus 4)

The genome of Epstein–Barr virus strain B95-8 contains 172,281 base pairs, and does not form isomers. The ends of the genome contain a different number of direct repeats of 538 base pairs (terminal repeat). The internal repeats (IR1, 3,072 base pairs), which – depending on the virus strain – are arranged in tandem in different numbers, divide the genome into a short and a long unique region (U_S or U1 and U_L), which comprise 12,000 and 134,000 base pairs, respectively (Fig. 19.25d). The U_L segment is interrupted by three additional short tandem repeats (IR2, IR3 and IR4), which subdivide this segment into the U2, U3 and U4 regions. Two additional conserved direct repeats of 125 base pairs (D_L and D_R) are located at the ends of the U_L segment. The two origins for the lytic replication cycle (oriLyt) are located in their neighbourhood. In contrast, the replication origin used for episomal genome replication during latency (oriP) is situated in the U1 region. Since each virus strain contains a specific number of different repeats, the length of the genome of Epstein-Barr viruses is heterogeneous. In addition, there are strains that contain large deletions. Among others, 11,835 base pairs are deleted in the right end of the U_L segment of Epstein-Barr virus B95-8, which infects B cells with a strong tendency to establish a latent state. This results in loss of the IR4 and DR sequences in the B95-8 genome. In contrast, the U2 region and part of the flanking IR1 and IR2 repeats are absent in the P3HR1 virus isolate, which is not able to infect B cells latently.

Human Herpesviruses 6 and 7

Human herpesvirus 6 differs in terms of genome structure from all other human pathogenic herpesviruses. The viral genome consists of approximately 160,000–162,000 base pairs. Direct repeats of about 8–9 kb in length are located at the ends of the linear DNA. These terminal repeats flank a unique region of nearly 143,000–145,000 base pairs, which is interrupted by three internal repeats (IR1, IR2 and IR3) within the coding region for the early gene products (Fig. 19.25e). Therefore, isomeric genomes cannot arise. A similar genome organization is found in human herpesvirus 7, as well as in herpesvirus species of New World monkeys, *Herpesvirus saimiri* and *Herpesvirus ateles*. In the sequences of terminal repeats DR_L and DR_R of human herpesvirus 6, there are seven open reading frames (DR1 to DR7) and the pac-1 and pac-2 sequence motifs, which are responsible for the cleavage of the concatemeric genomes into individual monomeric molecules and their packing into precursor capsids after DNA replication. The ends of the

genome resemble the telomere structures of mammalian chromosomes: they contain the characteristic nucleotide sequence motif GGGTTA in a multiply repeated succession; it is suspected that they are necessary for the preservation of genomes and their replication in latently infected cells. The DNA sequences of human herpesviruses 6 and 7 are collinear to each other, and the genome organization of the unique region is similar to that of the U_L region of human cytomegalovirus.

Human Herpesvirus 8 (Kaposi's Sarcoma-Associated Herpesvirus)
The genome of human herpesvirus 8 contains a unique sequence region of 133,600–137,500 base pairs. Various genotypes are known, and can be assigned to seven closely related strains or subtypes (A, B, C, D, M, N und Q). At the genome ends, there are differing numbers of terminal repeats of 801 base pairs in length (Fig. 19.25f). The genome structure of human herpesvirus 8 resembles to some extent to that of Epstein-Barr virus; furthermore, the genome also contains short GC-rich repeats which are similar to the IR2, IR3 and IR4 repeats of Epstein-Barr virus. However, it does not contain a region homologous to Epstein-Barr virus internal repeat IR1. Sequences with homology to the initiation sites for genome replication (oriLyt) of Epstein-Barr virus are located adjacent to IR1 and IR2 of human herpesvirus 8. Regarding the gene arrangement, the genome of human herpesvirus 8 is similar to the genomes of the herpesviruses of New World monkeys (saimiriine herpesvirus and ateline herpesvirus), as well as to those of other animal pathogenic viruses of the genus *Rhadinovirus*, such as murine gammaherpesvirus 68. For this reason, human herpesvirus 8 has also been assigned to this genus in the subfamily *Gammaherpesvirinae*.

19.5.3 Viral Proteins of the Lytic Cycle

Because of the high coding capacity of herpesvirus genomes, and the large number of encoded viral proteins, it is impossible to discuss individually all the molecular details and functions associated with them. Therefore, only some particularly important proteins involved in the replication cycle or pathogenesis will be discussed in this chapter. Readers who are interested in further details can find additional information in the review articles listed in "Further Reading" after Sect. 19.5.8.5.5.

19.5.3.1 Structural Proteins

Envelope Proteins
Different glycoproteins are embedded in the envelope of herpesviruses (Table 19.15). Some of them, namely glycoproteins gB, gH, gL and gM, are functionally conserved, and are present in all members of the different subfamilies. Other viral proteins are only found in the plasma membrane of infected cells and/or in the envelope of virus particles of certain herpesviruses; they characterize the members of the different subfamilies.

Twelve glycoproteins (gB to gN) have been identified in herpes simplex virus and animal pathogenic alphaherpesviruses such as pseudorabies virus.

Glycoproteins gB and gD and the complex gH/gL are involved in internalization of the virus into the cytoplasm. Each of these polypeptides induces the formation of neutralizing antibodies that inhibit the infection at the level of penetration. The gB and/or gC proteins bind to heparan sulphate proteoglycan on the cell surface (Sect. 19.5.6, Table 19.19). After removal of the amino-terminal signal peptide, the gB protein of herpes simplex virus 1 has 875 amino acids, and is modified at six residues with N-glycosidically linked carbohydrate groups. In its mature form, gB seems to be a homodimer. Most of the neutralizing antibodies which are produced during infection are directed against this protein. Herpes simplex virus 1 neutralizing antibodies are also directed against the gC protein. It contains 511 amino acids, whereby the 25 amino-terminal residues act as a signal peptide and mediate the synthesis on the ER. The gC protein is modified with sugar groups by both N- and O-glycosidic bonds, and is present as an oligomeric complex. The gC protein is present on the surface of infected cells, and binds to complement component C3b (▶ Chap. 7). Discontinuous regions of the protein are responsible for this. The function of C3b binding has not been fully elucidated. It is suspected that it enables infected cells or virus particles to evade further binding of components of the complement system and the complement-mediated elimination.

Besides the rather unspecific interaction with heparan sulphate proteoglycan, attachment of herpes simplex viruses is also performed by interaction of the gD protein with nectin-1 (cell receptor of the immunoglobulin superfamily) and a group of TNF-receptor-like proteins, which are commonly referred to as herpesvirus entry mediators. The gD protein is composed of 394 amino acids. Its 25 amino-terminal amino acids function as a signal peptide. It is modified by sugar groups by O- and N-glycosidic bonds. After a herpes simplex virus infection, not only gD-specific neutralizing antibodies can be detected in the patients, but also cytotoxic T cells, which recognize epitopes of this protein. Furthermore, the presence of the gD protein on the surface of infected cells prevents attachment and penetration of other herpesvirus particles. The human pathogenic varicella-zoster virus is the only known member of the alphaherpesviruses that does not possess the genetic information for a gD homologous protein.

In addition to the C3b-binding activity of the gC protein, herpes simplex viruses have another mechanism to evade the immune response: glycoproteins gE and gI form heterodimers that act as viral Fcγ receptors and bind to the γ chains of immunoglobulins. This prevents the interaction of antibodies with effector cells. Additionally, it seems possible that neutralizing B-cell epitopes of these surface proteins are masked by antibody binding, and cannot be recognized as foreign. Moreover, the gE and gI proteins are also involved in the dissemination of infection in the tissue. They accumulate during the late phase of infection at the lateral membrane domains of polarized cells and in cell junctions. They mediate the cell-to-cell transmission of progeny viruses. In this way, the infection spreads in cell cultures in vitro and in epithelial or neuronal tissue in vivo, without infectious particles being involved and released. Nevertheless, the complex of gE and gI proteins has another function in the early phase of infection. At this stage, it is anchored in the intracellular membrane compartments of the *trans*-Golgi network.

A sorting signal (Y–X–X–aromatic amino acid) in the carboxy-terminal cytoplasmic domain of the gE protein is responsible for anchoring. The gE and gI proteins mediate the enveloping of nucleocapsids with the membrane of the *trans*-Golgi network, and give rise to the formation of precursor virus particles, which are then transported to the lateral side of epithelial cells.

Varicella-zoster virus – also a member of the alphaherpesviruses – encodes only nine envelope proteins, eight of which correspond functionally to glycoproteins gB, gC, gE, gH, gI, gK, gL and gM of herpes simplex virus, and are termed as such. ORF1 of varicella-zoster virus encodes another envelope protein; a corresponding homologous gene product is found neither in herpes simplex viruses nor in other human pathogenic herpesviruses. The ORF1 protein has a molecular mass of about 14–17 kDa, is phosphorylated and is anchored in the cytoplasmic membrane and the membranes of the *trans*-Golgi network by a carboxy-terminal transmembrane domain. It is not essential for the production of infectious progeny viruses.

Like gB proteins, the gH/gL protein complex is also conserved in all herpesviruses, and is involved in both binding of virus particles to the cell surface and fusion between the cell membrane and the viral envelope. (Tables 19.15 and 19.19). Neutralizing antibodies are directed against epitopes of the gH/gL protein complex. The gH protein consists of more than 838 amino acids, and contains a carboxy-terminal transmembrane domain. A hydrophobic α-helix in the region of amino acids 377–397 of the gH protein has fusogenic activity, and is capable of inducing fusion of adjacent membranes. The gH proteins form complexes with the soluble gL proteins via disulphide bonds in the ER; gL comprises 224 amino acids in herpes simplex virus. Such covalent binding between the homologous gH and gL proteins does not occur in the members of beta herpesviruses and gammaherpesviruses. The gL proteins act as chaperones, and are essential for the transport of the gH/gL complexes to the cytoplasmic membrane. The gH/gL proteins of some herpesviruses interact with other glycoproteins (gO in human cytomegalovirus, gp42 in Epstein-Barr virus and gQ1/gQ2 in human herpesvirus 6). These large aggregates are involved in the interaction with cellular receptors and influence the cell tropism of the respective viruses.

The gB homologue of human cytomegalovirus is encoded in reading frame UL55, and is synthesized as a precursor protein of 906 amino acids. In contrast to the protein of herpes simplex virus, it is cleaved by the cellular protease furin at position 460. This processing creates a heterodimer composed of the amino-terminal gp93 and the carboxy-terminal gp55, which are linked by disulphide bridges. Most neutralizing antibodies are directed against this protein complex. The gB protein corresponds to gp110 of Epstein-Barr virus. It is encoded by the BALF4 reading frame, and is also proteolytically cleaved. A protein similar to gB, gp35–37, is encoded by ORFK8.1 in human herpesvirus 8. The gene product of human cytomegalovirus, gp86 (UL75), which is a homologue of the gH protein of herpes simplex virus, comprises 742 amino acids. It is homologous to gp85 of Epstein-Barr virus encoded by the BXLF2 reading frame, and the protein encoded by gene 37 of varicella-zoster virus. The homologous gH proteins of human herpesviruses 6 and 8 are encoded by the genes U48 and ORF22, respectively.

In Epstein-Barr virus, most neutralizing antibodies are directed against a glycoprotein which is present in two different forms on virions and infected cells. The gp350 protein is encoded by BLLF1, and translated from a non-spliced mRNA. For the synthesis of gp220, an intron is removed from the central region of the mRNA, so the resulting protein is identical to the amino- and carboxy-terminal regions of gp350. Both proteins have a high content of glycosidic modification. In addition to a protective antibody response, they also induce an antibody-dependent cytotoxic T-cell response in infected individuals (▶ Chap. 7). Epstein-Barr virus attaches to the cells by binding of the gp220/gp350 complex to CD21 proteins (CR2 complement receptor).

Cytomegaloviruses also Possess Immunoglobulin-Binding Surface Proteins
Like the gE/gI protein complex of herpes simplex virus, even cytomegaloviruses have glycoproteins that can bind to the heavy γ chains of antibody molecules. These are the human cytomegalovirus glycoproteins gp34 and gp68, which are encoded by the open reading frames TRL11/IRL11 and UL119/UL118, respectively. They are capable of binding to all four immunoglobulin subclasses (IgG1, IgG2, IgG3, IgG4), whereas the gE/gI proteins of herpes simplex virus cannot bind IgG3. The mouse cytomegalovirus also has a viral Fc γ receptor that has been termed fcr-1/m138. Although gE/gI, gp34, gp68 and fcr-1/m138 have a similar ability to interact with the γ chains of antibody molecules, their amino acid sequences do not exhibit significant homology. Their activities have presumably developed independently.

Tegument Proteins

The herpes simplex virus α-TIF protein (also VP16, 54 kDa) is encoded by open reading frame U_L48 and is synthesized the late phase of the lytic replication cycle. It corresponds to the varicella-zoster virus ORF10 gene product. It is incorporated into the infectious particles during morphogenesis, and it constitutes one of the tegument components. It is not necessarily required for viral replication: α-TIF-defective mutants are also able to replicate, albeit inefficiently and with prolonged replication times. The α-TIF protein consists of three functional domains. The first domain comprises residues 173–241. It is rich in basic amino acids and exhibits DNA binding activity. The second domain consists of amino acids 378–389, and interacts with the cellular transactivator Oct-1. The 80 acidic carboxy-terminal amino acids constitute the third domain, and harbour the transactivator function. α-TIF is transported into the nucleus of infected cells, where it enhances the expression of immediate early genes. The α-TIF protein does not bind to promoters, but rather interacts with the C1 factor, which is composed of two subunits (80 and 70 kDa). The α-TIF/C1 complex then binds to Oct-1, which in turn interacts with specific sequence motifs in the promoters of immediate early genes. This facilitates the interaction of the α-TIF transactivation domain with the TATA-box binding transcription factor TFIID, thereby enhancing transcription of immediate early

genes. The sequence 5′-GyATGnTAATGArATT-3′ (y is pyrimidine, n is purine or pyrimidine, r is purine) in the promoter region is essential for binding of the complex, and thus for transactivation. Oct-1 interacts preferentially with the nucleotide sequence ATGnTAAT. α-TIF interacts as part of the complex – but not in free form – with the GArAT residues (r is purine).

The tegument protein pp71 encoded by the UL83 gene of human cytomegalovirus has a transactivator function similar to that of the α-TIF protein of herpes simplex virus. It induces the expression of immediate early genes, and it is phosphorylated by the protein kinase associated with the virus particles. It binds to cellular factors and influences the AP-1 or CRE/ATF recognition sites within the enhancer that regulates the expression of the immediate early genes 1 and 2 (Fig. 19.28). It also causes SUMOylation, and thus the proteolytic degradation of Daxx, a protein component of the promyelocytic leukaemia nuclear bodies. Daxx functions as a co-repressor which impedes the expression of immediate early genes. The Epstein-Barr virus protein encoded by the BPLF1 gene has 30 % sequence homology with α-TIF. Whether it plays a similar role in initiating lytic infection is unknown.

α-TIF is not the only tegument protein that influences the expression of immediate early genes of herpes simplex virus: the products of the U_L46 and U_L47 genes (VP13 and VP14) also influence the transactivation process. However, it is unknown how this is done. VP13 and VP14 are phosphorylated by the viral protein kinase (U_L13), another component of the tegument. Whether and to what extent this protein modification influences immediate early gene expression remains to be elucidated. Protein kinases have also been found in the virions of betaherpesviruses and gammaherpesviruses. In human cytomegaloviruses, this activity seems to be associated with the protein pp65, the main component of the tegument. The protein pp65 promotes the incorporation of other viral proteins into the tegument of new developing cytomegaloviruses: these include the UL97 protein kinase and the gene products of UL25 and UL69. If the synthesis of pp65 is blocked by site-directed mutagenesis, this will prevent the in vitro infection of macrophages, but not that of fibroblasts. This is an indication that the tegument protein pp65 also affects the tropism of human cytomegaloviruses.

The herpes simplex virus protein encoded by the UL35 gene interacts with the protein Tctex1, the light dynein chain of microtubules, after the nucleocapsids have penetrated into the cytoplasm together with their adhering tegument proteins. This causes the intracellular transport of capsids to the nuclear pores. The U_S11 gene product is also a component of the tegument of herpes simplex virus, and it is synthesized at a late stage of the infection cycle. The U_S11 protein binds to double-stranded RNA, thereby inhibiting the activation of both protein kinase R (induced by double-stranded RNA) and 2′-5′-oligoadenylate synthetase. Hence, it inhibits the induction of the interferon class I dependent immune response. Furthermore, the U_S11 protein seems also to counteract transcription termination. It recognizes a hairpin structure in the mRNA transcribed from open reading frame U_L34. This interaction gives rise to the progression of mRNA synthesis, thus counteracting premature termination and formation of a non-polyadenylated mRNA.

The U_S11-regulated U_L34 protein is an envelope protein that is phosphorylated by the U_L13 kinase. It is present in the virions, but its function is unknown. Moreover, it has been reported that the U_S11 protein exerts an antiapoptotic effect.

Another tegument component of herpes simplex virus is responsible for the degradation and destabilization of cellular mRNA species during the early phase of infection, a process that is associated with a rapid decrease of host cell protein synthesis and modification. It causes – possibly by binding to the translation initiation factor eIF4H – the cleavage of 5′-cap structures, and thus the degradation of transcripts from the 5′ terminus. The Vhs protein (58 kDa), which induces the termination of host functions, is encoded by reading frame UL41, and is synthesized at a late stage of the infection cycle; a corresponding protein is encoded by ORF17 of varicella-zoster virus. In addition to the cellular effect, it also destabilizes viral mRNA molecules. Probably, this regulates the transition from early to late stages of replication. Vhs-like protein functions have not been identified in betaherpesviruses and gammaherpesviruses to date.

The Transactivators Oct-1 and Oct-2 Belong to the POU Homeobox Protein Family

The cellular transactivator Oct-1 (also known as NF-A1, NFIII, OTF-1 or OBPIOO) binds to the octamer motif ATGCAAAT, which is present in the promoter region of some genes such as those encoding histone 2B or the small nuclear RNA which are constitutively expressed in many different cells regardless of the cell type and stage of differentiation. The same octamer motif is also present in the promoters of immunoglobulin genes. However, it is bound by the Oct-2 protein. Both proteins belong, together with the factor Pit-1 and the UNC-86 protein of the nematode *Caenorhabditis elegans*, to the family of homeobox POU proteins (Pit-1, Oct, Unc-86). They have in common a POU domain of 150 amino acids, which is composed of the homeobox-like POU homeodomain and a POU-specific region. The former is responsible for the formation of the protein complex with α-TIF and also for binding to the DNA octamer motif. α-TIF specifically interacts with helix 2 of the POU homeodomain of Oct-1, but not with that of Oct-2. The function of the POU-specific domain is unclear. Presumably, it contributes to DNA binding.

19.5.3.2 Non-Structural Proteins

Enzymes and Functions Involved in Genome Replication

Apart from the previously mentioned enzymes such as the protease and the protein kinase that are components of the virions, herpesviruses encode a large number of enzymes and polypeptides that are active during genome replication, and catalyse several steps of nucleic acid metabolism and DNA synthesis (Table 19.16). The most are delayed early proteins, and must be present in their functionally active form in the infected cell before initiation of replication.

Table 19.16 Herpesvirus proteins involved in replication

Molecular mass (kDa)[a]/gene locus						
Herpes simplex virus	**Varicella–zoster virus**	**Human cytomegalovirus**	**Human herpesvirus 6**	**Epstein–Barr virus**	**Human herpesvirus 8**	**Function**
140/U_L30	ORF28	140/UL54	100/U38	110/BALF5	ORF9	DNA polymerase
124/U_L29	ORF29	140/UL57	U41	138/BALF2	110/ ORF6	Binds to single-stranded DNA
94/U_L9	ORF51	70/UL84	U73	38–40/ BZLF1	?	Binds to the replication origin oriLyt
62/U_L42	ORF16	52/UL44	41/U27	50/BMRF1	ORF59	Binds to double-stranded DNA (processivity factor)
99/U_L5	ORF55	115/UL105	U77	-/BSLF1	ORF56	5′-3′ helicase, DNA primase complex
114/U_L52	ORF6	110/UL70	U43	-/BBLF2/3	ORF40/41	5′-3′ helicase, DNA primase complex
80/U_L8	ORF52	-/UL102 ?	U74	-/BBLF4	ORF44	Stimulates primer synthesis
68/U_L12	ORF48	-/UL98	U70	70/BGLF5	ORF37	alkaline endonuclease/exonuclease
41/U_L23	ORF36	-/-	-/-	70/BXLF1	ORF21	Thymidine kinase
-/-	ORF1				ORF70	Thymidylate synthase
-/-		-/UL97	U69	-/-	-/-	Phosphotransferase, protein kinase monophosphorylates ganciclovir
140/U_L39	ORF19	-/UL45	U28	85/BORF2	ORF61	Large subunit of ribonucleotide reductase
38/U_L40	ORF18	-/-	-/-	34/BaRF1	ORF60	Small subunit of ribonucleotide reductase
39/U_L2	ORF59	-/UL114	U81	78–88/ BKRF3	ORF46	Uracil glycosylase
39/U_L50	ORF8	-/UL72	U45	-/BLLF3	ORF54	dUTPase

[a]The molecular masses, if known, are specified along with the reading frame encoding the proteins on the viral genomes

In addition to numerous cellular proteins, seven viral proteins are essential for herpes simplex virus genome replication:

1. The DNA polymerase (140 kDa), whose sequence is conserved in all herpesviruses. It is inhibited by phosphonoacetic acid and formic acid, in contrast to the cellular enzyme. In addition to the 5′–3′ polymerase function, like cellular enzymes, it possesses a 3′–5′ exonuclease activity, a proofreading function that contributes to the high fidelity of DNA synthesis.
2. A processivity factor (62-kDa protein) that forms a complex with the polymerase and binds to double-stranded DNA.
3. The helicase–primase complex, which is composed of three proteins (99, 80 and 114 kDa). It is responsible for unwinding and separating the double-stranded DNA helix and for the synthesis of RNA primers, which the DNA polymerase requires for the generation of Okazaki fragments during lagging-strand synthesis.
4. A 124-kDa protein that binds to single-stranded DNA and concomitantly interacts with the polymerase complex. It binds to single-stranded DNA at the replication fork, maintaining it in an extended and readable configuration for the polymerase.
5. An oriLyt-binding dimeric protein (94 kDa), which interacts with three sequence elements within the replication origin, and thus presumably facilitates the recruitment of the other components of the DNA replication machinery.

Similar proteins have been identified in human cytomegalovirus, Epstein-Barr virus and human herpesviruses 6 and 8 (Table 19.16). Presumably, these essential replication proteins assemble into a similar complex, as known from the DNA replication machinery of eukaryotic cells (Fig. 19.26).

In addition, herpesviruses have some other enzymes that intervene in nucleic acid metabolism whose function can be complemented by the corresponding cellular proteins. The best known of these enzymes is thymidine kinase, which has been detected in all herpesviruses, with the exception of cytomegalovirus. Herpes simplex virus mutants defective in thymidine kinase are able to replicate in vitro, but exhibit reduced virulence in mice. The enzyme catalyses the phosphorylation of thymidine as well as other pyrimidine and purine derivatives to monophosphates, or to diphosphates in the case of herpes simplex virus type 1. It has a significantly broader substrate spectrum than the cellular thymidine kinase. This enzyme activity is the basis of the antiviral effect of various nucleoside derivatives such as acyclovir, an acyclic analogue of guanosine lacking the 3′-OH group in its sugar moiety, which is essential for DNA chain elongation (▶ Chap. 9, ▶ Fig. 9.1). Its incorporation into nascent DNA strands leads to chain termination. The herpes simplex virus thymidine kinase monophosphorylates acyclovir, whereas the cellular thymidine kinase does not accept the guanosine analogue as a substrate. The further phosphorylation reactions that produce acyclovir triphosphate are performed by eukaryotic enzymes. Acyclovir triphosphate is then preferentially used by the herpesvirus DNA polymerase during viral genome replication (▶ Fig. 9.2). The fact that two viral enzymes preferentially accept the nucleoside analogue as a substrate guarantees its specific activation solely in virus-infected

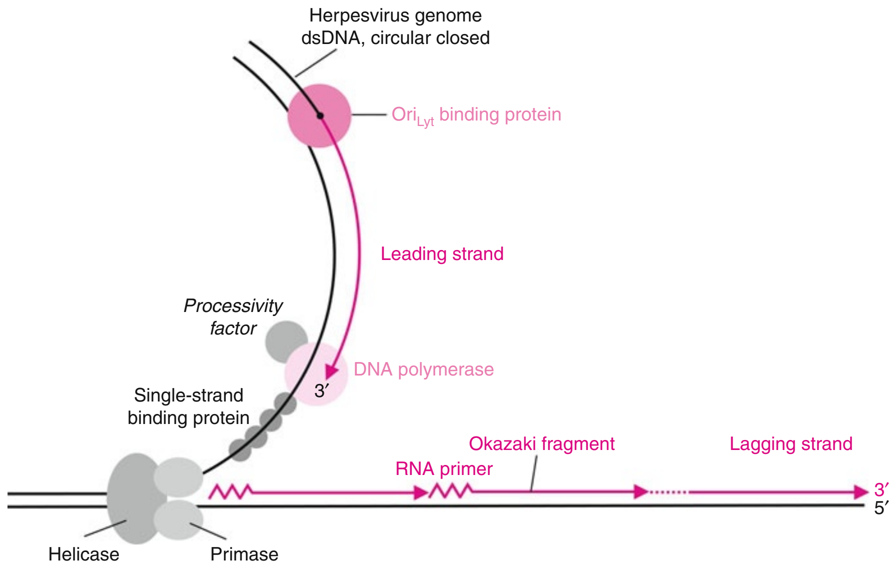


Fig. 19.26 Viral proteins involved in herpesvirus genome replication according to the rolling-circle mechanism. *dsDNA* double-stranded DNA

cells, and its exclusive incorporation into the viral DNA. Therefore, cellular processes are not impaired, or are only marginally affected. Ganciclovir is a similar nucleoside analogue (▶ Fig. 9.1). It is also phosphorylated by the viral thymidine kinase, and its incorporation into the viral DNA leads to chain termination. It also inhibits the replication of human cytomegalovirus and human herpesviruses 6 and 7, although these members of the betaherpesviruses do not encode their own thymidine kinase. The generation of the monophosphate is performed by viral protein kinases which are encoded by the UL97 and U69 genes of human cytomegalovirus and human herpesvirus 6, respectively. In addition to thymidine kinase, human herpesvirus 8 and the animal pathogenic rhadinoviruses of the gammaherpesvirus subfamily contain the genetic information for a thymidylate synthase. Although the viral enzymes are significantly smaller than cellular thymidylate synthases, they are functionally active in infected cells. Among the alphaherpesviruses, only varicella-zoster virus possesses a thymidylate synthase.

Furthermore, an alkaline DNase (exonuclease) and a uracil glycosylase have also been identified as additional enzymatic activities. It is believed that both enzymes are required for repair of incorrectly incorporated nucleotides. The viral uracil DNA glycosylase is a member of family 1 of this enzyme group, and is involved in repair of uracil residues from double-stranded DNA. Uracil-containing DNA arises either as a result of misincorporation of dUTP instead of dTTP during DNA synthesis or as a result of cytosine deamination. This results in mispairing uracil nucleotides, which are paired with guanosines instead with adenosines, leading to G:C to A:T transitions in the next replication rounds. The enzyme uracil

DNA glycosylase removes the uracil base from the nucleotide by hydrolysis of the N-glycosidic bond that links the base to the deoxyribose phosphate DNA backbone, leaving an apurinic/apyrimidinic site in the DNA. Subsequently, the base excision repair system corrects this DNA lesion by endonucleolytic removal of the remaining sugar and phosphate residues from the apurinic/apyrimidinic site and by closing the gap by DNA synthesis. Consequently, especially spontaneous cytosine deaminations are efficiently detected, removed and repaired by this DNA repair system. Since herpesvirus DNA has a high GC content, this enzyme is important to maintain the correct nucleotide composition in the viral genomes.

The ribonucleotide reductases of alphaherpesviruses and gammaherpesviruses are constituted of two large and two small subunits (140 and 38 kDa); however, betaherpesviruses do not contain the small subunit. The enzyme catalyses the conversion of ribonucleotides to deoxyribonucleotides, thus ensuring that sufficient substrates are available for the synthesis of viral genomes. Even this enzyme is a potential target for antiviral agents: peptides that prevent the interaction of the subunits, reduce viral replication.

Transactivators

Most of the immediate early proteins of herpesviruses are transactivators which regulate the course of the lytic infection by inducing the expression of delayed early and late genes. The sequences of these proteins are virus-specific, and only rarely exhibit short conserved regions.

Herpes simplex viruses encode five immediate early proteins (ICP0, ICP4, ICP22/U_S1, ICP27/UL54, ICP47/U_S12). The genes of ICP0 and ICP4 are located in the b and c sequences of the repeats, and thus are present in two copies (Figs. 19.25a and 19.27); the nomenclature of their loci does not follow the usual rules. The ICP0, ICP4 and ICP22 proteins are phosphorylated. The ICP4 protein (140 kDa) is additionally modified by UDP and ADP, and is essential for viral replication; the homologous protein (IE62) of varicella-zoster virus is encoded by ORF62. With the exception of immediate early genes, the ICP4 protein induces the expression of all the other reading frames, and binds relatively weakly to a variety of promoters. Presumably, it exerts its transactivator function in cooperation with cellular factors. In a negative-feedback loop, the ICP4 protein represses its own transcription by binding to the consensus sequence 5′-ATCGTC-3′, which constitutes the start site of mRNA synthesis. ICP4 also represses the expression of the genes encoding the ICP0 protein and the latency-associated transcript (LAT) via a similar *cis*-active element present in their promoters. LAT is the only viral gene product that can be detected during latency (Sects. 19.5.4 and 19.5.5). The ICP0 protein (79 kDa) – IE62 in varicella-zoster virus – is a pleiotropic activator of transcription; it acts synergistically with ICP4 and enhances its transactivation effect. It is not necessarily required for viral replication in vitro. Nevertheless, ICP0 possibly plays an important role in reactivation of the virus from latency into the lytic replication cycle. ICP0 has a RING finger domain and exhibits ubiquitin ligase activity. It interacts with several cellular E2 ubiquitination enzymes, and induces the ubiquitination of various cell proteins, including the protein Sp100,

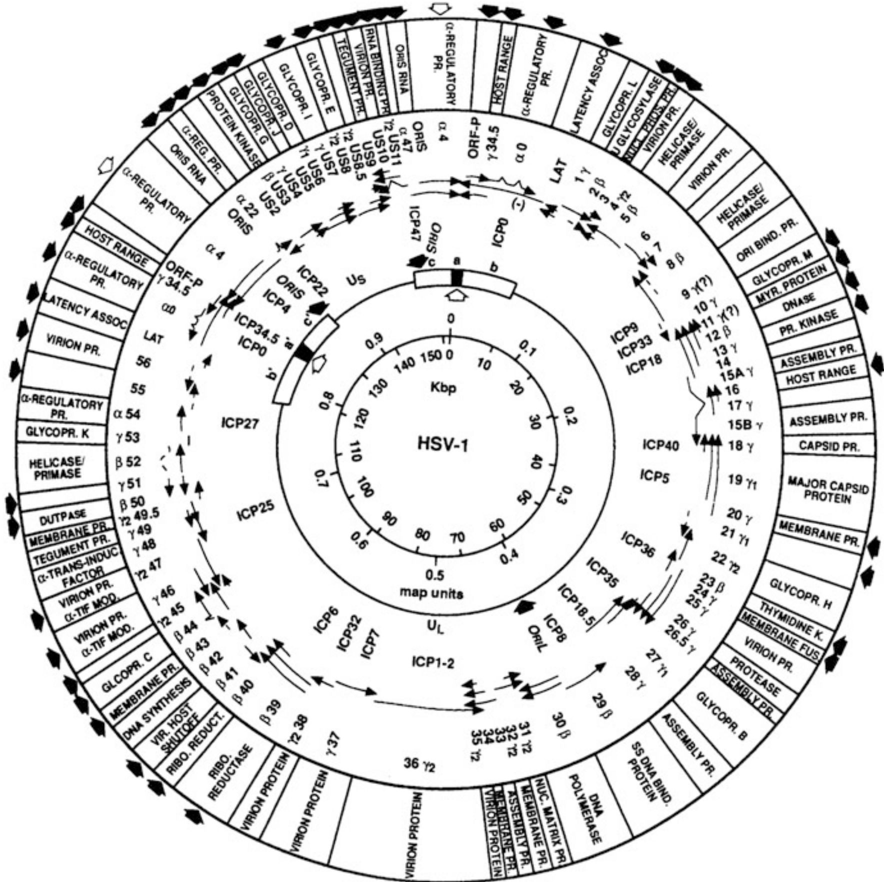


Fig. 19.27 Overview of the reading frames, transcripts and proteins that are synthesized during infection by herpes simplex virus type 1 (*HSV-1*). The innermost circle indicates the kilobases and the relative map units. The next circle describes the organization of the herpes simplex virus genome into unique (U_L and U_S) and repeated DNA sequences (depicted as *bars*, the lower-case letters *a*, *b* and *c* and *a′*, *b′* and *c′* denote the repeats present in different orientations). *Open arrows* indicate the nuclease cleavage sites at which the concatemeric DNA genomes are resolved into monomers after replication. *Black arrows* represent the origins of genome replication during the lytic infection cycle (oriS and oriL). The next circle represents a transcription map. The orientation of the various mRNAs is indicated by *arrows*. The gene products (infected cell protein, *ICP*) are indicated between the second and the third circle. *Arabic numerals* outside the third circle refer to the number of the open reading frame from which the respective mRNA is transcribed. The Greek letters α, β and γ indicate the stage of the lytic infection cycle (immediate early, early or late) at which the gene products are synthesized. The outermost circle indicates the function of the proteins encoded by the different reading frames. *Open arrows* indicate the localization of the gene encoding the ICP4 protein, which is essential for the initiation of the lytic cycle (see the text for details). *Black arrows* indicate the reading frames that can be deleted without affecting the infection cycle. (From Roizman and Sears 1996)

which is associated with nuclear domain ND10, and promyelocytic leukaemia protein. As a result, the ubiquitinated proteins are proteolytically degraded by the proteasome. The phosphorylated ICP27 protein (51 kDa) possesses a zinc finger domain, accumulates in the nucleus and is essential for replication. It increases the synthesis of late gene products, and represses the expression of immediate early proteins. This is presumably performed in different ways. It has been described that ICP27 exerts transactivating functions by binding to undefined promoter elements. However, the main regulatory function of ICP27 seems to be at the post-transcriptional level. It affects the use of alternative termination and polyadenylation sites during mRNA synthesis, and thus also the export of viral mRNA into the cytoplasm. In addition, ICP27 inhibits mRNA splicing. ICP22 and ICP47 are not required for lytic replication of herpes simplex viruses. Transactivating regulatory functions that have not been characterized further have been reported for ICP22. In this case, ICP22 interacts with the UL13 protein kinase, a component of the tegument, and alters the phosphorylation and consequently the activity of the cellular RNA polymerase II. ICP47 inhibits mRNA splicing during the early infection phase. In the late phase, ICP47 is involved in the transport of viral mRNAs from the nucleus into the cytoplasm; this property renders it functionally similar to the Rev protein of lentiviruses (▶ Sect. 18.1.3). Like most herpesvirus immediate early proteins, ICP47 exerts many different additional functions. An amino-terminal domain (amino acids 3–34) of ICP47 binds to the cytoplasmic region of the TAP transporter protein, which is anchored in the ER membrane. This protein transports peptides which are generated by degradation of cellular and viral proteins in the proteasomes into the ER lumen, where they interact with MHC class I proteins – a prerequisite for the presentation of complexes on the cell surface, and for the recognition by cytotoxic T lymphocytes (▶ Chap. 7). The interaction of ICP47 with the TAP transporter inhibits binding of peptides and, consequently, their transport to the ER. In this way, ICP47 affects the immunological recognition of infected cells. The effect of the human cytomegalovirus US6 protein is functionally similar, even though the molecular mechanism differs from that of ICP47 (Sect. 19.5.6).

The immediate early proteins of human cytomegalovirus also have transcriptional activation functions. Four regions of the genome are activated by the tegument protein pp71 very early during the lytic infection cycle:

1. The ie1/ie2 region, which contains the reading frames UL123 and UL122
2. UL36 to U38
3. US3
4. A gene of the US22 family (TRS1/IRS1) which is located within the c element of the repeats, and is thus present in two copies

The ie1/ie2 region is under the control of an enhancer composed of short, repetitive sequence elements, which is activated by pp71 and the cellular AP-1 or CRE/ATF factors after infection (Fig. 19.28). The IE1 phosphoprotein (68–72 kDa) is the most abundant immediate early protein of cytomegalovirus, and is translated from a multiply spliced mRNA constituted of four exons. The amino-terminal 85 amino acids are encoded in exons 2 and 3, and the remaining 406 residues are

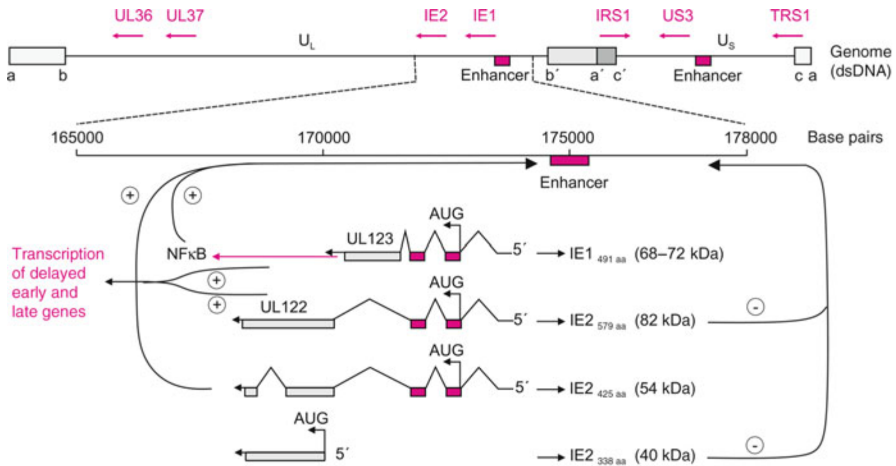


Fig. 19.28 Immediate early genes of cytomegalovirus, their position and their orientation in the genome. The viral genome is depicted in the *upper part*. *Red arrows* represent the five known genes which encode immediate early proteins. *Red boxes* indicate the enhancer regions that increase the expression of immediate early genes. The region of the genome encoding the various forms of IE1 and IE2 is magnified for clarity. The proteins are translated from reading frame UL123 or UL122 by using alternative splicing signals. The mRNA molecules and exons which are used for the synthesis of IE1 and IE2 proteins are also depicted. Translated sequences are shown as *bars*. The *arrows* indicate in what way the proteins affect their own expression, or that of other cytomegalovirus gene products. *aa* amino acids, *dsRNA* double stranded DNA, *NFκB* nuclear factor κB

encoded in exon 4, which corresponds to reading frame UL123. The IE1 protein stimulates the expression of the cellular transactivator NFκB and seems to increase its activity. In complex with NFκB, IE1 induces its own expression and that of delayed early and late genes. The extent of activation is cell-type dependent and not equally pronounced in all cells. IE2 is also phosphorylated and synthesized in different versions. The 85 amino-terminal residues are identical to those of IE1, and the coding sequences of reading frame UL122 are added by alternative splicing. The result is an IE2 protein comprising 579 amino acids (82 kDa). A shortened, 425 amino acid long IE2 form (54 kDa) arises by alternative splicing. An internal late promoter located in the UL122 reading frame induces the synthesis of an mRNA species from which a protein with 338 amino acids (40 kDa) is translated; it is identical to the carboxy-terminal region of the 82-kDa IE2 gene product. It binds to a *cis*-repression signal (crs) element upstream of the transcription start of IE1/IE2 mRNA, thus repressing its synthesis. This process can also be mediated by the 82-kDa IE2 protein. However, the principal function of this protein is the regulation of gene expression during early stages of the cytomegalovirus lytic infection cycle. It binds to delayed early and late promoters, and, probably by interacting with cellular transactivators induces the expression of the genes regulated by them. The IE1 and 82-kDa IE2 proteins act synergistically to increase their transactivating effect and also induce a large number of cellular promoters. There is evidence that the

products of other immediate early genes (UL36, UL37, U3 and TRS1/IRS1) also influence the expression of later viral genes. However, their effects have not been investigated as thoroughly as those of IE1/IE2 proteins.

The transactivator genes of human herpesvirus 6, which – like cytomegaloviruses – belongs to the betaherpesvirus subfamily, encode similar immediate early proteins with transactivator function: the US3 gene resembles members of the human cytomegalovirus US22 protein family. The IE-A and IE-B proteins of human herpesvirus 6, which are encoded by the genes UL86/87–90 and U16–U19, respectively, correspond to the immediate early proteins IE1 and IE2 as well as UL36 to UL38 of human cytomegalovirus, respectively. However, open reading frame U94 of human herpesvirus 6 encodes an additional transactivating protein. It has homology to the Rep proteins of adeno-associated viruses and interacts with the cellular TATA-binding protein, a transcription factor (▶ Sect. 20.1.3). The pU94 protein transactivates not only promoters of human herpes virus 6, but also various cellular and viral promoters, including the long terminal repeat promoter of HIV.

Three immediate early genes have been identified in Epstein-Barr virus and induce the expression of delayed early and late proteins. They are encoded by open reading frames BZLF1, BRLF1 and BI′LF4 (Fig. 19.29). Inasmuch as there is no suitable cell culture system in which the lytic replication cycle of this virus can be exclusively investigated, the immediate early genes were initially defined as those whose expression is activated after induction of the lytic cycle in latently infected B cells. This can be achieved by addition of specific chemicals (phorbol esters, butyric acid) to latently infected B cell lines, or by transduction with the non-transforming virus strain P3HR-1. Meanwhile, it has been demonstrated that infection of primary umbilical cord lymphocytes leads to the same expression pattern of immediate early proteins. The BZLF1 or Zta transactivator protein is of particular importance: its presence is sufficient to induce a productive replication cycle of Epstein-Barr viruses in permissive cells. It is a phosphoprotein (38–40 kDa) with homology to the AP-1 family of cellular transcription factors, to which the products of the proto-oncogenes c-*fos* and c-*jun* also belong. Like these transcription factors, BZLF1 also dimerizes by a leucine-zipper motif at its carboxy-terminal region. A basic amino acid sequence in the central region mediates DNA binding, and the transactivation domain is located in the amino-terminal region (Fig. 19.30). The gene is transcribed into three different, differentially spliced mRNAs. One is controlled by the complex promoter upstream of the BZLF1 gene, and its activity is influenced by several, hitherto only partially characterized cellular factors (such as c-*fos*, c-*jun*, the transcription factor YY1 and the Ku protein). The other two mRNAs are bicistronic, and differ by alternative splicing in the untranslated regions. Their synthesis is induced by the promoter of the BRLF1 gene. The genetic information encoding the BRFL1 protein (94–98 kDa) is located in the 5′ half, and that of the BZLF1 protein is located in the opposite side. Presumably, only the BRLF1 protein is translated from this mRNA. The BZLF1 protein has transactivating effects on most viral promoters. It also binds to oriLyt sequences, and probably induces the initiation of lytic genome replication.

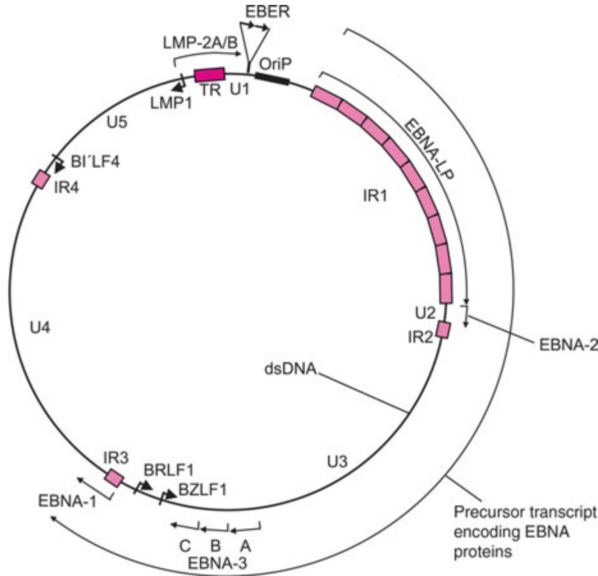


Fig. 19.29 Immediate early and latency genes of Epstein-Barr virus, their position and their orientation in the genome. The genome of Epstein-Barr virus is shown in its episomal form, as it exists in the nucleoplasm. The precursor transcripts encoding the different Epstein-Barr virus nuclear antigen (*EBNA*) proteins start at the same promoters; the coding sequences of different reading frames are appropriately assembled by alternative splicing. Latent membrane protein (LMP) 1 and LMP2 possess distinct transcriptional start sites. The LMP2A and LMP2B polypeptides are translated from a multiply spliced mRNA, which spans the terminal repeats (*TR*), and thus can only be read from the circularized viral genome. *dsDNA* double-stranded DNA, *EBER* Epstein-Barr-virus-encoded RNA, *LP* leader protein

Furthermore, it interacts with cellular proteins such as p53, NFκB, the Ku protein and retinoic acid receptors, inhibiting their activities. It has also been reported that an E3 ubiquitin ligase associated with BZLF1 mediates ubiquitination of p53, and targets it for proteasomal degradation. The BRLF1 protein, also known as Rta, is presumably also an immediate early phosphoprotein. It cooperates with BZLF1 and enhances its transactivating properties. Expression of the BI′LF4 gene is dependent on the differentiation stage of the cell. This gene is deleted in the transforming virus strain B95-8. The BI′LF4 gene encodes a transcriptional activator protein (68 kDa) which induces its own expression and that of BSMLF1 by a positive-feedback mechanism. The amino-terminal region of BSMLF1 (60 kDa) is encoded in an exon of the BSLF2 gene, which is connected to that of the BMLF1 gene by splicing. BSMLF1 expression is additionally enhanced by the BZLF1 transactivator. The protein probably affects predominantly post-transcriptional processes. There are indications that it regulates polyadenylation of viral mRNA species, and thus their export into the cytoplasm.

In human herpesvirus 8 the situation is similar to that in Epstein-Barr virus. In B lymphocytes, the virus exists in a latency state from which it can be reactivated

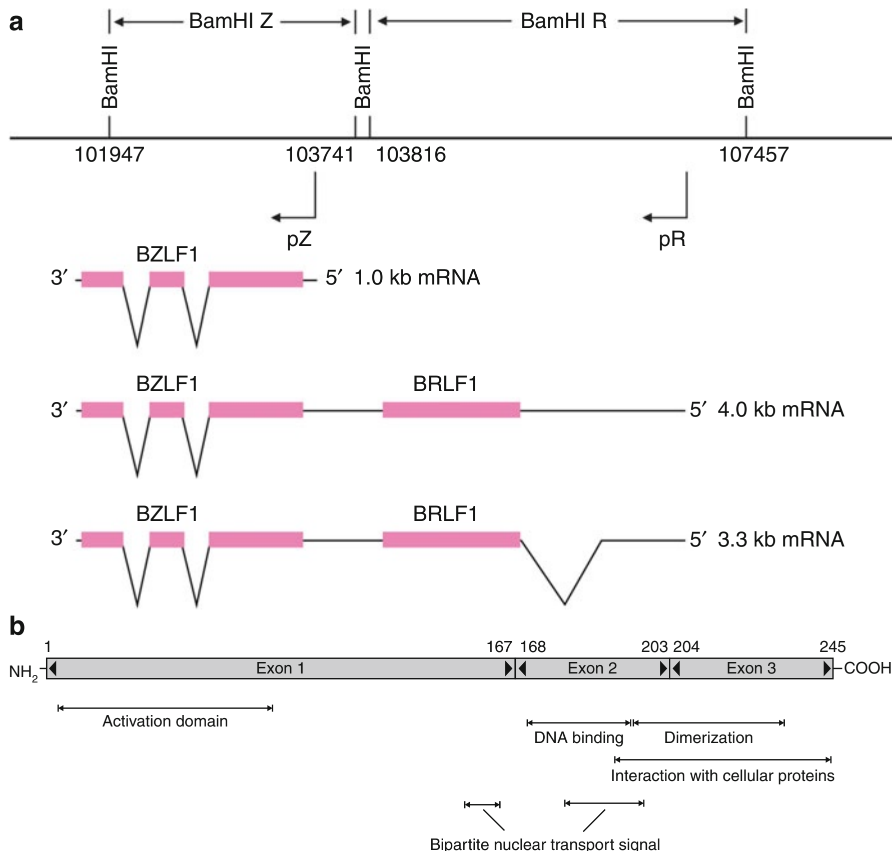


Fig. 19.30 Functional domains of the Epstein-Barr virus BZLF1 protein. (**a**) The transcripts from which the immediate early proteins BZLF1 and BRLF1 are translated. The BZLF1 mRNA is composed of several exons and starts on a promoter that is located upstream of the coding sequences. A different promoter is used to transcribe the BRLF1 mRNA, but using the same termination site, so a bicistronic mRNA is produced, which is probably used exclusively for the translation of the BRLF1 gene product. The functional domains of the BZLF1 protein are shown in (**b**)

into the lytic replication cycle either spontaneously or by induction by certain compounds (such as phorbol esters). A protein has also been found, K-bZIP, which is homologous to the BZLF1 protein of Epstein-Barr virus, and is translated from a spliced mRNA very early during the lytic replication cycle. The synthesis of early and late mRNAs correlates directly with the amount of K-bZIP. The amino-terminal region of K-bZIP is encoded by ORFK8, whereas the carboxy-terminal region is encoded by three exons located further upstream. The protein consists of 237 amino acid residues, and contains a leucine-zipper domain in its carboxy-terminal region, which causes the formation of K-bZIP homodimers. Besides this protein variant, there are two other forms of K-bZIP lacking the

carboxy-terminal dimerization domain. They are translated from spliced mRNAs. ORF50 encodes a polypeptide similar to the BRLF1 protein of Epstein-Barr virus, and is located near ORFK8 in the human herpesvirus 8 genome. It seems to be a strong transactivator protein which induces various viral promoters.

Proteins with Homologies to Cellular Gene Products

All herpesviruses encode proteins with homologies to cellular gene products. The homology of some glycoproteins of herpes simplex virus with Fc and C3b receptors has already been briefly discussed. A gene (UL18) has been identified in human cytomegalovirus whose gene product is homologous to the heavy chain of MHC class I proteins. The UL18 protein can interact with β_2-microglobulin, which is normally present as a complex with the MHC class I heavy chain and is involved in epitope presentation (▶ Chap. 7). The UL18 gene is not essential for cytomegalovirus infection. It may be involved in the initiation of the viral latency phase. Since UL18 is complexed with β_2-microglobulin, the cells are depleted of structures that are recognized by cytotoxic T cells, and thus evade the immune response. This process is supported by another glycoprotein, gp47-52 (gCII), which is encoded by the US6 gene of human cytomegalovirus. It acts as an inhibitor of the peptide transporter complex TAP, through which peptides generated by the proteasome are transported into the ER lumen. Since this process is interrupted, loading of MHC class I antigens with peptides does not occur, and consequently neither does their transport to the cell surface. This does not affect the MHC class I homologue UL18 product itself: this protein is transported to and anchored in the cytoplasmic membrane, and counteracts lysis mediated by natural killer cells, which would be directed against these cells owing to their reduced amount of MHC proteins. In doing so, cytomegaloviruses are capable of evading both the unspecific and the specific immune response. Three other viral genes (UL33, US27 and US28) are similar to chemokine receptors, and are expressed in the late phase of the replication cycle. The US28 gene product is similar to G-protein-coupled receptor proteins with seven transmembrane regions. It interacts with the chemokine CX3CL1 (fractalkine) and this process facilitates cell–cell and cell–virus contacts. The chemokine-receptor-like proteins are not essential for infection, but confer a growth advantage to infected cells. The UL29 gene product is homologous to the γ chain of T-cell receptor.

Proteins with homology to chemokine receptors are also found in the human herpesvirus 6 genome: the U12 gene encodes a β-chemokine receptor with homology to the receptors CCR1, CCR3 and CCR5, which bind the chemokines RANTES, macrophage inflammatory protein (MIP)-1α, MIP-1β und MCP-1, but not IL-8. Open reading frame U51 encodes a chemokine receptor homologue that interacts with RANTES. The U83 gene of human herpesvirus 6 does not have a corresponding homologue in human cytomegalovirus: it codes for a chemokine that interacts with the CCR2 receptor on the surface of monocytes and macrophages, acting on them in a chemotactic manner. This possibly attracts potential target cells for human herpesvirus 6, a process that promotes dissemination of the virus in the organism.

Even Epstein-Barr virus possesses and expresses several proteins which allow an effective adaptation to the organism owing to their similarity to eukaryotic proteins. The BCRF gene product is a homologue of IL-10 which inhibits the induction of cytotoxic T cells. It is secreted by infected cells and acts like IL-10. The BHRF1 protein is related to the cellular protein Bcl-2 and retards the induction of apoptosis in cells infected with Epstein-Barr virus. Even Epstein-Barr virus nuclear antigen (EBNA) 1 has a homologous cellular protein, but its function is unknown.

Analysis of the genome of human herpesvirus 8 has revealed that this virus also has many open reading frames with homologies to cellular genes. The viral genes are clustered in the regions of the genome termed blocks B, D3 and E, which have no resemblance to those of related viruses such as Epstein-Barr virus and saimiriine herpesvirus. For example, several genes of block B encode proteins with homology to cellular growth factors, including a viral IL-6 (vIL-6), two proteins with homology to MIP-α, and one polypeptide resembling MIP-1β, which are encoded by ORFK2, ORFK4, ORFK6, and ORF4.1, respectively. Furthermore, this region also contains gene products that are homologous to the cellular enzymes thymidylate synthetase (ORF70) and dihydrofolate reductase (ORF2). Block D3 carries the genetic information to direct the synthesis of various proteins with homology to cellular interferon regulatory factors (IRFs). Viral IRF-1 (vIRF-1) is a protein of 449 amino acids and is encoded by ORFK9; viral IRF-2 (vIRF-2; 163 amino acids, ORFK11.1) is similar to the amino-terminal domain of vIRF-1. A protein encoded by ORFK10.5 and ORFK10.6 is expressed very early during lytic replication of human herpesvirus 8. After splicing, both reading frames are interconnected in the mRNA, which directs the synthesis of viral IRF-3 (vIRF-3; 566 amino acids, 73 kDa). An additional form of viral IRFs, IRF-4, has been identified recently. Cellular IRFs usually induce the expression of IFR-regulated genes by binding to ISRE sequences (▶ Chap. 8). Although vIRF-1 and vIRF-3 bind to other DNA sequence elements, they probably function competitively and prevent the expression of the cellular IRFs. In contrast, vIRF-2 appears to interact with NFκB binding sites, hence interfering with its transactivation activity. Moreover, a homologue of cellular cyclin D (ORF72), a homologue of G-protein-coupled receptors (ORF74) and a product similar to cellular Fas-associated death domain (FADD)-like IL-1β-converting enzyme (FLICE)-inhibitory proteins (FLIPs) (viral FLIP, ORFK13) are encoded in the block E. The latter inhibits the interaction of death effector domains in the FADD adapter molecules with FLICE (caspase 8) during apoptosis induced by Fas or TNF receptor 1, thereby preventing the induction of apoptosis. The protein encoded in ORF16, a viral homologue of the cellular Bcl-2 protein, has a similar antiapoptotic function.

19.5.4 RNA Products

19.5.4.1 Latency-Associated Transcripts

All herpesviruses have developed mechanisms that allow them, after the initial infection associated with the production of infectious progeny viruses, to transit

into a latent stage in the infected organism. No viral particles are produced in this phase. In the case of herpes simplex viruses, it has not yet been shown that viral proteins are synthesized in latently infected neuronal cells. The only product that has been found is the spliced LAT (2 kDa), which is transcribed from the TR_L and IR_L regions, arises from a 8.3-kDa-long precursor RNA and extends in antisense orientation to the ICP0 gene (Fig. 19.27). Because of its opposite orientation, it seems conceivable that this LAT forms RNA duplexes with the transcripts encoding the immediate early ICP0 protein, giving rise to degradation of the ICP0-specific mRNA via RNA interference, and thus preventing the synthesis of the transactivator protein. Whether such a process contributes to the establishment and maintenance of latency is unclear. There are also indications of an antiapoptotic effect of LAT which promotes survival of latently infected neurons. The LAT intron contains several open reading frames. However, the pertinent proteins have not yet been discovered in vivo. If the putative protein from the largest reading frame is produced by genetic engineering methods in cell culture, it is found in phosphorylated form within the nucleus, but its function is unknown. The animal pathogenic members of alphaherpesviruses, such as bovine herpesvirus 1, equine herpes virus 1 and pseudorabies virus, also produce a LAT during latency. In bovine herpesvirus 1, it is significantly shorter than in herpes simplex virus, and does not reach into the antisense region of the ICP0 gene. A transcript equivalent to the LAT of herpes simplex virus has not been identified in the human pathogenic varicella-zoster virus so far.

19.5.4.2 Epstein-Barr-Virus-Encoded RNA

The genes encoding Epstein-Barr-virus-encoded RNA (EBER) 1 and EBER2 are arranged in tandem, and are located in the U1 region of the Epstein-Barr virus genome (Fig. 19.29). They are short, uncapped, non-polyadenylated, non-coding transcripts synthesized by RNA polymerase III, which are present both in the nucleus and in the cytoplasm, and form extended intramolecular double-stranded regions. They are similar to the virus-associated RNAI and RNAII species of adenoviruses (Sect. 19.4), and can functionally replace them. EBERs also induce degradation of mRNAs by an RNA interference mechanism, and bind to protein kinase R, which plays a crucial role in interferon-mediated inhibition of protein synthesis by phosphorylating the translation initiation factor eIF2, and thus inhibiting protein synthesis. Especially, EBER1 binds to protein kinase R and inhibits its activity. This prevents the protective effect of interferon in latently infected cells (▶ Chap. 8). In addition, protein kinase R seems to have important functions in the induction of apoptosis, and these are inhibited by interaction with EBER1. This antiapoptotic effect might promote cell immortalization by Epstein-Barr virus. Whether EBERs are also involved in other activities, such as affecting splicing, is unclear.

19.5.4.3 T1.1/nut RNA

Human herpesvirus 8 produces a nuclear RNA too; however, the synthesis of the T1.1/nut1 RNA occurs not during latency, but during the immediate early or early

phase of the productive replication cycle. The T1.1/nut1 RNA is synthesized by the cellular RNA polymerase II, does not contain a 5′-cap group, but it is polyadenylated. It exhibits sequence similarity to the cellular U1 and U11 small nuclear RNAs, which are involved in splicing. Probably, the T1.1/nut1 RNA regulates and influences alternative splicing during viral replication.

19.5.5 Latency Proteins

19.5.5.1 Epstein-Barr Virus

In contrast to herpes simplex viruses, Epstein-Barr virus synthesizes up to nine different proteins during latent infection. Six of them are necessary for the establishment and maintenance of the non-productive cycle in B lymphocytes in vitro. B lymphocytes are immortalized by the activity of these proteins. The cells gain the ability of unrestrained division, and grow indefinitely as continuous cell lines in vitro. These proteins are EBNA1 and EBNA2, the related proteins EBNA3A and EBNA3C, EBNA leader protein (EBNA-LP) and latent membrane protein (LMP) 1 (Fig. 19.29, Table 19.17). EBNA3B and LMP2A/LMP2B are not essential for immortalization of B cells in cell culture.

Epstein-Barr Virus Nuclear Antigens

The genes of latent EBNAs are widely scattered throughout the viral genome. Nevertheless, the EBNA genes are transcribed in a common precursor RNA of more than 100,000 nucleotides in length, from which the different mRNA species are generated by alternative splicing and using different polyadenylation signals. Two promoters (Cp and Wp) control the synthesis of the precursor RNAs that are responsible for the synthesis of all EBNAs. Cp is located within the *Bam*HI C fragment in the U1 region, whereas Wp resides within the *Bam*HI W fragment in IR1 (Figs. 19.29 and 19.31). Furthermore, two additional promoters (Fp and Qp) have also been found which control the synthesis of transcripts that direct only the translation of EBNA1. These control elements are situated in the *Bam*HI fragments F and Q. The Fp promoter seems to be preferentially active in cells derived from tumours such as Burkitt's or Hodgkin's lymphoma which are associated with Epstein-Barr virus.

EBNA-LP is encoded primarily in two exons of 66 and 132 base pairs which are located in the sequence elements of the IR1 repeats. To facilitate expression, the first exon must be linked to the second in such a way that an AUG initiation codon arises. Since each Epstein-Barr virus isolate has a different number of IR1 repeats, EBNA-LP consists of a corresponding number of repeated protein domains of 66 amino acids. Only 45 residues of the carboxy-terminal region are unique because they are encoded by an exon in the U2 region of the genome. EBNA-LP is phosphorylated by cellular kinases. In this modified form, it enhances the transactivation effect of EBNA2 and induces together with EBNA2 the transition from the G_0 phase into the G_1 phase of the cell cycle in resting B lymphocytes. The presence of both proteins is sufficient for immortalization. They activate the expression of cyclin D2, and thus

Table 19.17 Properties and functions of the Epstein-Barr virus proteins synthesized during virus latency

Protein	Molecular mass (kDa)	Modification	Function
LMP1	70	Phosphorylated	Latent membrane protein; integral membrane protein; is similar in its effect to the CD40 receptor; induces expression of CD23, LFA-1, LFA-3, ICAM and Bcl-2 via a TNF-receptor-like signalling cascade
LMP2A	53	Phosphorylated	Latent membrane protein; phosphorylated on tyrosine residues, Src kinases; modulation of signal transduction
LMP2B	40		Latent membrane protein; amino-terminally truncated version of LMP2A; very hydrophobic; inhibits function of LMP2A
EBNA1	70–80	Phosphorylated	Latent nuclear protein; binds to oriP and host chromosomes, which promotes episomal replication; transactivator for the EBNA promoter; glycine–alanine repeats prevent MHC class I presentation
EBNA2	75–88	Phosphorylated	Latent nuclear protein; transactivator of EBNA and LMP1 promoters; causes increased expression of CD21, CD23, various cytokines, cyclin D2, c-Myc and c-Frg
EBNA3	90–110		Latent nuclear proteins, three forms A–C; EBNA2A induces EBNA3C synthesis, EBNA3B induces CD40 and vimentin, EBNA3C represses gene expression, binds to histone deacetylases
EBNA-LP	20–50	Phosphorylated	Latent nuclear protein; activates expression of cyclin D2; together with EBNA2 induces the transition from the G_0 to the G_1 phase of the cell cycle

EBNA Epstein–Barr virus nuclear antigen, *ICAM* intercellular adhesion molecule, *LFA* lymphocyte-function-associated antigen

induce continuous cell proliferation. In addition, it has also been described that EBNA-LP binds to the Hsp70 protein (heat shock protein, 70 kDa). The complex is associated with promyelocytic leukaemia nuclear bodies, also known as ND10 or POD domains (promyelocytic leukaemia-associated/promyelocytic leukaemia protein oncogenic domains). Upon cellular stress, they are transported into the nucleolus. It is believed that the POD domain and its associated components are involved in regulation of transcription, the cell cycle and apoptosis. EBNA-LP also binds to the cytoplasmic HS1-associated protein X-1 (HAX-1), which in turn interacts with the HS1 protein. The latter is synthesized exclusively in haematopoietic cells, and seems to be involved in signal transduction processes in B lymphocytes. However, the significance of the EBNA-LP interaction with these cellular proteins is currently as poorly understood as that with the tumour-suppressor proteins p53 and RB105.

EBNA1 is translated from an mRNA that is composed of several short, non-coding exons, to which the exon containing the BKRF1 reading frame is fused by splicing. Since this reading frame encompasses the IR3 repeats, EBNA1 also contains repetitive sequences; in this case, the IR3-encoded amino acids glycine

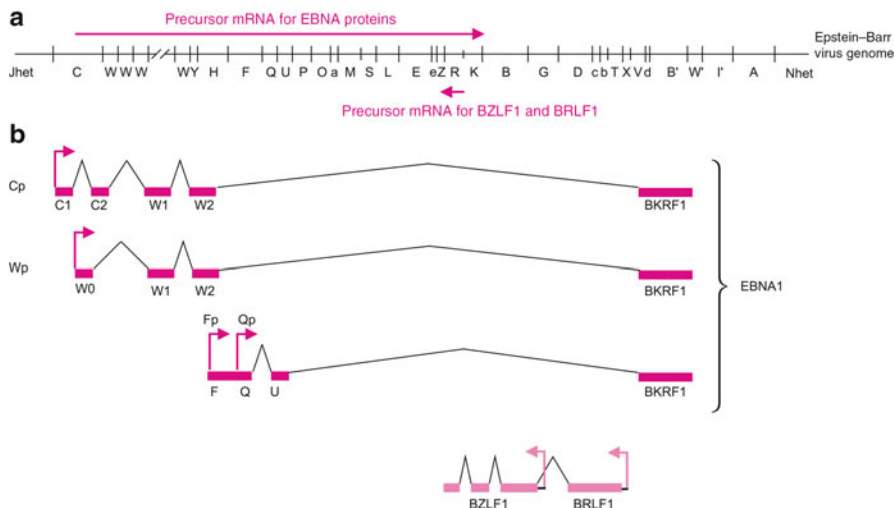


Fig. 19.31 The immediate early genes of Epstein-Barr virus are transcribed in antisense orientation to the mRNA encoding EBNA1. (**a**) Overview of the entire Epstein-Barr virus genome represented in a linear form. *Letters* indicate the DNA fragments that arise after digestion of the viral genomic DNA with the restriction enzyme *Bam*HI. The denomination is based on their size. (A is the largest fragment. Since there are more fragments than letters in the alphabet, after Z, fragment designation is continued using *lower-case letters*. Fragments with *prime* are located within the deletion found in the genome of the virus isolate B95-8). Shown in red are the orientation and position of the precursor transcripts encoding the EBNA and immediate early proteins, which are located in the *Bam*H1 fragments R and Z, and are synthesized during latency. (**b**) Enlarged representation of the precursor transcripts and their orientation. The mRNA for EBNA1 begins at promoters which are located in the *Bam*H1 fragments C, W, F and Q (for details, see the text). The coding sequences for EBNA1 are in the BKRF1 reading frame, which is situated in the *Bam*HI K fragment. A very large intron comprising more than 100,000 nucleotides is removed from the precursor transcript during splicing of the mRNA. The reading frames encoding the immediate early proteins BZLF1 and BRLF1 are located within this intron, but in opposite orientation. The BRLF1 protein is translated from a bicistronic mRNA that also contains the BZLF1 open reading frame, which, however, is transcribed from an independent promoter

and alanine, whose number corresponds to that in the IR3 elements. Therefore, EBNA1 has a specific molecular mass ranging between 70 and 80 kDa, depending on the virus isolate. The phosphorylated EBNA1 has the following structure: the amino-terminal domain consists of 89 mainly basic amino acids; this is followed by about 240 residues of the repetitive glycine–alanine region, which are followed by a short basic region; the carboxy-terminal region is hydrophilic and contains both basic and acidic amino acids. EBNA1 is synthesized in every transformed cell, and is essential for maintaining both latency of the virus and immortalization of the cell. It functions as a transcriptional activator, and induces its own expression, as well as that of latent genes by activating the Cp promoter. Furthermore, EBNA1 is also important for replication of the episomal viral genome during latency. It binds to the initiation site of DNA synthesis, oriP, which is active in the latent cycle. This is

a region of 20 symmetrically arranged sequences of 30 base pairs in length which is bound by several units of EBNA1. Similar sequence elements are also found in the chromosomal DNA of the cell, and EBNA1 interacts with them as well. Hence, EBNA1 constitutes a connection between the DNA of Epstein-Barr virus and that of the cell. This ensures that the viral genetic information is passed on with the chromosomes to daughter cells during mitosis. Therefore, EBNA1 is essential for both the inheritance of the episomal viral genome in dividing cells and the maintenance of latency. Although all transformed cells synthesize EBNA1, $CD4^+$ T lymphocytes that recognize EBNA1-specific epitopes have only been shown in an in vitro system so far. The glycine–alanine repeats are responsible for the extensive immunological failure to recognize Epstein-Barr virus immortalized cells: They prevent EBNA1 degradation by the proteasome complex, and thus the emergence of peptides that may be presented by MHC class I proteins. Fusion products of the glycine–alanine repeats with other types of easily identifiable T-cell epitopes can also not be presented by MHC class I antigens.

EBNA2 is essential for establishing latency and immortalization. A first indication was the observation that the virus strain P3HR-1 carrying a deletion of the EBNA2-coding region is not able to immortalize B cells in vitro. The transforming capability can be restored by complementation with the lacking sequences. There are two Epstein-Barr virus subtypes, and they differ in their EBNA2 sequences. Type 1 possesses a high transformation rate and corresponds to the EBNA2 variant of the B95-8 strain, whereas type 2 (strain Jijoye) exhibits a reduced transformation capability in B lymphocytes. Both subtypes are found in ex vivo cell lines derived from B-cell lymphomas. Therefore, a different tumorigenic potential seems rather unlikely. The two EBNA2 variants are based on different DNA sequences in the BYRF1 reading frame, which is added to the noncoding regions of the precursor RNA by splicing, thus generating the latent EBNA products. EBNA2 type 1 possesses 491 amino acids, whereas EBNA2 type 2 comprises 443 amino acids. The differences reside especially in a relatively hydrophobic domain in the centre of the protein. On the other hand, both EBNA2 variants contain a proline-rich region and repeats of arginine/lysine–glycine residues. The proteins are phosphorylated, have molecular masses between 75 and 88 kDa, form oligomeric complexes by their amino-terminal domains and accumulate in the nucleus. EBNA2 acts as a transactivator. It probably does not bind directly to the promoters, but mediates this effect indirectly in cooperation with cellular factors such as Pu-1 and RBP-Jκ (a recombination signal binding protein). The latter is a component of the Notch1 receptor signalling pathway which binds to the sequence motif 5′-GTGGGAAA-3′ found in various viral and cellular regulatory elements. EBNA2 binds to this complex and induces the viral Cp promoter, which regulates the expression of the EBNA gene products; it also binds to the promoter of LMP1. In addition, it increases the synthesis of various cytokines and growth factors (TNF-α, lymphotoxin, granulocyte colony-stimulating factor), as well as that of the cellular proteins CD21 and CD23, cyclin D2 and the proto-oncogenes c-*Myc* and c-*Frg*. CD23 is a low-affinity IgE receptor. It is expressed in antigen-stimulated, Epstein-Barr-virus-transformed B cells and primary B lymphocytes. A secreted form of

CD23 acts as a B-cell growth factor, and contributes to the activation and proliferation of B lymphocytes during Epstein-Barr virus infection.

EBNA3 proteins (EBNA3-A, EBNA3-B and EBNA3-C) are translated from mRNA species which contain the exons of reading frames BLRF3, BERF1, BERF2, BERF3 and BERF4, and arise by alternative splicing of the precursor RNA. Open reading frames BLRF3 and BERF1 encode EBNA2-A, BERF2a and BERF2b genes encode EBNA2-B, and BERF3 and BERF4 direct the translation of EBNA3-C. These genes are related to each other, and have probably evolved by gene duplication. Depending on the virus strain, the proteins are between 925 and 1,069 amino acids long. The mode of action of EBNA3 proteins is unclear; EBNA3-C seems to repress the expression of certain genes, whereas EBNA3-B activates the promoters of the cellular CD40 and vimentin genes. The repression effect of EBNA3-C appears to be related to its ability to bind to histone deacetylase proteins, and to mediate their association with chromosomal histone proteins. If histones are deacetylated, then the histone–DNA complex is stabilized and represses gene transcription unspecifically. EBNA3-A regulates the expression of EBNA3-C, which in turn induces the synthesis of CD23 and LMP. EBNA3-B is not required for immortalization.

Latent Membrane Proteins

LMP1 is encoded by the BNLF1 open reading frame. Its expression is regulated by its own promoter, which is active during latency. The amino-terminal half of LMP1 consists of six transmembrane regions that anchor the protein in the plasma membrane of infected cells, where it forms multimeric aggregates. A signal peptide is not present, and the amino- and carboxy-terminal domains are oriented to the cytoplasm. LMP1 is post-translationally phosphorylated at serine residues in the carboxy-terminal region. It is associated with the cytoskeleton via vimentin, and thus is present in high local concentrations in specific areas of the cell surface. LMP1 has some properties that characterize it as a potential oncogene: its sole expression alters the growth properties and morphology of rodent fibroblast cell lines, which lose their contact inhibition and their growth dependence on high serum concentrations in the culture medium. Furthermore, it inhibits the differentiation of epithelial cells, and LMP1-synthesizing cells develop tumours in immunodeficient mice. LMP1 behaves like a constitutively activated receptor, resembling the cellular CD40 receptor in its mode of action: both proteins induce a signalling cascade that leads to the upregulation of the transcription factor NFκB, which, as a result, activates all promoters whose expression is dependent on this transactivator. When the LMP1 gene is introduced into transgenic mice that do not have CD40 genes, then the CD40 function can be partially suppressed. It has also been described that the cytoplasmic-oriented, carboxy-terminal domain of LMP1 (carboxy-terminal activating region, CTAR) has a function similar to that of the TNF receptor, although they share only modest sequence homology. The factors of the TNF-receptor-associated factor (TRAF) family bind to the CTAR domains in LMP1, like to the corresponding domain of the activated TNF receptor. TRAF-1 and

TRAF-2 induce phosphorylation and activation of various cell proteins, including NFκB. This leads to the activation of all genes that are regulated by NFκB-dependent promoters. LMP1 induces the expression of CD23 – in this case it acts synergistically with EBNA2 – and the synthesis of the adhesion molecules lymphocyte-function-associated antigen 1, lymphocyte-function-associated antigen 3 and intercellular adhesion molecule as well as the transferrin receptor. These cells exhibit increased concentrations of the proteins Bcl-2 and A20, which suppress apoptosis.

With use of an internal promoter, an amino-terminal truncated variant of LMP1 (40 kDa) is synthesized in the late phase of lytic infection, and consists of only two amino-terminal transmembrane regions and the carboxy-terminal domain. This protein can not induce any of the processes described above.

The synthesis of LMP2A and LMP2B is controlled by promoters which are located in the *Bam*HI N fragment of the Epstein-Barr virus genome. They induce the synthesis of multiply spliced mRNA species, which span the terminal repeat region. Therefore, these genes can be transcribed only when the genome is present as a closed circular episome. The very hydrophobic LMP2B protein consists exclusively of 12 potential transmembrane regions, and its ends are oriented to the cytoplasm. LMP2A has an additional hydrophilic cytoplasmic domain of 119 amino acids at the amino-terminus. The proteins are in complex with cellular protein kinases of the Src and Syk families, and are phosphorylated at tyrosine residues present in the amino-terminal domain. The Src and Syk kinases usually phosphorylate the intracellular domain of the B-cell receptor; thus, they regulate the signal transduction processes that are triggered upon receptor activation. Kinase binding to LMP2 proteins can repress this function. This prevents intracellular Ca^{2+} ions from being mobilized and B cells from being stimulated by interaction of their membrane-associated immunoglobulins with antigens. This results in B cells being present in a dormant state with a reduced cell division rate. The synthesis of B7 proteins, which in addition to MHC antigens are necessary co-receptors for the recognition of cells by cytotoxic T lymphocytes, is repressed. LMP2B inhibits the activity of LMP2A, and blocks these mechanisms.

19.5.5.2 Human Herpesvirus 8

Cell lines latently infected with human herpesvirus 8 can be isolated from certain lymphomas (body-cavity-based lymphoma, BCBL, and primary effusion lymphoma, PEL). These cells do not produce infectious viruses, but – like B cells latently infected with Epstein-Barr virus – can be stimulated to produce viruses by treatment with phorbol esters. BCBL and PEL cells have multiple copies of the episomal viral genome during latency, from which a limited number of genes are transcribed (Table 19.18).

Latency-Associated Nuclear Antigen

Latency-associated nuclear antigen (LANA) (222–234 kDa) is encoded by ORF73. It is by far the most abundant gene product of latency and is translated from a 6,000-base-long tricistronic mRNA, which also contains the information

Table 19.18 Properties and functions of the human herpesvirus 8 proteins synthesized during virus latency

Protein	Molecular mass (kDa)	Modification	Function
LANA	222–234	Phosphorylated	Latent nuclear protein; binds to oriP; promotes episomal replication; mediates interaction of the viral genome with the chromosomes of the host cell and ensures its transmission during cell division; functionally similar to EBNA1
Kaposins			Latent proteins synthesized in several isoforms
Kaposin A	6		Very hydrophobic
Kaposin B	38	Phosphorylated	Localized in different cellular compartments; associated with intracellular membranes; induces the activity of various cellular kinases; transforms rodent cells
Kaposin C	54		
LAMP	35–60	Phosphorylated	–
K1	46	Glycosylated, phosphorylated	Latent membrane protein; aggregates to form dimers and induces interaction of SH2-domain-containing proteins and cell activation via Src-dependent tyrosine phosphorylation; transforms rodent cells
Viral cyclin D2	~30		Homologous to cellular cyclin D2; together with cyclin-dependent kinase 6 induces the phosphorylation of histone H1 and RB105/RB107 proteins resulting in the release of E2F transactivators; promotes entry into the S phase of the cell cycle
Viral FLIP			Acts as a FLICE (caspase 8) inhibitor protein and inhibits CD95/Fas-induced apoptosis
Viral IL-6	~20		Homologous to cellular cytokine IL-6; is secreted by the cells; binds to the β subunit of the IL-6 receptor and stimulates proliferation of plasma cells
Viral IRF1–IRF4	~20–73		Homologous to the cellular IRF proteins; prevents their interaction with ISRE promoter elements and the expression of the genes controlled by them; viral IRF-2 inhibits the transactivation activity of NFκB

FLICE Fas-associated death domain like IL-1β-converting enzyme, *FLIP* Fas-associated death domain like IL-1β-converting enzyme inhibitory protein, *IRF* interferon regulatory factor, *ISRE* interferon-stimulated response element, *LAMP* latency-associated membrane protein, *LANA* latency-associated nuclear antigen, *NFκB* nuclear factor κB

for the viral cyclin D2 and FLIP; however, the latter are probably translated from their own transcripts. LANA contains 1,162 amino acids; it is localized in the nucleus, where it causes the mottled staining pattern observed in immunofluorescence tests with sera from patients, also known as LANA fluorescence. Whether other latent proteins are also involved in its formation has not yet been conclusively clarified. LANA has an acidic domain characterized by a differing number of repeats, depending on the virus isolate. It contains a leucine zipper at its

Table 19.19 Herpesvirus proteins involved in attachment and interaction with cell surface components

Cell surface component	Herpes simplex virus 1	Herpes simplex virus 2	Varicella–zoster virus	Human cytomegalovirus	Human herpesvirus 6	Human herpesvirus 7	Epstein–Barr virus	Human herpesvirus 8
Heparan sulphate proteoglycan	gB, gC	gB, gC	gB	gB	–	gB	–	gB, K8.1
Nectin-1	gD	gD	–	–	–	–	–	–
Herpesvirus entry mediator	gD	gD	–	–	–	–	–	–
Human insulin-degrading enzyme	–	–	gE	–	–	–	–	–
Mannose 6-phosphate receptor	–	–	gB	–	–	–	–	–
CD21 (complement receptor 2)	–	–	–	–	–	–	gp250/350	–
CD46	–	–	–	–	gH/gL/gQ	–	–	–
CD4	–	–	–	–	–	?*	–	–
MHC class II	–	–	–	–	–	–	gH/gL/gp42	–
Unidentified surface protein on epithelial cells	–	–	–	–	–	–	gH/gL	–
Integrins	gH/gL	?[a]	–	gB gH/gL/gO	–	–	BMRF2	gB
DC-SIGN	–	–	–	–	–	–	–	?[a]
xCT (cystine/glutamate transporter, light chain)	–	–	–	–	–	–	–	?[a]
Epidermal growth factor receptor	–	–	–	gB	–	–	–	–
Unidentified surface protein	–	–	–	gH/gL/UL128/UL130/UL131	–	–	–	–

Interactions between viral surface proteins and cellular components that are described in the literature are indicated

DC-SIGN dendritic-cell-specific intercellular adhesion molecule 3 grabbing non-integrin

[a]The specified cellular membrane protein was described to be involved in the interaction between the virus and the cell; however, the nature of the viral surface protein has not been conclusively resolved

carboxy terminus. The carboxy-terminal domain is also responsible for the formation of homodimeric, and possibly multimeric protein complexes. LANA has a function similar to that of EBNA1 of Epstein-Barr virus: it binds to oriP and to the chromosomal DNA of host cells, and is responsible for the episomal persistence of the viral genome in cells latently infected with human herpesvirus 8. Moreover, LANA is also capable of activating or repressing a large number of viral and cellular promoters. LANA has an important function in the regulation of latent infection and reactivation of the virus: During latency, LANA represses the expression of the viral transactivator K-Rta, a homologue of the immediate early protein Rta of Epstein-Barr virus. LANA is phosphorylated by the cellular serine/threonine kinases Pim1 and Pim3. Phosphorylated LANA does not act as a repressor of the K-Rta promoter. As a result, K-Rta is produced, inducing productive viral replication.

Kaposin

Latently infected spindle cells of Kaposi's sarcoma and PEL cells express the ORF-K12 gene products and those of adjacent regions of the genome. They span a region that is characterized by two direct repeat clusters of 23 nucleotides (DR1 and DR2). The kaposin-encoding mRNAs are formed from a common precursor transcript by alternative splicing. Kaposin A is encoded by ORF-K12; it consists of 60 amino acids (6 kDa), and is very hydrophobic; it is anchored in the membrane compartments of the ER and the Golgi apparatus, as well as in the cytoplasmic membrane. When it is heterologously expressed in rodent cells, it is able to transform them. The activity of several serine/threonine kinases, such as protein kinase C, Cdc2 kinase and calcium/calmodulin-dependent kinase II, is significantly increased in these cells. If such cells are injected into immune-defective mice, the mice develop vascular, undifferentiated sarcomas. There are indications that kaposin may have a function similar to that of the E5 protein of bovine papillomavirus (Sect. 19.3.3). In addition to kaposin A, two other proteins (kaposin B and kaposin C) are translated using multiple unusual start codons (CUG) and different reading frames. They span the DR1 and DR2 repeats. Kaposin B is a phosphorylated, proline-rich protein that is found in many compartments of latently infected cells. It binds to and activates mitogen-activated protein kinase associated protein kinase 2 (MK2 kinase). This prevents the degradation of relatively unstable mRNAs in the cytoplasm which are characterized by AU-rich elements in their non-coding sequences at the 3′ ends. These AU-rich element RNAs are responsible, inter alia, for the synthesis of several proinflammatory cytokines (IL-6, granulocyte–macrophage colony-stimulating factor). There are only few data concerning the function of kaposin C.

Latency-Associated Membrane Protein

This highly variable protein (35–60 kDa, 60–70 % difference in amino acid sequence) is translated from a multiply spliced mRNA which is composed of eight exons. The last exon is derived from ORF-K15. The resulting protein has similarity with both LMP2A and LMP1 of Epstein-Barr virus. It has 12 putative

transmembrane regions; it is found in the plasma membrane and in intracellular membrane compartments. Phosphorylation consensus sequences for tyrosine residues are situated in the likely cytoplasm-oriented carboxy-terminal domain, as found in LMP2A of Epstein-Barr virus. Like LMP1, this domain also interacts with TRAF-1, TRAF-2 and TRAF-3, which are involved in signal transduction. A truncated version of latency-associated membrane protein (LAMP) which lacks the sequences of exons 4 and 5 is synthesized upon induction of the lytic replication cycle.

K1 Protein

This protein is encoded in ORF-K1. Several virus isolates differ considerably in their K1 sequences, which exhibit variability of up to 40 % at the amino acid level. The K1 protein is a membrane type I protein that is anchored in the cytoplasmic membrane. It is a glycoprotein of 46 kDa. In the short conserved, cytoplasmically oriented carboxy-terminal domain there is a so-called immunoreceptor tyrosine-based activation motif, which has proved to be functionally active. Usually, the tyrosine residues located in the immunoreceptor tyrosine-based activation motif are phosphorylated by Src kinases upon exogenous stimulation, thus mediating attachment of proteins with SH2 domains such as Syk, Vav and Lyn. As a consequence, a signal transduction pathway which is associated with mobilization of Ca^{2+} ions is triggered, and leads to activation of lymphocytes. The K1 protein is able to induce cell activation independently of external stimuli; it is probably mediated by homodimerization of K1 proteins by the amino-terminal domain on the cell surface. If the K1 gene is introduced into rodent cells, then they will develop a transformed phenotype. However, the K1 gene does not behave as a classic latency gene: it is probably expressed at low levels in PEL and spindle cells, but upon induction of the lytic cycle by phorbol esters, synthesis of K1 protein is significantly increased.

Viral IRFs

Four viral proteins with homology to cellular IRFs have been identified. They are synthesized early during the lytic infection cycle as well as during latency. They act competitively to the cellular IRF proteins, and prevent the expression of genes that are regulated by the ISRE, or they inhibit the transactivator function of NFκB (► Chap. 8, Sect. 19.5.3).

Other Proteins

It is not entirely clear whether the three proteins described below really constitute genuine latency proteins. They are synthesized in tumour cells of Kaposi's sarcoma, the multicentric Castleman's disease as well as in BCBL and PEL cells, in which no productive viral replication occurs. However, since they are important for the pathogenesis of malignant diseases, they are described together with the classic latency proteins.

Viral cyclin D2 The homologue of the cellular cyclin D2 is encoded by ORF72; it contains 257 amino acids. This gene is expressed in tumour cells (Kaposi's sarcoma and PEL). It forms a complex with cyclin-dependent kinase 6, and gives

rise to the phosphorylation of histone H1 and RB105/RB107 proteins. This changes the degree of interaction between the histones and DNA, and leads to the release of E2F transcription factors in their active form (▶ Chap. 6, Sects. 19.2.3, 19.3.3 and 19.4.3). This enables the cells to enter the S phase, promoting cell division.

Viral FLIP This protein is encoded by ORFK13. It is translated from a bicistronic mRNA, which also contains the genetic information for viral cyclin D2. Viral FLIP has homology to death effector domains in the amino-terminal domain of caspase 8 (FLICE protease) and to the cytoplasmic adapter molecule FADD, which mutually interact via death effector domains. This is an essential process during Fas-induced apoptosis. Viral FLIP inhibits this step, and acts as an antiapoptotic FLIP. A protein with similar function is synthesized by equine herpesvirus 2.

Viral IL-6 This protein is a secreted homologue of the cellular IL-6 and contains 204 amino acids. The corresponding gene is ORFK2. vIL-6 binds to the β subunit (gp130) of the cellular IL-6 receptor. This gives rise to a signal transduction mechanism which leads to phosphorylation of STAT1, STAT3 and Jak1 proteins (▶ Chap. 8), and induces proliferation of plasma cells in vitro. If mice are inoculated with vIL-6-producing cells, they show increased haematopoiesis and angiogenesis. Therefore, it is assumed that the production of this protein might be causally involved in tumour vascularization in humans.

19.5.6 Replication

19.5.6.1 Lytic Infection Cycle

Herpes simplex virus, varicella-zoster virus and cytomegalovirus can proliferate lytically by a productive replication cycle in a number of primary or established fibroblast and epithelial cell lines in vitro; human herpesvirus 6 and human herpesvirus 7 perform a productive infection cycle in activated $CD4^+$ T lymphocytes and in a number of established T-cell lines. Currently, there is no cell culture system to simulate the productive replication of Epstein-Barr virus, which reproduces lytically in epithelial cells of the throat, nose and pharynx in vivo. As mentioned already, the lytic cycle can be induced only by treatment of latently infected B cells with various compounds or by superinfection. The same applies for the productive replication of human herpesvirus 8.

Attachment of herpesvirus is a complex multistep process that is performed by binding of viral surface proteins to specific structures on the cell surface

The first step in most herpesviruses (herpes simplex virus types 1 and 2, varicella-zoster virus, human cytomegalovirus, human herpesviruses 7 and 8) is a relatively non-specific binding to heparan sulphate or to the 3-*O*-sulphotransferase-modified heparan sulphate moiety of proteoglycans on the cell surface, which is mediated – mainly but not exclusively – by the viral surface protein gB. In varicella-zoster virus, mannose 6-phosphate residues, which are part of the complex carbohydrate modifications of gB proteins, additionally bind to mannose 6-phosphate receptors on the cell surface.

Concomitantly, gD proteins of herpes simplex virus interact specifically with nectin-1 and herpesvirus entry mediator, members of the immunoglobulin superfamily and the TNF receptor family, respectively. In Epstein-Barr virus, gp220/gp350 specifically interacts with CD21 (complement receptor CR2). In addition, herpes simplex viruses also bind through their gH/gL proteins to a still unknown receptor on epithelial cells. In Epstein-Barr virus, the complex of gH/gL proteins and gp42 – a glycoprotein encoded by the BZLF2 reading frame – bind to MHC class II proteins on the surface of B lymphocytes. Furthermore, the gH/gL complex of human cytomegalovirus along with other viral proteins such as gO or the gene products of reading frames UL128, UL130 and UL131 bind to not yet completely characterized cellular components or integrins. Integrins have also been identified as cellular interaction partners of other herpesviruses. Human herpesvirus 6 binds to CD46 by a complex of surface proteins composed of gH/gL and gQ_1/gQ_2 (gQ_1 is an 80-kDa glycoprotein and gQ_2 is a 37-kDa glycoprotein, both encoded by the U100 reading frame). CD46 is also used as a receptor by some adenoviruses and attenuated measles vaccine viruses (▶ Sects. 15.3 and 19.4). Human herpesvirus 7 interacts with the CD4 receptor, which is also used by human immunodeficiency viruses to interact with T-helper cells (▶ Sect. 18.1). Whether this interaction is also mediated by gH/gL protein complexes is unknown. However, it is known that the gB proteins of human herpesvirus 8 interact with integrins. Furthermore, these viruses bind to DC-SIGN – a lectin on the surface of macrophages and dendritic cells – as well as to the light chain of the cystine–glutamate transporter system xCT, but it is still unknown which viral proteins are involved in this interaction.

The viral envelope merges with the cell membrane. Whether a specific viral protein or the combination of the different glycoprotein interactions is responsible for the fusion process has not been definitively solved. However, the activity of gB proteins seems to be important; they have structural and possibly functional similarities to the G protein of vesicular stomatitis virus (▶ Sect. 15.1). In addition to the direct fusion of the viral envelope and the cell membrane, virus particles also seem to penetrate by receptor-mediated endocytosis, during which the endosomal membrane and the viral envelope fuse by a pH-dependent mechanism, followed by the release of capsids into the cytoplasm. Notwithstanding, membrane fusions can also occur between non-infected and infected tissue cells whose cell membranes contain viral glycoproteins. This enables the herpesviruses to spread within the cell tissue without producing free virus particles. A similar cell-mediated dissemination of infection is also found in paramyxoviruses and lentiviruses (▶ Sects. 15.3 and ▶ 18.1). Among herpesviruses, only human herpesvirus 6 does not seem to have the ability to induce cell fusion: no viral surface proteins are found on the surface of infected T lymphocytes. Instead, they accumulate in the membranes of Golgi vesicles and the annulate lamellae. The latter are cellular organelles that are located in the cytoplasm, and consist of several membrane layers. They possess pores which are very similar to nuclear pores.

After fusion of the viral envelope with the cytoplasmic or endosomal membrane, the capsid arrives with the tegument in the cytoplasm. The capsids bind to microtubules, and are transported to the nuclear pores, through which the genome is

released into the nucleoplasm. Even viral proteins arrive there, insofar as they possess nuclear transport signals, such as α-TIF.

The linear DNA genome is circularized and is present as an episome. Expression of the viral genome is regulated in a cascade-like process. First, immediate early proteins are synthesized. In herpes simplex virus and cytomegalovirus, their expression is enhanced by the transactivators α-TIF and pp71, which bind in cooperation with specific cellular factors to immediate early gene promoters, and induce their transcription by RNA polymerase II. This leads to 5′-capped partially spliced, polyadenylated RNAs, which are transported into the cytoplasm, where they are translated. Immediate early proteins are important regulators in the cell. They are transported into the nucleus, where they activate the promoters of viral delayed early genes, thus inducing their transcription. Delayed early proteins include enzymes and nucleic acid binding polypeptides that are required for replication of the viral genome.

Replication of viral DNA during the lytic cycle occurs in the nucleus, more precisely within the ND10 subnuclear structures, which are characterized by the SUMOylated cellular proteins promyelocytic leukaemia protein and Sp100. The ND10 regions are dissolved during replication of herpesvirus genomes; there is evidence that in the case of herpes simplex virus, the ICP0 protein is responsible for this process; it directs the SUMO-modified proteins to proteasomal degradation. The actual replication of the viral genome is performed following the rolling-circle replication mechanism. The genome contains sequence elements (oriLyt) which function as start sites for DNA synthesis (Fig. 19.25). It is unclear why they are present in multiple copies in most herpesviruses, as only one is used for initiation. OriLyt-binding proteins bind to the origin of replication (UL9 gene product in herpes simplex virus, the BZLF1 protein in Epstein-Barr virus), and facilitate recruitment and access of the viral DNA polymerase/helicase/primase complex (Table 19.16). A DNA strand is cleaved, thus creating a 3′-OH end and a 5′-phosphate terminus. It is not known which protein acts as a single-strand-specific endonuclease. The 3′-OH end serves as a primer for the polymerization reaction. The nucleotides are continuously added to the growing DNA chain (leading strand synthesis) using the circular DNA strand as a template (Fig. 19.26). During this process, the 5′ end is progressively displaced from the template strand. Its single-stranded DNA is converted into double-stranded DNA by discontinuous synthesis of short RNA primers and Okazaki fragments (lagging DNA synthesis) (Fig. 19.26). This process results in a DNA molecule that contains four copies of the viral genome, and is arranged in a concatemeric structure.

Late viral genes are expressed concurrently with DNA replication. Synthesis of glycoproteins occurs on the ER membrane. They are subject to modifications with carbohydrate groups, and are partially proteolytically cleaved in both the *trans*-Golgi network and Golgi vesicles. Some glycoproteins possess consensus sequences in their carboxy-terminal, cytoplasmic domains which determine their association with the membranes of the *trans*-Golgi network; the others are transported to the cell surface and are inserted into the cytoplasmic membrane. The antibody-mediated, cytotoxic killer cell response, which leads to lysis of

infected cells, is directed against these polypeptides. Cellular and viral chaperones induce in part the return of glycoproteins to the membrane compartment of the *trans*-Golgi network.

After their synthesis, the various capsid protein components are transported into the nucleus, where they are assembled into precursor particles. Initially, the capsid proteins interact to form particulate precursors, which do not contain DNA. The viral protease (U_L26 in herpes simplex virus, UL80/assemblin in human cytomegalovirus) is associated them, and produces the scaffolding proteins by autocatalytic cleavage and proteolytic processing of the U_L26.5 and UL80a gene products, which are involved in the assembly of precursor capsids. Proteins on the inner side of capsid faces (VP19C/U_L38 in herpes simplex virus) allow interaction with the U_C element in the a-repeat element at the end of the double-stranded DNA concatemers (pac sequence) or with equivalent packaging signals in other herpesviruses. One genome unit is introduced into the precapsid through the pore that is formed by the portal proteins. The scaffolding proteins are degraded during this process. An additional interaction with the pac element occurs at the transition site to the subsequent genome unit, and the concatemeric DNA molecule is cleaved by a viral endonuclease, leading to concatemer resolution and monomerization. Now, the DNA-containing capsids accumulate on the inner nuclear membrane, which envelopes them by budding, releasing them into the perinuclear spaces and cisterns. These pass into the ER lumen. Once there, the transient viral envelope fuses with the ER membrane and the non-enveloped nucleocapsids enter the cytoplasm. The next step, which eventually leads to accumulation of the tegument proteins and final envelopment, occurs in the cisternae of the *trans*-Golgi network. Viral glycoproteins accumulate at the concave sides of cisternae, their cytoplasmic domains interact with the tegument proteins and the capsids interact with these structures. In the next step, the membrane of the *trans*-Golgi cisternae surrounds the attached capsids and envelopes them along with the tegument components. The newly enveloped capsids are released into the lumen of the *trans*-Golgi network; subsequently, they are transported to the cell surface via Golgi vesicles, and are released into the environment. During the transport in the Golgi vesicles, the surface proteins are modified again, and the particles mature into infectious virions. Alternatively, the virus may also spread as tegument-containing nucleocapsids by cell-to-cell contact or cell fusion.

Herpesviruses Induce Cell Cycle Arrest at the Beginning of Lytic Replication
In contrast to all other viruses, except lentiviruses (▶ Sect. 18.1.4), lytic replication of herpesviruses does not occur in cells that are in the S phase of the cell cycle. Whereas, for instance, parvoviruses (▶ Sect. 20.1) infect only cells that are in the S phase, and polyomaviruses, papillomaviruses or adenoviruses (Sects. 19.2–19.4) induce entry to the S phase after penetration, herpesviruses have developed mechanisms that induce cell cycle arrest in the G_1/G_0 phase, preventing progression to the S phase. Such processes have been described in cells infected with herpes simplex virus or human

cytomegalovirus, or during activation of the lytic replication cycle in B lymphocytes latently infected with Epstein-Barr virus. For example, the ICP0 protein of herpes simplex viruses is able to bind to the cellular protein p53, stabilizing it in its active form. The transcription factors of the E2F family are retained in their inactive form in complex with the proteins RB105 and RB107. The IE2 protein of cytomegalovirus and the BZLF1 protein of Epstein-Barr virus have similar functions even though they do not have sequence homology. The transactivation functions of immediate early proteins are not involved in these regulatory processes. However, the ICP27 protein of herpes simplex virus and the functionally homologous UL69 protein of cytomegalovirus and the BMLF1 polypeptide of Epstein-Barr virus are involved in these processes. During evolution, it has apparently proven to be advantageous for the complex herpesviruses, which are endowed with plenty of regulatory functions and enzymes, to be able to replicate the genome during the G_1 phase in order to avoid competition with the cellular DNA replication for the required nucleotides. However, the herpesvirus-induced cell cycle arrest seems to be restricted only to the cellular genome. In respect of other issues, such as energy production, infected cells are largely similar to replicating cells.

19.5.6.2 Latent Infection Cycle

During latency, the viral genome is present as an extrachromosomal episome in the nucleoplasm. It exhibits different, but stable copy numbers, whereby the episomes are associated with the host cell chromosomes. They are replicated concomitantly with the cellular genome by the cellular DNA polymerase, and are inherited by daughter cells. In this phase, different mechanisms among the various herpesviruses repress the production of infectious particles. The molecular processes and regulatory mechanisms involved in establishing and maintaining latency as well as those governing the reactivation into the lytic replication cycle are largely not well understood.

Alphaherpesviruses

Herpes simplex virus is latently present in the neurons of spinal ganglia, exhibiting copy numbers of up to 100 episomes per cell. During primary infection, the virus migrates from lytically infected epithelial cells with nerve endings into the nerve fibres by cell-to-cell contact; it is axonally further transmitted as a tegument-containing capsid from cell to cell until it reaches the ganglia. The immediate early genes are not expressed permanently in the neurons because these cells express the Oct-2 protein in addition to the cellular transactivator Oct-1. This is produced by alternative splicing and also binds to the promoters of immediate early genes. However, owing to it having an α-helix in its POU domain, it cannot interact with α-TIF, which is introduced into the cell as a component of the viral tegument. The Oct-2 protein has amino acid sequences to which a cellular repressor binds.

Consequently, the expression of immediate early proteins does not occur, preventing the induction of the lytic replication cycle. Ganglion cells express LAT only during latency. It acts as an antisense RNA to the ICP0 transcripts and contains sequences for the formation of 21–24-base-long microRNAs. These microRNAs (miR-I to miR-III), induce the degradation of ICP0 and ICP34.5 coding mRNAs via RNA interference, thus contributing to the maintenance of latency. However, it is unclear how the balance between the transcription of latent and immediate early genes is changed during reactivation of the virus. It is presumed that a transient induction of Oct-1 occurs in the cells, which in turn induces the transcription of early genes. Subsequently, the immediate early protein ICP4 represses the synthesis of LAT by binding to a sequence located near the transcription start site. Similar mechanisms are also found in animal pathogenic alphaherpesviruses.

Apparently, varicella-zoster viruses follow alternative approaches for the establishment and maintenance of latency. These viruses also infect neurons during primary infection, and are latently present particularly in the sensory ganglia of the spinal cord. In rodent models, the IE63 protein has been found to be indispensable for establishing latency. IE63 is one of the immediate early proteins; it acts as a transactivator and is a homologue of ICP22/U_S1 of herpes simplex virus. During latency, there are no RNA molecules that correspond to the LAT of herpes simplex virus, but there is a highly restricted viral expression of only seven viral genes (ORF4, ORF18, ORF21, ORF29, ORF62, ORF63 and ORF66). These genes encode either early transactivator proteins (ORF4, ORF62, ORF63) or enzymes (ORF18, ORF29) which are essential for replication of the genome of varicella-zoster virus. However, not all of these RNAs are translated; in cases in which the corresponding viral proteins are synthesized, they remain localized in the cytoplasm, and are not transported to the nucleus, thus remaining inactive.

Betaherpesviruses

The molecular processes required for the establishment and maintenance of latency of cytomegaloviruses are still unsolved. During primary infection, the virus replicates in many organs and in bone marrow stem cells. The induction of an immune reaction leads to a control of productive viral replication, and to the establishment of latency, which is maintained primarily in the lungs and spleen. It is not known which cell types are latently infected in these organs. A sporadic, modest synthesis of viral immediate early proteins is found in these tissues. Cytotoxic T lymphocytes that recognize epitopes of these proteins are also present in the latently infected organs. This also suggests an occasional synthesis of immediate early proteins. How the reactivation of the virus proceeds and whether this is influenced by cell differentiation processes is unclear.

Human herpesvirus 6 establishes a latent infection in monocytes and in undifferentiated haematopoietic progenitor cells. The viral genome is present as an extrachromosomal episome or is integrated into the cellular genome, namely in the terminal sequences of chromosomes 1, 17 and 22. Possibly, the telomere-like sequence elements in the DR_R and DR_L repeats mediate the integration into the

chromosomal telomeres. In addition, the pU94 gene product seems to be functionally involved in the establishment of latency and integration of the viral genome into the cellular DNA: the protein pU94 is synthesized at low levels during the productive infection cycle; it has activity similar to that of the Rep protein of adeno-associated virus 2, a parvovirus, which also integrates its genome in the chromosomes of the host cell (▶ Sect. 20.1): the Rep protein binds to both specific sequence elements on the viral and chromosomal DNA, thus mediating a close proximity of both nucleic acid molecules, and leading to integration.

Gammaherpesviruses

As already mentioned, Epstein-Barr virus can latently infect and transform B lymphocytes, in which the viral genome is present in a copy number of 40–100. The activity of EBNA1 supports only the latent DNA replication origin, oriP, and the genome is replicated by the cellular polymerase complex. EBNA1 is also responsible for equal segregation of the episomal viral genome during division of latently infected cells. The other gene products of the latent infection cycle, whose properties are described in Sect. 19.5.5, promote the establishment of the latent state. Even in the case of Epstein-Barr virus, there is evidence that the expression of immediate early genes, particularly that of the BZLF1 gene, is inhibited by cell-specific factors. In addition, antisense RNA and RNA-interference-mediated inhibitory mechanisms seem to play a role as well. The intron sequences of the 100-kb precursor RNA of the EBNA proteins are present in antisense orientation to the immediate early BZLF1, BRLF1 and BSMLF1 transcripts, and can form double-stranded RNA regions with them (Figs. 19.29 and 19.31).

The processes that establish the latent state of human herpesvirus 8 are largely not understood. This virus is able to replicate lytically in monocytes, macrophages and presumably also in mononuclear cells of the peripheral blood. The viral genome is also episomally present in BCBL and PEL cells as well as in spindle cells of Kaposi's sarcoma, which are latently infected with human herpesvirus 8, and express only a few viral genes. The proteins and their possible functions are described in Sect. 19.5.5. Some proteins, such as LANA and LAMP, seem to have functions similar to those of EBNA1 and LMP2A of Epstein-Barr virus. Two proteins, K-bZIP and KSHV-Rta, are also necessary to express the gene products of the lytic cycle during reactivation; their homologues also induce productive replication of Epstein-Barr virus.

Bacterial Artificial Chromosomes Facilitate Directed Mutation of Herpesvirus Genomes

New genetic methods facilitate the generation of targeted mutations in the very large DNA genomes of herpesviruses in the laboratory, and make possible the investigation of specific genes and the functions of their gene products during the infection cycle. For this purpose, the entire herpesvirus

genome is cloned into so-called bacterial artificial chromosomes (BAC) and propagated in *Escherichia coli*. Subsequently, the cloned herpesvirus genomes can be subjected to mutagenesis by various methods. It is possible to generate whole mutant libraries by transposon mutagenesis. This system is also accessible to site-directed mutagenesis. In both cases, BACs carrying mutated viral genomes can be introduced into eukaryotic host cells by transfection to reconstitute the corresponding herpesvirus mutants. The phenotype of virus mutants can be subsequently determined in cell culture. Instead of transfection methods, a trick to "resuscitate" the mutant genomes into viruses is the use of invasive *Escherichia coli* strains for BAC propagation which contain the bacterial genes invasin and listeriolysin in their genetic information and express them. These mutants are able to penetrate permissive eukaryotic cells, and to release the BACs with the herpesvirus genomes.

19.5.7 Human Pathogenic Herpesviruses

19.5.7.1 Herpes Simplex Viruses

Epidemiology and Transmission

Herpes simplex virus types 1 and 2 have long been adapted to humans. Skin blistering diseases were known as herpes in ancient times. Even William Shakespeare describes in his play *Romeo and Juliet* apparently recurrent herpes blisters on the lips. In addition, Jean Astruc, physician to the King Louis XV of France, found evidence for genital herpesvirus infections around 1763. In Marburg in 1913–1914, the ophthalmologist Wilhelm Grüter showed that human herpetic keratitis, i.e. the corneal infection caused by herpes simplex virus type 1, can be transmitted to the rabbit eye. In 1919, Ernst Loewenstein also transmitted the pathogen of fever blisters to the rabbit eye. In 1920–1921, Robert Doerr reported that encephalitis can occur after a corneal infection. Klaus Munk and Wilbur Ackermann pictured the herpes simplex virus using an electron microscope for the first time in 1953. At the end of the 1960s, Andre Nahmias and Karl Schneweis demonstrated that two different viruses with distinguishable antigenic properties are responsible for the development of labial and genital herpesvirus infections.

Herpes simplex viruses have a worldwide distribution, and belong to the most frequent pathogens. More than 90 % of adults are infected with them for life. The primary infection with herpes simplex virus type 1 commonly occurs in children through contact with virus-containing blisters or secretions of the oral cavity.

Thereafter, the virus retrogradely colonizes the trigeminal ganglion in the facial region, and can be reactivated at irregular intervals. These reactivations are manifested by recurrence of skin blisters, usually on the same sites as in the primary infection, and are caused by anterograde migration of the virus along the nerves from the trigeminal ganglion into the oral mucosa. The viruses are excreted with the content of the skin blisters, which contain high concentrations of infectious virions. Transfer is also possible by asymptomatic carriers, which secrete only small

quantities of the virus. Herpes simplex virus type 2 is primarily transmitted by genital secretions during sexual contact. This infection occurs mainly in young adults. The strict classification of herpes simplex virus type 1 infection as “oral” and type 2 infection as “genital” has become blurred because the two viruses are increasingly found at both sites. Nevertheless, the risk of infection with herpes simplex virus type 2 is primarily determined by the sexual behaviour: only about 3 % of members of monasteries have antibodies against this virus type; in contrast, more than 70 % of prostitutes have antibodies. Women infected with herpes simplex virus in the genital region can transmit the pathogen to the newborn at birth, especially in manifest primary infections. In such cases, the virus may cause severe generalized and formerly often fatal disease (neonatal herpes). To clarify epidemiological questions and to elucidate chains of infection, intratypic strain differences can be determined by analysing the viral DNA with restriction enzymes or by sequencing.

Clinical Features

Clinically, herpes simplex virus types differ in the locations of apparent primary infections and the sites of latent infections as well as reactivation: herpes simplex virus type 1 infections predominantly manifest themselves in the orofacial region. Primary infections are usually inapparent, but herpetic gingivostomatitis is frequently found as the predominant symptom in young children. This very painful inflammation of the mucosa of the mouth and throat should not to be mistaken for the herpangina caused by Coxsackie A viruses. The incubation period lasts, on average, 6–8 days. Symptomatic primary infection with herpes simplex virus type 1 can be characterized by fever and ulcerative or vesicular lesions on the lips and the mucosa of the mouth (herpes labialis and herpetic gingivostomatitis). In addition, oedematous swelling of the face, lymph node swelling and corneal infections are observed. Patients are viraemic at this stage of primary infection. The symptoms usually subside after 2–3 weeks. Meningitis and encephalitis are associated with this infection only in rare cases. Immunocompromised patients can also develop hepatitis, oesophagitis, severe skin ulcerations, and especially pneumonia.

Infections with herpes simplex virus type 2 are similar, but are predominantly manifested in the genital mucosa (herpes genitalis). Whereas herpes simplex virus type 1 establishes a latent state in the cells of the trigeminal and ciliary ganglion following primary infection, the typical site of latency of herpes simplex virus type 2 is the sacral ganglia. Complications include meningitis and a generalized infection, herpes neonatorum, which may occur in newborn children by transmission of herpes simplex virus type 2 due to a primary maternal infection. Recurrence is manifested in both infections by the appearance of vesicular rash, usually in an attenuated form (herpes labialis or genitalis as reactivation). They can be manifested without inflammation (recurrence), or may be associated with it (recrudescence). Recurrent corneal inflammations can lead to blindness. The viruses can be reactivated by different factors, such as ultraviolet rays, fever, psychological factors, stress, hormonal factors (menstruation) and certain chemicals and drugs, e.g. adrenaline, and particularly

immunosuppressive agents. Pneumonias associated with herpes simplex virus are not uncommon in intensive care units, even in non-medicamentous immunosuppressed patients.

Pathogenesis

During primary infection, the virus infects the oral or genital mucosal cells, where it replicates lytically. Histological examinations of skin blisters reveal degenerated keratinocytes and multinucleated giant cells, which are generated by cell fusion, and contain eosinophilic intranuclear inclusion bodies. Apart from blistering and epithelial necrosis, migration of granulocytes into the infected regions is a first sign of the developing inflammation. Necroses do not break through the basal lamina. However, $CD4^+$ T lymphocytes accumulate in the dermis and release IFN-γ. In this way, macrophages are activated, resulting in further stimulation of the immune system. The first contact of the virus with the immune system occurs in the Langerhans cells, which subsequently migrate into local lymph nodes and differentiate into mature dendritic cells. Probably, Langerhans cells and keratinocytes secrete IL-1, TNF-α and other cytokines that trigger inflammation. Concurrent with the onset of inflammation, an escape of tissue fluid occurs, which leads to the formation of blisters, and to an increased migration of cells in the infected region. The viruses differ in their neuroinvasivity and neurovirulence. It has been found that herpes simplex virus ICP34.5 mutants lose their ability to replicate in the central nervous system in experiments in mice. In humans, the viruses reach into free nerve endings via cell-to-cell contact during the acute infection, and migrate retrogradely through the axons of sensory nerves into the corresponding ganglia, infecting them latently and remaining there for life. Inflammation processes occur in the neurons of the ganglia with the participation of $CD4^+$ and $CD8^+$ T lymphocytes. Even in this case, TNF-α and IL-1 are released. Depending on the neurovirulence of virus isolates, neurons become necrotic sooner or later. Whether apoptotic processes play a role is unclear. Little is known about the duration of inflammation, and how replication is completed.

After primary infection, episomal viral genomes are found in the neurons of the ganglia (herpes simplex virus type 1 in trigeminal and ciliary ganglia, herpes simplex virus type 2 in sacral ganglia). There are indications that herpes simplex virus persists in experimentally infected animals in other tissues, such as the cornea. Viral DNA has been detected even in the brainstem.

After reactivation, tegument-containing capsids migrate anterogradely from the ganglia along the nerve fibres back into the skin. There, herpesvirus causes new inflammations with blister formation; these are delimited by the activity of macrophages and interferon production. It is unclear why recurrences occasionally occur without tangible signs of inflammation, and why about 50 % of people infected with herpes simplex virus never develop recurrences.

Herpes simplex virus associated encephalitis is an acute necrotizing inflammation. It is characterized by viral replication, cell necrosis and granulocyte infiltration, and partially occurs as haemorrhagic encephalitis. The neonatal herpes can be

associated with encephalitis, generalized infections or forms that only involve the eyes and skin. It is conceivable that herpesvirus infections do not lead to IFN-γ and TNF-α synthesis in the immunologically immature organism. Herpes simplex virus encephalitis can occur in two ways: the virus may move ascending through the dorsal root ganglion or the olfactory bulb into the brain. In experimental animals, herpes simplex virus migrates through the ganglion into the spinal cord and thence to the brain; starting from the primarily infected ganglion, it moves along the nerve fibres into other dorsal root ganglia, including the ganglia of the autonomic nervous system.

Immune Response and Diagnosis

IgM, IgG and IgA antibodies are produced during a herpes simplex virus infection. Immunoglobulins directed against non-structural proteins are initially found, whereas antibodies against glycoproteins are produced later. Neutralizing antibodies are mainly directed against glycoproteins gB, gC and gD. They protect against new infections with the same virus type. Especially immunoglobulins directed against the gD protein prevent haematogenous spread of the virus, and are involved in the elimination of infected cells by the antibody-mediated cytotoxic cell response of granulocytes and macrophages. Cytotoxic, $CD8^+$ T lymphocytes can be detected especially in epitopes of glycoproteins. Almost no changes in antibody concentration are observed in recurrent herpes simplex virus infections. Since recurrent viruses move along the nerves to the skin, where they are transmitted by cell contact, they are probably not accessible to the immune system. Although neutralizing antibodies are present in the organism, they cannot prevent de novo viral replication and the development of inflammation. After a primary infection with herpes simplex virus type 1, the organism is not completely protected from infection with type 2. However, the infection is usually less severe, and is less prone to the formation of recurrences. This is also true even if the infections occur in reverse order. This partial cross-immunity is regarded as evidence that a protection against recurrent herpesvirus infections could also be achieved by appropriate vaccination.

In addition to the antibody-mediated cellular response, $CD4^+$ and especially $CD8^+$ cytotoxic T lymphocytes are essential for control of the lytic viral infection in the skin. Elimination of the virus from the central nervous system is delayed in CD8-deficient mice. In particular, the immediate early proteins ICP4 and ICP27 appear to possess epitopes that are recognized by cytotoxic T cells.

The diagnosis is based on the serological determination of antibody levels (IgM, IgG), and permits a distinction between primary infections and reactivations. In clinically manifested primary infections or reactivations with organ manifestations, the pathogen can be isolated from the corresponding blister material, cerebrospinal fluid or tissue by using cell culture. The cytopathic effect in cell culture appears after 1–3 days. The quantitative detection of viral DNA by PCR is a better method, which can be performed with almost all possible materials. Quantification also allows differentiation between primary infections and reactivations which may occur concomitantly with other infections. Differentiation between herpes simplex

virus types 1 and 2 can be performed by determining type-specific IgG antibodies or by type-specific PCR, but is clinically and therapeutically irrelevant.

Therapy and Prophylaxis

Herpes simplex virus infections can be treated locally or systemically (orally or intravenously) with acyclovir, which is accepted as a substrate by both the viral thymidine kinase and the DNA polymerase, leading to chain termination during viral DNA replication (▶ Chap. 9, Sect. 19.5.3). This therapy has high selectivity by using these two viral enzymes as therapeutic targets, and is associated with few side effects. Therapy should be started as soon as possible in cases with meningitis/encephalitis, and at the first suspicion of neonatal herpes. However, an exclusively local application is recommended only for limited, recurrent infections. However, modern acyclovir derivatives are oral prodrugs, have a considerably longer biological half-life and must be taken only once or twice daily. Drug-resistant virus mutants can emerge during systemic therapy, representing a major problem mainly in immunosuppressed patients. The mutations are generally located in the thymidine kinase gene, and only in rare cases are found in the gene encoding the DNA polymerase. However, these resistant viruses do not play an epidemiological role. Probably, they are much less infectious in vivo. Vaccination against herpes simplex virus infections is not currently possible. Several vaccines based on glycoproteins gB and gD as an antigen are being tested in clinical trials.

19.5.7.2 Varicella-Zoster Virus

Epidemiology and Transmission

Chickenpox (varicella) has been known as a human disease since the early Middle Ages. In 1875, Rudolf Steiner demonstrated that an infectious agent is the trigger of the disease. He was able to transmit the disease to healthy individuals by application of the content of blisters. In 1909, Janos Bokay described chickenpox and shingles (zoster) as similar diseases. This was experimentally verified by Karl Kundratitz and E. Bruusgaard in the 1920s and 1930s, when they induced chickenpox by the content of zoster blisters. The virus was first cultivated in cell cultures by Thomas H. Weller and Marguerite B. Stoddard independently of each other in 1952/1953. This demonstrated that the causative agent of chickenpox and shingles is identical. Subsequent restriction enzyme analysis of the viral genomes by Stephen E. Straus and colleagues demonstrated that the "chickenpox" virus is able to cause shingles after various periods of time. Therefore, shingles is not an infection with a related virus type, but is an endogenous recurrence. Varicella-zoster virus affects only humans, and has a worldwide distribution; there are several genotypes with different regional distributions. More than 95 % of people are seropositive at the age of 15 years. The virus is transmitted by contact with the contents of the vesicular rash of chickenpox and – in rare cases – by people with shingles, or by droplet infection of patients during the viraemic phase. After primary infection, the virus remains latent for life in the paravertebral sensory ganglia of the spinal cord. Shingles, which usually occurs in elderly or immunocompromised

individuals, is attributed to the reactivation of latent varicella-zoster virus. It is estimated that about 10–20 % of seropositive individuals develop shingles during their lifetime.

Clinical Features

Varicella-zoster virus infection is asymptomatic in only about 5 % of cases. The incubation period lasts on average 2 weeks. Chickenpox begins with fever, followed by blister-shaped, vesicular rash. This occurs initially in the mouth, on the head and trunk and spreads from there to the extremities. New blisters are formed for about 3–5 days.

Subsequently, the rash subsides, forming crusts, and the lesions heal after 2–3 weeks. Severe, generalized infections are found especially in newborns and immunocompromised patients. Such patients can develop hepatitis, pneumonia, encephalitis and thrombocytopenia. If women are infected in early pregnancy, then varicella-zoster virus can be transmitted transplacentally to the embryo in very rare cases, causing embryopathy, the congenital varicella syndrome. This can lead to encephalitis, with the consequence of microcephaly. If the mother contracts chickenpox just before or after delivery, the virus can be transmitted to the neonate and cause a generalized infection, which has a high lethality when untreated. Chickenpox is frequently severe in pregnant women and may be associated with fatal lung infections.

Reactivation of the virus is usually announced by pain and increased skin sensitivity a few days before the onset of shingles. The rash is usually limited to one area of the skin on the head or torso. It runs like a belt from the spine around the thorax or lumbar region, and is often painful and accompanied by fever. The vesicles form a crust within 1–2 weeks. Prolonged and painful postherpetic neuralgia frequently occur in the skin area that was affected by the shingles. Other complications such as encephalitis and pneumonia are very rare in reactivations. Generalized and chronic forms of herpes zoster can emerge in immunocompromised patients, in particular varicella pneumonia. The involvement of cranial nerves in shingles is also dangerous, and can lead to blindness (ophtalmic zoster).

Pathogenesis

The virus enters the oral mucosa via airborne transmission. During primary infection, it replicates in the upper respiratory tract and the oropharynx, from where it infects peripheral blood mononuclear cells. These transport the virus to the lymph nodes, from which a small first viraemia starts after 4–6 days. The virus spreads via the reticulohistiocytic system and endothelial cells into the organism. Thereafter, the virus arrives at the periphery via the bloodstream during the subsequent second viraemia. It remains associated with mononuclear cells, and is not released into the environment. Even during cultivation in cell culture, virtually no infectious particles can be found in the medium. In the body, free viruses are only released at the apical side of epithelial cells. Mononuclear cells migrate into the infected areas and secrete cytokines such as IFN-y. Skin lesions appear when the infection spreads from endothelial cells in the blood capillaries of the skin into epithelial cells, and

when tissue fluid is released as a result of the inflammatory response. For example, the non-infected layer of the stratum corneum is separated from the underlying infected basal cells of the epidermis, and forms blisters. Initially, they do not contain cell-free viruses. Later, macrophages, lymphocytes and cytokines can additionally be found in the vesicles. Infected cells fuse, and form multinucleated giant cells with eosinophilic intranuclear inclusion bodies. During infection, varicella-zoster virus enters the nerve endings of peripheral nerves especially by direct cell-to-cell contact, and migrates retrogradely from there into the sensory ganglia of the spinal cord. The virus remains latent in neurons. Viral DNA is also found in the olfactory bulb and in the geniculate body of the central nervous system.

The productive infection with varicella-zoster virus induces apoptosis in vitro. Apoptotic processes are prevented in neurons by the activity of the immediate early protein IE63, a transactivator with homology to the ICP22/U_S1 protein of herpes simplex virus, which contributes to the establishment of latency. Varicella-zoster virus has evolved ingenious mechanisms to evade the host immune response. ORF66 encodes a serine/threonine kinase which prevents the transport of MHC class I antigens to the cell surface in infected T lymphocytes and fibroblasts. As a result, viral peptides, which are presented in complex with MHC class I antigens, can not be recognized by cytotoxic T cells. Presumably by similar molecular processes, infected keratinocytes become depleted of intercellular adhesion molecule 1 on the cell surface. Additionally, the amount of MHC class II proteins is reduced in infected dendritic cells. Possibly, all these processes are based on the inhibition of phosphorylation of the cellular STAT1α and Jak2 proteins by the ORF66 kinase. As a result, the synthesis of IFN-α does not occur, and consequently, cellular, immunologically important surface proteins are synthesized only in reduced concentrations.

Little is known about the molecular processes involved in the reactivation of the virus, which replicates in the ganglia, destroying large parts of them. Immunosuppression, trauma to the spinal cord, psychological factors and certain medications may contribute to trigger reactivation of the virus. Reactivation is preponderantly found in older people who exhibit a decreased number of $CD4^+$ and $CD8^+$ T lymphocytes that recognize varicella-zoster-virus-specific epitopes. Like herpes simplex viruses, reactivated varicella-zoster viruses also reach the affected skin area by anterograde migration along the nerve fibres of the corresponding ganglion. In the epidermis, they cause the formation of a recurrent vesicular rash, shingles. Shingles also induces inflammation and necrosis of the affected ganglia and nerves during reactivation of the virus.

Immune Response and Diagnosis

IgM, IgA and IgG antibodies against viral proteins are produced during primary infection. IgG antibodies remain detectable for life. They are directed against glycoproteins; hence, they are partially neutralizing and confer protection against new infections, but they do not protect from reactivation of the virus. The cellular immune response probably contributes significantly to elimination of the virus from the infected skin areas during primary infection. Cytotoxic $CD8^+$ T lymphocytes,

which lyse infected cells, are detectable early during infection. $CD4^+$ T lymphocytes recognize particularly epitopes of glycoproteins and epitopes of the immediate early IE63 protein. A decrease of humoral and cellular immune responses can be observed during the course of infection, and they are only regenerated by the renewed replication of the virus during reactivation, i.e. during the development of shingles. Apart from serological diagnosis (IgG negative, IgM positive), the virus is detected by PCR from the content of the vesicles or pharyngeal lavage in primary infections. This also applies to manifestations in the context of reactivation. Serologically, IgG levels increase, and IgM can be detected in such cases.

Therapy and Prophylaxis

Varicella-zoster virus infection can be prevented by administration of a live vaccine. It contains attenuated viruses (OKA strain) that were obtained by continuous cultivation of the wild-type virus in cell culture. The vaccine was first recommended for use in children with leukaemia prior to immunosuppression. Varicella-zoster virus infections are often very severe in such patients because their immune system is weakened by the disease and the associated therapy. In addition, seronegative women with a desire to become pregnant have been vaccinated, and so have workers with who have contact with children. Today, combination vaccines to protect against measles, mumps, rubella and chickenpox are generally recommended in most countries worldwide. Since the vaccination occurs early in the second year of life, it protects against chickenpox infection and from the possible reactivation with shingles. In contrast, vaccination of older children and adults who are already infected protects only against reactivation of shingles, or mitigates its course. Chickenpox is usually not treated in children. Immunoglobulins with high concentrations of neutralizing antibodies against varicella-zoster virus are applied after exposure especially in people who have high risk of complications: these include neonates, pregnant women, immunosuppressed patients, and children. These immunoglobulins prevent infection or reduce its progression when they are applied as quickly as possible after contact with infected individuals, i.e. within 96 h. Shingles is treated with brivudine, famciclovir or other acyclovir derivatives which exhibit significantly better pharmacokinetics than acyclovir, and thus frequently show a better effect, especially in the treatment of neuralgias associated with shingles. The duration of generalized infections is reduced, and the symptoms can be prevented. Drug-resistant varicella-zoster virus strains have been isolated from AIDS patients treated with acyclovir. However, they seem to have a selective disadvantage because of mutations in the thymidine kinase gene, and are without significance for the human population.

19.5.7.3 Human Cytomegalovirus

Epidemiology and Transmission

Cytomegaloviruses were originally known in connection with prenatal infections leading to damage to the fetus or newborn children. However, serological studies

revealed that cytomegalovirus infections are widespread. Whereas about 40 % of the population is infected with cytomegalovirus in Western countries, the level may reach more than 90 % in developing countries.

After a usually asymptomatic primary infection, the pathogen persists lifelong in humans, and reactivates sporadically and in phases of immunosuppression. In adults, especially in immunosuppressed patients, e.g., transplant recipients, cancer patients and AIDS patients, cytomegalovirus frequently causes severe diseases. Clinically, it is the most important virus in transplant recipients. Cytomegaloviruses are excreted intermittently after primary infection. Primary cytomegalovirus infection is most frequent in children aged up to 3 years. The virus is transmitted by infected cells present in the saliva, urine and breast milk or by contamination with virus-containing secretions. Frequently, infants and young children are postnatally infected by breast milk because almost all seropositive mothers reactivate cytomegaloviruses during lactation, and these are subsequently excreted in the breast milk. The virus is also found in cervical secretions and in semen, so sexual intercourse constitutes a source of infection. Immunocompromised patients excrete far larger quantities of virus. Transmission is also possible by organ transplants, blood transfusions and blood products.

Transplacentary transmission from primarily infected pregnant women to the unborn child plays an important role. Pregnant women are primarily infected in approximately 2–6 % of pregnancies. In such cases, up to 40 % of fetuses are transplacentary infected. It is estimated that an average of one in a 1,000 is born with a congenital cytomegalovirus infection, which can later be manifested as neurological damage such as mental retardation, calcification in the brain, visual impairment and deafness. Hence, human cytomegalovirus is the commonest viral pathogen for embryopathies and fetopathies. The hormonal changes during pregnancy are associated with reactivation of the virus from latency, and 10 % of all pregnant women excrete cytomegaloviruses; however, transplacental transmission leading to damage to the unborn child is rare during reactivation of the virus and its prevalence is only 0.2–2 %.

Clinical Features

The incubation period lasts on average 4–8 weeks. The infection is commonly asymptomatic. Symptoms similar to those of mononucleosis, namely fever, lymph node swelling, gastritis, oesophagitis, and flu-like symptoms, are only rarely observed. Leucocytopenia and thrombocytopenia and atypical $CD8^+$ T lymphocytes as well as a decreased titre of $CD4^+$ T lymphocytes are occasionally observed. Transfusion mononucleosis is additionally characterized by angina. Prenatal infections, especially those that occur in early pregnancy, lead to cytomegaly in approximately 10 % of cases, and this is manifested as hepatosplenomegaly, thrombocytopenia, hearing loss and developmental defects owing to the infection of the central nervous system. The rare perinatal or postnatal transmission of the virus to neonates can lead to similar, but usually attenuated symptoms. Because of the typical organ locations of the virus, hepatitis, chorioretinitis, gastrointestinal ulceration, colitis with pain and

diarrhoea and relatively rarely encephalitis can be observed in immunosuppressed patients. Interstitial cytomegalovirus pneumonia often exhibits severe courses, and is the major cause of death in AIDS patients and bone marrow transplant recipients. In the former, the virus is found in the adrenal glands, lungs, gastrointestinal tract, central nervous system and retina (chorioretinitis). In patients with organ transplants, cytomegalovirus leads to inflammation of blood vessels in the graft (post-transplant vasculitis), which histologically resembles a rejection reaction and may lead to graft loss. Therefore, cytomegaloviruses play an important role in transplant patients during primary infection and reactivation, and still cause high mortality rates in spite of treatment.

Pathogenesis

During the apparent and probably also during the asymptomatic primary infection of immunocompetent patients, cytomegalovirus reaches the salivary glands commonly via oral transmission, and then spreads haematogenously and also in a cell-associated manner. Vascular endothelial cells play an important role in spread of the virus to various organs. Cytomegalic cells are observed in many organs, particularly in the salivary glands, kidneys and suprarenal glands. Viral DNA can also be detected in histopathologically inconspicuous cells of the myocardium, liver, spleen, lung, bone marrow and kidneys. Furthermore, the virus can also be present in free or cell-bound form in the blood, especially in endothelial cells and granulocytes. Antigenaemia and viraemia suggest a generalized infection. The virus presumably remains latent in many organs in a state that is not characterized at the molecular level (Sect. 19.5.6). As cytomegalovirus is detectable in the urine and saliva, cervical secretion and semen, the cells of the respective organs must be latently infected.

Spread of cytomegalovirus infection is largely determined by the functional state of the immune system, since the postnatal infection and that of young children and elderly people are usually asymptomatic. When transmitted to unborn children, the virus encounters a still undeveloped immune system. The damage rate of infections between the second and sixth months of pregnancy is about 50 %. Depending on the development stage, the following organs may be damaged: brain (microcephaly), eye (chorioretinitis), ear and liver (hepatosplenomegaly). Infected children may die in utero or after birth. A distinction must be made between primary infection and reactivation in individuals with immune deficiencies, since the former is severer. Those severe courses are based on a generalized deficiency in cellular immunity. As a result, the virus can spread to many organs, causing the corresponding, histopathologically detectable organ damage. AIDS patients often develop chorioretinitis and gastrointestinal ulcers. Without antiviral or antiretroviral medication, 20–30 % of these patients die because of interstitial cytomegalovirus pneumonia.

Immune Response and Diagnosis

During primary infection, the organism produces IgM and IgG antibodies. In addition, cytotoxic T cells are induced. The cellular immune response is responsible

for elimination of the virus and cessation of symptoms; antibodies control the viraemic spread and protect against new infections. Neutralizing antibodies are principally directed against the gB protein. Most detectable immunoglobulins recognize the phosphoprotein pp65, the major component of the tegument. However, neither antibodies nor the cellular immune responses are able to prevent reactivation of the virus from latency. Owing to the existence of a prestimulated immune system, reinfections and multiple infections are rarely observed in organ transplants. The following observations evidence that the cellular immune system is important in controlling lytic infection: in prenatal or perinatal infections, excretion of the virus lasts much longer than in infections that occur later in life. It can be inferred that more cells have been infected and, possibly, the not yet developed immune system of the fetus or newborn permits enhanced viral replication. Furthermore, excretion of the virus is increased in patients with immunodeficiency; in part, a persistent, cell-bound viraemia can be observed. Since these patients have an impaired cellular immune system, but the humoral response is usually intact, $CD4^+$ and $CD8^+$ T lymphocytes as well as natural killer cells probably have an important function in the control of infection during latency. The risk of prenatal infection appears to be increased when the cellular immune system of the mother is harmed. It is known that cytotoxic T cells recognize epitopes of immediate early proteins of murine cytomegalovirus. Similar mechanisms might be important for the control of lytic viral replication in humans.

The serological diagnosis of primary infection is performed by detecting specific IgM antibodies by ELISA. The virus can be isolated from blood, saliva, urine and cervical secretion, and its detection is performed by quantitative PCR. The cultivation of human cytomegalovirus is possible only in human or chimpanzee cells, especially in embryonic lung fibroblast cells or foreskin cells of neonates. The infected cells enlarge (cytomegaly), and owl-eye-like inclusion bodies can be observed in the nuclei after fixation. Because of the slow replication rate of the virus in cell culture, and the long time to the onset of the cytopathic effect, viral replication is determined by the detection of immediate early proteins (pp72) by immunofluorescence after 1–2 days. The tegument protein pp65 is synthesized in large amounts in infected cells, and is released into the blood after destruction of the cells. It is ingested as an immune complex with antibody molecules by granulocytes and can be detected by immunofluorescence in these cells (pp65 proteinaemia). Moreover, the viral load is determined by quantitative PCR.

Cytomegaloviruses Possess Various Mechanisms to Evade the Immune Response

All herpesviruses have evolved plenty of sophisticated strategies to evade the host immune response. These mechanisms are especially well studied in cytomegaloviruses. They possess the genetic information for proteins that resemble key cellular polypeptides. These include a protein with homology to MHC class I antigens which is encoded by open reading frame UL18.

It interacts with β_2-microglobulin, thus depriving MHC class I antigens of their interaction partner, and resulting in a reduced concentration of MHC complexes for presentation of peptide antigens. Concurrently, UL18 proteins on the cell surface prevent the attack of natural killer cells which recognize cells with low amounts of MHC on their surfaces (▶ Chap. 7). The gene product of the US28 open reading frame is a homologue of chemokine receptors and binds to the chemokines RANTES and MCP-1, which are secreted, inter alia, by activated T-helper cells (▶ Chap. 8). This interaction with US28 gives rise to internalization and degradation of the complex. In this way, binding of these chemokines to their natural receptors is prevented, blocking the respective signalling pathways. Furthermore, cytomegaloviruses have additional proteins that interfere with immunological regulatory mechanisms. These include the proteins US2 and US11, which bind to MHC class I antigens that are anchored in the ER membrane, thus inducing their transport to the proteasomes in the cytoplasm and their degradation. This process also results in cellular depletion of MHC class I antigens. Moreover, these viruses are also able to interfere with MHC peptide loading. The viral US6 protein, which is anchored in the ER membrane, is responsible for this effect. The lumenal domain of the US6 protein inhibits the ATP-dependent transport of peptides by TAP transporters into the ER, and inhibits ATP hydrolysis by a still unknown mechanism that blocks the transport process. Furthermore, cytomegaloviruses are also able to inhibit the transport of MHC class I peptide–β_2-microglobulin complexes from the ER to the Golgi vesicles and then to the cell surface. This process is inhibited by the viral US3 protein.

Therapy and Prophylaxis

Treatment of cytomegalovirus infections is possible by using ganciclovir, cidofovir and foscarnet (phosphonoformic acid) (▶ Chap. 9). However, all these substances cause some serious side effects. In contrast to other herpesviruses, members of the subfamily *Betaherpesvirinae*, and thus also cytomegaloviruses, do not have the genetic information coding for a thymidine kinase. Ganciclovir is monophosphorylated by the viral protein kinase (UL97), whereas its triphosphorylation is performed by the cellular dGMP and NDP kinases. It is incorporated into elongating DNA strands by the viral DNA polymerase during genome replication (Sect. 19.5.3). Acyclovir cannot be used, as it is not recognized as a substrate by the UL97 kinase. Foscarnet inhibits the viral DNA polymerase non-competitively: it is applied only after administration of nucleotide analogues because of its severe side effects. Hyperimmunoglobulin preparations are administered in acute infections during early pregnancy and in cases of suspected perinatal infections, as well as during haematogenous dissemination of the virus in transplant patients (in such cases, in combination with the aforementioned antiviral agents). A vaccine is not yet available.

19.5.7.4 Human Herpesviruses 6 and 7

Epidemiology and Transmission

Although Syed Zaki Salahuddin and colleagues originally isolated human herpesvirus 6 as human B-lymphotropic virus from patients with lymphoproliferative disorders in 1986, it was later proven that it infects primarily T cells, in which it replicates. Four years later, another virus related to human herpesvirus 6 was found in $CD4^{+}$ T lymphocytes; it is referred to as human herpesvirus 7 today. Human herpesvirus 6 is divided into two subtypes, A and B: their genome sequences differ by about 10 %, and subtype B has nine additional open reading frames (B1 to B9) in comparison with subtype A. The highest degree of variance is found in the immediate early gene products and in the region of the genome encoding the surface protein gQ. So far, no disease has been associated with subtype A in immunocompetent individuals. Human herpesvirus 6 subtype B causes exanthema subitum in children, which is also called "three day fever". The prevalence of the virus is high; at the age of 2 years, virtually all children are seropositive. Human herpesvirus 7 infections occur a while later, namely from the third year of life. Human herpesvirus 7 has only sporadically been isolated from patients with exanthema subitum. Viral DNA has been found in the gastric mucosa, and in 5 % of central nervous system biopsies. After primary infection, both viruses remain latent in the organism; they are excreted in the saliva, through which they are transmitted. Both human herpesvirus types are frequently found in the blood plasma of bone-marrow-grafted patients, in particular human herpesvirus 6 type B.

Clinical Features

The primary infection with human herpesvirus 6 usually occurs in children aged under 2 years, is often asymptomatic and the average incubation period is 1–2 weeks. The main symptom of exanthema subitum, also known as roseola infantum or sixth disease, is characterized by a sudden high temperature that usually lasts 3 days, and is accompanied by rash. Swollen lymph nodes, liver impairment and encephalitis are occasionally found. Thereafter, the virus is detectable in T cells for life. Acute infections in adults are extremely rare because of the widespread infection in childhood. In the few documented cases, symptoms have been described that are similar to those of infectious mononucleosis. There is evidence that this viral infection may damage bone marrow. Whether reactivations are associated with illnesses in immunocompetent individuals is unknown. Evidence suggesting an association between human herpesvirus 6 and multiple sclerosis or chronic fatigue syndrome in later life has not been verified. Immunosuppressed transplant patients frequently exhibit reactivation of human herpesvirus 6 approximately 2–4 weeks after bone marrow or organ transplant. However, since the virus is present in latent form in the cells of many organs, in such cases, re-infection of patients may occur from the donor organs. Patients develop fever, rash, and occasionally pneumonia, encephalitis and hepatitis. Generally, the clinical symptoms are similar to those of cytomegalovirus infection.

Pathogenesis

Human herpesvirus 6 and human herpesvirus 7 replicate in vivo in activated T lymphocytes: human herpesvirus 7 shares a common receptor with HIV, the CD4 protein on the surface of T-helper cells, whereas human herpesvirus 6 attaches to CD46. Inasmuch as CD46 is present on the surface of various cells types (macrophages, dendritic cells, fibroblasts, epithelial cells and bone marrow cells), many of them can be infected in vitro, but not all of them support productive replication of human herpesvirus 6. After primary infection, low amounts of virus are continuously produced and excreted in the salivary glands. A similar low level of persistent viral infection may also be found in the brain. In addition, human herpesvirus 6 also induces a latent infection in monocytes and undifferentiated bone marrow cells without producing virus particles. In this state, LATs are transcribed from the IE-A (U86-90) region of the genome, which encodes the early gene products IE1 and IE2. However, LATs differ from the immediate early transcripts by different initiation sites and sequences in the 5′ untranslated region.

Since human herpesvirus 6 is found in the same cells that are infected by HIV, it has been considered as possible cofactor in the pathogenesis of AIDS. The finding that some of the immediate early and early proteins of human herpesvirus 6 are capable of inducing the long terminal repeat promoter of HIV supports the hypothesis that suggests the involvement of a cofactor function in AIDS. The proteins involved are encoded by reading frames U86-90 (immediate early protein A), U16-19 (immediate early protein B), PU3, DR7 and U94, which exhibits homology to the Rep protein of adeno-associated virus 2. Additionally, the expression of the cellular CD4 gene is increased by infection with human herpesvirus 6. This results in an augmented number of $CD4^{+}$ cells, which can serve as additional targets for replication of HIV. Whether both infectious agents act synergistically in vivo has not yet been definitely elucidated.

Immune Response and Diagnosis

The organism produces IgM and IgG antibodies during primary infection. IgG remains detectable for life, but its concentration gradually decreases in the course of ageing. Like in all other herpesviruses, reactivation of human herpesvirus 6 leads to a reincrease of antibody concentrations. The cellular immune response has been scarcely investigated so far. Because of the high prevalence already in childhood, serological diagnosis plays virtually no role. The virus can be isolated from the saliva or pharyngeal lavage, and is cultivable in primary umbilical cord lymphocytes. Quantitative detection of viral DNA is performed by PCR from relevant materials or blood to diagnose reactivations, particularly in immunocompromised patients.

Therapy and Prophylaxis

There is no vaccine to prevent cytomegalovirus infections with human herpesvirus 6 and human herpesvirus 7. Infections with human herpesviruses 6 and 7 are mild and self-limiting in immunocompetent individuals; treatment is not necessary.

Severe and in individual cases life-threatening diseases can be found in association with reactivations in immunosuppressed patients. In analogy to human cytomegalovirus, therapy is possible with ganciclovir (orally with valganciclovir), cidofovir or foscarnet. Human herpesvirus 6 is a member of the subfamily *Betaherpesvirinae*; hence, it does not have a thymidine kinase for monophosphorylation of nucleoside analogues. This reaction is catalysed by the viral phosphotransferase pU69, which is similar to the pUL97 protein of human cytomegalovirus in terms of function and operation. The cellular enzymes dGMP and NDP kinase catalyse the triphosphorylation reactions of nucleoside analogues, which are incorporated into newly synthesized viral DNA genomes by the viral DNA polymerase, causing chain termination.

19.5.7.5 Epstein-Barr Virus

Epidemiology and Transmission

In 1958, Dennis Burkitt described a lymphoma that was the commonest cancer among children and adolescents in East Africa and Central Africa. Its distribution matched that of malaria. In 1964, Anthony Epstein, Budd Achong and Yvonne Barr as well as Robert Pulvertaft discovered herpesvirus-like particles in B-cell lines from monoclonal Burkitt's lymphomas. The pathogen was identified as a new virus genus by Gertrude and Werner Henle, who named it Epstein-Barr virus after its discoverers. Burkitt's lymphoma is the commonest childhood cancer in Africa, and has an incidence of one case per 10,000 people per year. In marmosets (*Callitrichidae*), the virus causes a Burkitt's lymphoma-like, but polyclonal tumour – a reference to the causal involvement of the virus in oncogenesis. In 1968, Gertrude and Werner Henle as well as Volker Diehl found antibodies against Epstein-Barr virus in the blood of patients who had been overcome by an infectious mononucleosis. Meanwhile, it has been found that the virus has a worldwide distribution, and about 95 % of adults are seropositive. All infected individuals have Epstein-Barr-virus-positive B cells in the blood lifelong after primary infection. These quiescent B lymphocytes contain the virus and have the ability to develop into immortalized cell lines in vitro. An efficiently functioning cellular immune system probably prevents uncontrolled proliferation of these cells in vivo. Apart from Burkitt's lymphoma, the virus is associated with other human cancers: Hodgkin's lymphoma and nasopharyngeal carcinoma, which has an incidence of approximately two cases per 10,000 people per year, and constitutes the commonest cancer in China and Southeast Asia. Besides Epstein-Barr virus, also behavioural and environmental factors appear to play an important role in the development of these tumours.

After primary infection, Epstein-Barr virus is excreted and transmitted lifelong in different amounts in the saliva. It has a worldwide distribution. In Europe and the USA, infections usually occur in adolescents and early adulthood. The main way of transmission is the reason for the popular name of kissing disease. In China and developing countries, all children aged about 2 years are seropositive owing to poor hygiene standards and the still conventional mouth-to-mouth feeding of young children.

Clinical Features

The incubation period of Epstein-Barr virus primary infections lasts, on average, 4–6 weeks. The infection is commonly asymptomatic during childhood. If it affects adolescents or adults; the symptoms of infectious mononucleosis occur more frequently. This self-limiting lymphoproliferative disease was described by the paediatrician Emil Pfeiffer in the second half of the nineteenth century; it is also known as glandular fever. It manifests itself as angina with sore throat, fever and significantly swollen lymph nodes. A common side effect is watery eyes. Spleen swelling can be observed in approximately half of patients 2–3 weeks after the onset of the symptomatic phase. Skin rash appears in 20–25 % of cases, and increased transaminase levels indicate hepatitis. Meningitis or arthritis is rarely observed. The symptoms usually disappear after a few weeks, the virus remains in the organism and is excreted from the nasopharyngeal region in the saliva. In addition, the Epstein-Barr virus genome is latently present in about one of 10,000 B cells of the peripheral blood. In rare cases, the symptoms of infectious mononucleosis do not subside completely, and patients develop a chronic, persistent infection in which the virus increasingly replicates lytically, in addition to the latency stage. Free viruses are frequently detected also in the serum. The symptoms of this chronic active infection such as fatigue, malaise and lymph node swelling are less pronounced than in infectious mononucleosis. They can last for months and years. High-performance athletes are particularly affected.

Fatal infectious mononucleosis occurs with familial incidence in men and is correlated with genetic defects on the X chromosome (Xq25-27). This so-called X-linked lymphoproliferative (XLP) syndrome or Duncan's syndrome (named after the first family described by David Purtilo as a carrier of the gene defect) is characterized by hypogammaglobulinaemia, aplastic anaemia and massive infiltration of $CD8^{+}$ T and B lymphocytes in the lung, kidney, liver and bone marrow. The consequence is multiple organ failure. About 30 % of patients also develop polyclonal B-cell lymphomas. A defect has been found in patients in a gene (SAP/SH2D1A) which encodes a member of the SAP-like protein family. SAP proteins interact with signalling lymphocytic activation molecule (SLAM) receptors, which are located on the surface of T and B lymphocytes and other haematopoietic cells. These include the CD150 protein, which is uses as a receptor by measles virus and various animal pathogenic paramyxoviruses (▶ Sects. 15.3.4 and ▶ 15.3.5). Binding of SAP proteins probably regulates the SLAM-receptor-mediated signalling pathway, which is important for the interaction of T lymphocytes with antigen-presenting cells during the immune response. In XLP patients, the function of SAP/SH2D1A is impaired by the genetic defect; therefore, these patients cannot control the acute Epstein-Barr virus infection. Similar symptoms have been found in XLP patients with other infectious diseases. Epstein-Barr-virus-associated polyclonal B-cell lymphomas are frequently also found in immunosuppressed patients. In AIDS patients, the risk rises to 60 %. Accordingly, post-transplant lymphoproliferative disorder (PTLD) is deemed to be a new Epstein-Barr-virus-associated disease. B-cell lymphoma typically occurs as a secondary disease in immunosuppressed children after an Epstein-Barr virus infection. This is clearly correlated with the degree of immunosuppression and is especially common in lung, heart and bone marrow transplant recipients. However,

Epstein-Barr-virus-positive adults also develop a PTLD, albeit less frequently. Therapeutic approaches attempt to reduce immunosuppression as much as possible.

Burkitt's lymphoma occurs endemically in children in Africa and Equatorial Guinea at the age of about 7–9 years. Roughly 95 % of Burkitt's lymphomas contain Epstein-Barr viruses in the transformed cells. About 20 % of the sporadic form of this tumour, which is found worldwide especially in adults, is associated with Epstein-Barr virus. The endemic form is manifested predominantly as a tumour of the jaw and face area, or the abdomen. By contrast, the bone marrow is usually involved in sporadic Burkitt's lymphoma. All Burkitt's lymphoma cells have chromosomal translocations. Usually, the long arm of chromosome 8 is translocated to chromosome 14. Parts of chromosome 2 or chromosome 22 are rarely translocated to the long arm of chromosome 8. Hodgkin's lymphoma is another malignant disease that is associated with Epstein-Barr virus infection. This B-cell lymphoma is histologically characterized by Reed–Sternberg cells, the typical Hodgkin's cells, which are mixed with a large number of lymphocytes and other immunologically active cells. In Western countries, viral genomes are present in 20–50 % of Hodgkin's lymphomas, and the cells produce EBERs and the latent proteins LMP1, LMP2A and EBNA1. This situation is found in almost all Hodgkin's lymphomas in developing countries. Monoclonal nasopharyngeal carcinoma, another disease caused by Epstein-Barr virus, develops as a primary tumour in the pharyngeal recess (Rosenmüller's fossa). It manifests itself as hearing limitations, nose bleeding and blockage of the ear tubes. This kind of tumour metastasizes quickly, so most patients develop secondary tumours by spreading of tumour cells in the lymph system. In recent years, other lymphoepithelial carcinomas which have morphological characteristics similar to those of nasopharyngeal carcinoma have been studied for their possible association with Epstein-Barr virus. It has been found that the Epstein-Barr virus genome is present in 80 % of gastric lymphoepithelial carcinomas, and that the latency gene products are synthesized in these cells. A similar situation concerns the salivary gland and lung carcinomas in countries in which Epstein-Barr-virus-associated nasopharyngeal cancer is endemic. In these cases, it is not clear to what extent a causal relationship exists between viral infection and tumour formation.

Pathogenesis

Primary Infection

By salivary transmission, the virus reaches the mucosa of the mouth and throat, where B lymphocytes are present. On their surfaces they have high concentrations of the CD21 protein, to which the Epstein-Barr virus gp220/gp350 glycoprotein complex binds to mediate penetration into B lymphocytes. The infection causes a polyclonal activation of B lymphocytes, resulting in a massive immune response. This process leads to infectious mononucleosis and its characteristic manifestations, including swelling of the lymphatic tissue such as lymph nodes, spleen, liver and tonsils. Presumably, the virus replicates lytically in some B cells in the early phase of infection, releasing infectious virus particles. The subsequent cellular immune response is directed especially against cells that synthesize the proteins

of the productive cycle. In contrast, a latent infection is established in other infected B lymphocytes. The proteins responsible for this are probably cell factors, such as the Ku protein, which together with still unknown factors, repress the expression of the BZLF1 immediate early gene, and thus prevent the lytic cycle. As a result, EBNA proteins become active: EBNA1 promotes episomal replication of the viral genome. EBNA-2, EBNA-3A, EBNA-3C and EBNA-LP and the LMPs are responsible for the immortalization of the cells and facilitate their continuous division. EBERs prevent activation of apoptotic processes and interferon-mediated immune mechanisms (Sects. 19.5.4 and 19.5.5). After establishment of latency, the resting B lymphocytes express LMP2A as the only viral protein. In this resting stage, the cells have only small amounts of MHC antigens on their surfaces. In addition, they lack the factor B7, which exerts a costimulatory effect in activating the cellular immune system. Therefore, Epstein-Barr-virus-positive B lymphocytes evade the immune response, remain in the body and constitute the reservoir of the virus. The lytic replication cycle and the concomitant production of infectious viruses are only very rarely induced in them. This process leads to the elimination of these cells by cytotoxic T lymphocytes that recognize epitopes of immediate early proteins. Important T-cell epitopes that are bound by several HLA subtypes and are recognized by cytotoxic T cells have been identified in functionally important regions of the BZLF1 protein. If the immunological elimination fails to function, then the cells will die owing to viral replication.

In addition to B lymphocytes, a further cell population can be infected by Epstein-Barr virus at its entry site in the oral and pharyngeal region: the epithelial cells of the oropharynx and nasopharynx, in particular those of the salivary glands. Small amounts of CD21 have been detected on their surfaces. Thus, they are possibly infected by Epstein-Barr viruses which are produced by some B cells in the early phase of infection as described above. The virus may also enter through fusion of infected B cells with epithelial cells in the pertinent tissue. The lytic infection cycle occurs preferentially in epithelial cells. The differentiation state of infected cells seems to be of crucial importance for its complete execution. In the basal cells of the epithelium, only the synthesis of BZLF1 has been detected. However, it is present in the cytoplasm, and thus cannot exert its transactivating and regulatory functions. With increasing cellular differentiation, which gradually progresses towards the surface of the mucosa, the viral transactivators are transported into the nucleus, where they induce the viral gene expression cascade of the lytic cycle. Finally, the cells exposed on the surface release viruses, which are secreted in the saliva. The cells of the nasopharynx are continuously infected with Epstein-Barr viruses from B lymphocytes, leading to a constant secretion of the virus.

It is not known how the chronic active Epstein-Barr virus infection occurs. Apparently, the virus is not able to infect B cells latently in these patients, so they continuously produce small amounts of the virus. These patients do not have antibodies against EBNAs, which is usually an indication of establishment of a latent infection cycle. On the other hand, a defect of the cellular immune response means that the organism cannot completely eliminate lytically infected B cells.

Secondary Diseases

Although all Epstein-Barr-virus-positive individuals have latently infected B lymphocytes in the peripheral blood, they rarely develop Epstein-Barr-virus-associated tumours. In an immunologically healthy organism, the cellular immune system prevents the uncontrolled proliferation of infected B cells. Therefore, Epstein-Barr-virus-associated polyclonal B-cell lymphomas are especially found in AIDS patients or organ transplant recipients, whose cellular immune system is impaired. Cytotoxic T-cell epitopes have been identified in all latent gene products, especially in EBNA-3C. Only EBNA1 can repress epitope presentation by the function of its glycine–alanine repeats (Sect. 19.5.5). However, even if the expression of latent gene products occurs only sporadically and is temporally limited, it still allows the cellular immune system to prevent the uncontrolled proliferation of cells.

Several factors contribute to pathogenesis of monoclonal Burkitt's lymphoma. The correlation of this tumour with endemic areas of malaria is striking. In these regions of Central Africa, children are infected at a very young age, and sometimes they may have had contact with Epstein-Barr virus during their embryonic development. The virus is probably transmitted to the embryo by the pregnant woman in the course of malaria-induced fever attacks. Furthermore, infections with *Plasmodium falciparum*, the causative agent of malaria tropica, suppress T-cell responses and stimulate B-cell proliferation. On the one hand, Epstein-Barr virus finds an immunologically immature organism, and on the other hand, it infects a larger number of cells. Unknown factors cause chromosome translocations in the cells. In all cases, the c-*myc* gene is translocated into regions of the genome that are highly expressed in B lymphocytes. In most cases, the c-*myc* gene, which is normally located in a genetically not very active region of chromosome 8, is translocated to chromosome 14. The relevant section on chromosome 14 is responsible for the synthesis of the immunoglobulin heavy chain. Translocation to chromosome 2 causes the c-*myc* expression to come under the control of the strong enhancer of the light κ chain. Translocation to chromosome 22 places c-*myc* expression under regulation of the enhancer of the light κ chain. Hence, both translocation processes lead to the production of high concentrations of the DNA-binding, *trans*-active c-Myc protein. Possibly, c-Myc is also involved in another property of Burkitt's lymphoma cells: there is evidence that c-Myc represses the expression of MHC class I genes, thus reducing the concentration of the corresponding proteins on the cell surface. This and the previously mentioned mechanisms might lead to a failure of the cellular immune system to recognize unrestrained proliferating cells.

How nasopharyngeal carcinoma occurs is unclear. The investigation of its pathogenesis is especially difficult because the tumour cells cannot be cultivated in vitro. Furthermore, there is no cell culture system that permits one to investigate the lytic replication cycle. Nasopharyngeal carcinoma is a monoclonal tumour. All cells, including those of the usually very small primary tumours, contain Epstein-Barr virus genomes. Although the tumour has a low incidence worldwide, its geographical concentration in Southeast Asia and China is striking. The coincidence of a number of factors probably promotes its development. In China, Epstein-Barr virus infections occur in infants, so the pathogen encounters a still immature

immune system. In high-risk areas, it is also usual to consume certain foods and medicines, such as permanently preserved foods and salted fish, which have a high content of nitrosamines. High concentrations of phorbol ester like substances have also been detected in herbal teas, which are used especially for treatment of colds. These chemicals induce the lytic cycle and production of progeny viruses in latently infected B-cell lines in vitro. It can be assumed that – owing to the lifestyle – the population of Southeast Asia has significantly higher levels of the virus in the mucosa of the nasopharynx than populations in other countries. Thus, there is a higher probability that Epstein-Barr virus infects cells in the nose and throat, in which the lytic replication cycle is inhibited (if it occurs, it would led to cell death). These cells can be immortalized and develop into a tumour. There are also indications that certain genetic constellations of HLA subtypes facilitate the emergence of the tumour. Possibly, individuals with the MHC class I combination A33/B58 or A2/Bw46 are not able to adequately present certain viral epitopes, so the cells evade the immune control.

Furthermore, Epstein-Barr virus DNA is found in about 40 % of Hodgkin's lymphomas, in tumours of the smooth muscle tissue and in certain stomach carcinomas. Only some latency gene products are synthesized in these tumour cells (EBNA1 and EBNA2). Whether they contribute to carcinogenesis is not clear.

Immune Response and Diagnosis

The polyclonal B-cell activation during infectious mononucleosis induces the production of autoantibodies and heterophilic immunoglobulins which are directed against many different, often unknown antigens. This "immunological chaos" is an initial diagnostic indication for a fresh Epstein-Barr virus infection. It can be detected in the so-called Paul–Bunnell test, which is, however, often false-negative. During primary infection, the organism produce virus-specific IgM, IgA and IgG antibodies directed against the delayed early and structural proteins, which in diagnostic detection methods (immunofluorescence tests and ELISA) are also known as EA (for "early antigen") and VCA (for "viral capsid antigen") (Fig. 19.32). The presence of IgM and IgA indicates an acute primary infection. IgG antibodies against early proteins are detectable shortly thereafter and their levels decline during the following months and years. IgG antibodies against structural proteins remain present for life. IgA and IgG antibodies against glycoproteins gp220/gp350 (MA) are neutralizing and protect against new infections. IgG antibodies against EBNAs emerge concurrently with the transition of Epstein-Barr virus into the latent phase. They are a diagnostic indication for a previous Epstein-Barr virus infection (Fig. 19.32). In patients with chronic active Epstein-Barr virus infections, they are usually not present. This indicates that the virus in these individuals does not transit, or only marginally transits, into the latent cycle, and thus EBNAs are synthesized only in low concentrations. IgA antibodies directed against early and also late proteins have an additional diagnostic significance. Their concentration is strongly increased in patients with nasopharyngeal carcinomas, even when the tumour is at an early stage and the patients still have no symptoms. Since early diagnosed nasopharyngeal carcinomas (before metastasis) can be treated very well by irradiation, these

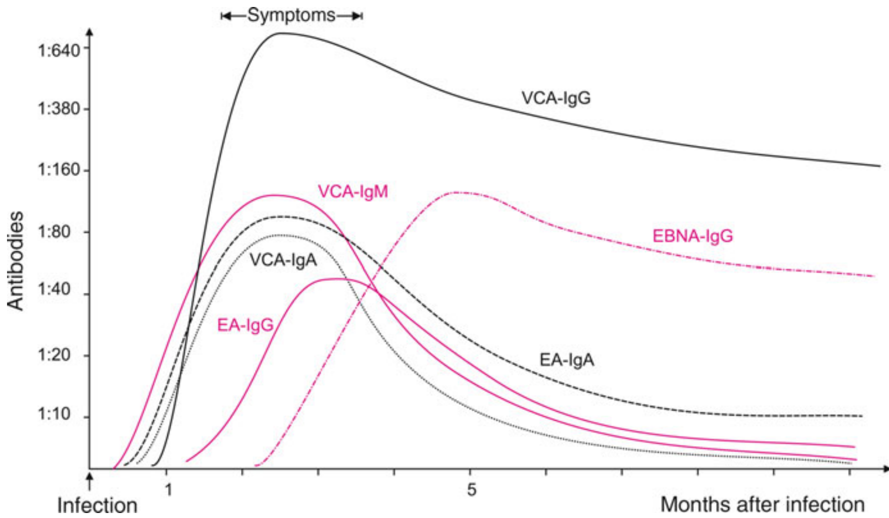


Fig. 19.32 Time course of antibody formation during an Epstein-Barr virus infection. The antibody classes (IgM, IgG and IgA) which can be detected by the immunofluorescence test at different times during a primary infection with Epstein-Barr virus are indicated. *EA* early antigen (early viral protein), *EBNA* Epstein-Barr virus nuclear antigen, *VCA* viral capsid antigen (structural protein)

antibodies are of particular importance also for determining the progression of the disease. Furthermore, their presence is a further clue to the causal involvement of Epstein-Barr virus in the pathogenesis of this tumour. The presence of viral genomes in B cells or free virus particles in the peripheral blood can be quantitatively detected by PCR in individuals with a fresh or reactivated infection.

The importance of cytotoxic T cells in controlling viral production during primary infection was described in the previous section. In this context, T lymphocytes which recognize immediate early protein epitopes and eliminate the infected cells from the organism before they can produce and release progeny viruses seem to be especially important. On the other hand, the T-cell response against latent viral proteins particularly prevents the uncontrolled proliferation of latently infected B lymphocytes.

Therapy and Prophylaxis

Although acyclovir is a good inhibitor of viral replication in vitro, its use has no significant effect on the clinical course of infectious mononucleosis and XLP syndrome. However, acyclovir, ganciclovir and cidofovir are therapeutically used in severely ill immunocompromised patients or PTLD patients, in combination with the greatest possible reduction of immunosuppression. Therefore, the therapy is primarily limited to the cancers that are caused by Epstein-Barr virus infection. Early diagnosed nasopharyngeal carcinomas (the importance of IgA antibodies and their early detection have already been pointed out) can be treated

very well by local irradiation or surgery. However, the chances of successful treatment are inversely correlated with increasing metastasis. Burkitt's lymphomas can be treated by chemotherapy. Vaccines against Epstein-Barr virus are not yet available.

19.5.7.6 Human Herpesvirus 8

Epidemiology and Transmission

In the autumn of 1994, Yuan Chang and co-workers described a previously unknown herpesvirus in tissues of Kaposi's sarcomas originating from AIDS patients. It is now known that the double-stranded DNA genome of human herpesvirus 8 comprises approximately 135,000 base pairs. Its genome organization is similar to that of saimiriine herpesvirus and Epstein-Barr virus. Because of phylogenetic sequence homologies, human herpesvirus 8 has been assigned to the genus *Rhadinovirus* in the subfamily *Gammaherpesvirinae*. The virus is present in all forms of Kaposi's sarcoma, even in tumours of patients who have not been infected with HIV. Human herpesvirus 8 is the causative agent of two other diseases: primary effusion lymphoma (PEL), also known as primary body-cavity-based lymphoma (BCBL), and the HIV-associated variant of multicentric Castleman's disease, a hyperplastic lymphoproliferative B-cell lymphoma. Diagnostic tests for the detection of antibodies against viral proteins and viral genomes have revealed that the population of the USA and western, northern and central Europe is hardly infected with human herpesvirus 8. On average, virus-specific antibodies can be detected – taking into account regional differences – in about 3–10 % of examined subjects. A similar situation probably applies to South America and Asia. The prevalence in the countries of the Mediterranean region (4–35 % with notable regional differences) and Africa (30–60 %) is significantly higher. Countries and regions with high prevalence also show a higher frequency of the classic form of Kaposi's sarcoma. Homosexual men in the USA and central Europe also have an increased risk of infection with human herpesvirus 8: virus-specific antibodies can be detected in the blood of 20–40 % of patients. In 50 % of cases, those who are infected with both human herpesvirus 8 and HIV develop a Kaposi's sarcoma within 5–10 years. Therefore, HIV infection can be considered an important cofactor for the development of this tumour.

The virus has been detected in infected individuals in modest concentrations in the semen and saliva. Epidemiological studies in highly endemic regions in Africa have revealed that children are infected with human herpesvirus 8 from the age of 2 years. In these cases, the virus seems to be transmitted by the close contact between family members, and is aggravated by the general poor hygiene and living conditions. In other regions, particularly certain risk groups (homosexual and HIV-infected men) are infected with human herpesvirus 8. In rare cases, the virus can also be transmitted by bone marrow and organ transplants.

Clinical Features

It is not known whether human herpesvirus 8 causes a disease during primary infection. In some cases, it has been shown that the primary infection is associated

with an increase of the levels of plasma cells in the blood, bone marrow and various tissues (plasmocytosis). All epidemiological data suggest that the virus is causally involved in the development of Kaposi's sarcoma. The classic form of Kaposi's sarcoma, which is endemic mainly in Africa and the Mediterranean region, can be distinguished from the epidemic forms, which are primarily found in HIV-infected subjects. The duration of the incubation period is unclear. Patients who are concurrently infected with HIV-1 and human herpesvirus 8 develop Kaposi's sarcomas within 5–10 years. In addition, human herpesvirus 8 has also been detected in PELs (primary BCBL), tumours that emerge in the late stage of AIDS, and in patients with multicentric Castleman's disease. The latter is a multicentric, angiogenic, follicular hyperplasia of the lymph nodes, which is accompanied with high probability by Kaposi's sarcoma.

Pathogenesis

Human herpesvirus 8 enters the organism probably by contact with mucous membranes. What cells are attacked in the early phase of infection has hardly been investigated. Small amounts of the virus can be detected in semen and in the salivary glands. The viruses that are present in the saliva probably originated also from B lymphocytes which are released from the tonsils and the lymphoid tissue of the submucosa. The virus can be cultivated in primary keratinocytes in vitro. Kaposi's sarcoma develops from vascular endothelial cells, from which the fibrocytes are infected with human herpesvirus 8. Presumably, some latent viral proteins are involved in the transformation process. Their functions are described in Sect. 19.5.5. Moreover, similar to cytomegalovirus, some of the viral gene products with homology to cellular growth factors, growth factor receptors and antiapoptotic proteins also contribute to strategies for the virus to evade the host immune response.

Immune Response and Diagnosis

IgG antibodies against LANA can be detected by immunofluorescence in sera from people infected with human herpesvirus 8. For this purpose, phorbol ester inducible cell lines from PEL are used as an antigen. Furthermore, there are Western blot tests and ELISAs to detect antibodies in which purified viruses or genetically engineered capsid and membrane proteins are used as the antigen. Viral DNA can be detected in mononuclear cells of the peripheral blood in only 20 % of individuals infected with human herpesvirus 8. The percentage of viral DNA detected in the blood cells increases with the progressive development of the symptoms associated with the infection. Nevertheless, detection of viral DNA in blood cells is not always reliable. Viral genomes are regularly detectable only in these tumour cells.

Therapy and Prophylaxis

To date, there is neither a treatment nor a vaccine for protection against infection with human herpesvirus 8. Productive viral replication can be inhibited with ganciclovir, cidofovir and phosphonoacetic acid, but the latent infection remains

unaffected. To date, there have been no clinical studies to answer the question of whether these antiviral substances may affect the disease.

19.5.8 Animal Pathogenic Herpesviruses

Animal pathogenic herpesviruses are represented in all subfamilies of the family *Herpesviridae*. Veterinarily relevant prototypes are found primarily within alphaherpesviruses: these include bovine herpesvirus 1, bovine herpesvirus 2, equine herpesvirus 1, equine herpesvirus 4, porcine herpesvirus 1 and feline and canine herpesviruses. In contrast, bovine herpesvirus 3 (alcephaline herpesvirus 1) is a gammaherpesvirus, and causes a severe disease in cattle, namely bovine malignant catarrhal fever.

Only a few animal cytomegaloviruses (betaherpesvirus) have been described. They are considered non-pathogenic, such as porcine cytomegalovirus, and have been scarcely investigated. An exception is murine cytomegalovirus. Fundamental facts on and insights into immunity to cytomegaloviruses have been ascertained with this system.

Koi Herpesvirus Infections Cause Severe Damage to Fish Farming

A herpesvirus emerged in koi carps for the first time in the 1990s which caused epidemic outbreaks with a high mortality. It soon became clear that farmed carps are susceptible to this virus, and can become severely ill. Cyprinid herpes virus 3, also known as koi herpesvirus, has been identified as the causative agent of this disease, and it has been assigned to the genus *Cyprinivirus* in the family *Alloherpesviridae*. Like all members of the order *Herpesvirales*, it induces a latent infection. Its optimal reproduction temperature is between 22 and 26 °C. The virus enters its host through gills, skin and gut, followed by systemic spread. The main symptoms are lethargy and occasional disorientation, skin lesions, exophthalmos and respiratory distress. Pathologically and anatomically, nephritis, necrosis of the gills and petechial haemorrhages in the internal organs are the principal consequences.

The epidemiological aspects of koi herpesvirus are not completely understood. It is currently found almost worldwide; it infects not only carps, but also numerous other fish species. There is even evidence that molluscs (snails) may be infected. Therefore, control of the virus in natural ponds is nearly impossible. Since routine detection of the virus is possible only by PCR analyses, and not by serological diagnosis, control of this viral infection in fish farms is very difficult. Frequently, only individual fish are tested, often giving a negative result; however, the symptoms of a general outbreak throughout the fish population (pond) can be observed later. Koi herpesvirus infection is an economically important disease in countries and regions with intensive carp farming. Therefore, this is a notifiable animal disease in many countries.

19.5.8.1 Bovine Herpesvirus 1

Epidemiology and Transmission

Bovine herpesvirus 1 causes diverse clinical pictures in cattle. Infectious bovine rhinotracheitis is a highly acute respiratory disease which especially affects young animals. Furthermore, diseases of the genital mucosa caused by bovine herpesvirus 1 have also been described, and are often associated with infertility, and are known as infectious pustular vulvovaginitis or infectious balanoposthitis. These manifestations are caused by different bovine herpesvirus 1 subtypes, which can be distinguished only by molecular biological methods.

The virus is transmitted especially by direct animal contact. Starting from a single animal with infectious bovine rhinotracheitis, the infection spreads rapidly in a susceptible herd, which is completely infected within a few days. The pathogen is frequently introduced into herds by apparently healthy carriers of the virus which intermittently excrete bovine herpesvirus 1. The virus induces a latent infection in sensory ganglia, such as the trigeminal ganglion. From there, it can be reactivated, replicating lytically in the mucosa. Immunosuppressive drugs such as cortisone can reproducibly induce reactivation. The immunity does not prevent latency, but only prevents the clinically manifest disease.

The genital manifestation of bovine herpesvirus 1 infection has been significantly reduced by increased breeding hygiene and selection of seronegative breeding animals. The virus can be transmitted by mating. Similarly, a virus-positive bull may pass the virus along with the preputial secretion in the semen collected for artificial insemination.

Clinical Features

Infectious bovine rhinotracheitis is an acute systemic infection in cattle that is primarily associated with respiratory symptoms. The disease often develops subclinically, but it may have a morbidity rate of nearly 100 % in some occasional cases. By contrast, the mortality rate is low. The symptoms of infectious bovine rhinotracheitis are fever, anorexia, severe respiratory dyspnoea with chest breathing, abundant salivation and coughing. Cases without secondary infections resolve completely after 5–10 days. An infected animal remains a virus carrier for life.

The genital manifestations, the infectious pustular vulvovaginitis or the infectious balanoposthitis, also result from a systemic infection. Fever and anorexia are principal symptoms as well. The mucous membrane lesions are painful, and this is manifested in an abnormal tail position. The mucosa is highly reddened, and is covered with multiple pustules. Infected bulls refuse to mate. In uncomplicated cases, complete recovery can also be expected after about 2 weeks.

Bovine Herpesvirus 2 Causes Mammilitis in Cattle

Bovine herpesvirus 2 (mammilitis virus) plays only a minor role as a pathogen in cattle. It causes an ulcerative dermatitis of the teats and udder and a generalized dermatitis, also known as pseudo-lumpy skin disease. It is

primarily transmitted by flies (*Musca* spp.). The virus is not very contagious. The diagnosis can be easily achieved by isolation of the virus in primary cell lines. The animals develop immunity, but a vaccine is not available.

Pathogenesis

The virus can be transmitted by mating during primary infection, or by droplet infection in the case of infectious bovine rhinotracheitis. After initial replication in fibroblasts of mucous membranes, the viruses infect B lymphocytes, which distribute them throughout the body. The most important pathogenetic event during primary infection is the infection of the trigeminal nerve, as well as the sciatic nerve and sensory ganglia. There, the virus establishes latency, during which viral protein synthesis and viral DNA replication are shut off. However, the viral genome is present as an episome in the nucleus. Normally, only LATs are detectable (Sect. 19.5.4); they possibly block the transcription of early genes. The stage of latency can be interrupted by external stimuli (stress), the virus returns to the lytic replication cycle and recurrences develop. The originally latently infected ganglion cell is destroyed, and neighbouring cells are infected. The virus migrates along the nerves to the mucosal tissues, where it replicates lytically, and is finally excreted.

Immune Response and Diagnosis

The immunity against animal pathogenic herpesviruses is generally less durable and resilient; therefore, re-infections after natural infection are the rule. It is assumed that an immunity lasts for about 1 year or less. Immunity prevents the development of clinical symptoms, but not the establishment of viral latency.

The diagnosis of clinically ill animals is not difficult; the virus can be easily isolated from nasal or vaginal swabs. Alternatively, the viral DNA can be detected by PCR. The infection can be verified serologically by detecting antibodies in the serum or milk. For this purpose, ELISAs containing purified virus or the surface proteins gB and gE as an antigen are routinely employed. The gB ELISA is currently regarded as the most sensitive test. Furthermore, neutralizing antibodies can be detected in the sera of infected animals as an indication of immunity. The monitoring of virus-free herds is usually performed by milk screening using whole-virus ELISA.

Control and Prophylaxis

Infections with bovine herpesvirus 1 are controlled in many European countries. Switzerland, Denmark and parts of Austria are virus free. In Germany, the eradication of the pathogen is also being intensively pursued. The motivation is less the economic losses that are directly caused by the disease, but rather the trade restrictions that are imposed by the virus-free countries of the European Union. Two strategies are being pursued: all cattle are tested for the presence of antibodies as an indication of a past bovine herpesvirus 1 infection. Seropositive animals are separated or removed. This is possible in slightly contaminated farms. If many

animals of a herd are infected, all seropositive cattle are vaccinated in order to induce and enhance an immunological reaction. These are able to suppress reactivation and excretion of the virus. Vaccinated animals are then gradually removed from the herd, and non-vaccinated, seronegative animals are permanently monitored. This strategy has proven to be successful, and has led to the virtual eradication of bovine herpesvirus 1 infection.

Alternatively, all animals are vaccinated in moderately infected herds. However, only marker vaccines based on inactivated viruses in which glycoprotein gE is deleted are used. The principle of this marker vaccine was developed for the vaccine against the virus of Aujeszky's disease (porcine herpesvirus 1, see later), and has been successfully used. It was subsequently adopted for the control of bovine herpesvirus 1 infection. Marker vaccines enable one to distinguish between vaccinated animals and those that are infected with the wild-type virus: cattle infected with bovine herpesvirus 1 have antibodies against the gE protein, whereas vaccinated animals do not. However, the fact that the gE protein is not immunodominant aggravates this distinction. Many cattle infected with bovine herpesvirus 1 develop only a weak gE-specific antibody response. Therefore, cattle with low gE-specific antibody levels may appear gE-negative in individual cases. The situation is additionally complicated by the fact that, according to animal health requirements, vaccinated gE-negative cattle are classified as virus-free and can be freely traded.

19.5.8.2 Porcine Herpesvirus 1 (Suid Herpesvirus 1)

Epidemiology and Transmission

Porcine herpesvirus 1, also known as pseudorabies virus, is the causative agent of Aujeszky's disease. It is an exception among herpesviruses because it has a broad host spectrum. Besides its main host, it also infects ruminants, carnivora and rodents. Horses and humans are not susceptible. As a prototype alphaherpesvirus, porcine herpesvirus 1 establishes a latent infection in ganglia cells of infected pigs. These swine are virus carriers for life, and can reactivate the virus. The virus is mainly excreted and transmitted by respiratory secretions. Piglets are usually infected intrauterine by the sows or during the first few days of life. Dogs and cats are especially infected when they are fed with raw meat from pigs infected with porcine herpesvirus 1. Like infected cattle, dogs and cats become severely sick very fast, so they generally do not play a significant role in disseminating the virus. However, infected rodents may carry the virus from farm to farm. Therefore, according to animal health regulations, disinfestation of the affected population is compulsorily required in the case of epizootic outbreaks. Aujeszky's disease has become very rare in western Europe because of the rigorous control of porcine herpesvirus 1 infection.

Clinical Features

The clinical features of porcine herpesvirus 1 infection are strongly dependent on the age of the affected pigs. Adult pigs develop either a subclinical form of infection or only mild symptoms. However, pregnant sows abort in most cases.

In contrast, young piglets suffer from severe central nervous system symptoms. They tremble, convulse and die within hours after the onset of symptoms. If piglets aged between 8 and 20 weeks are infected, they show mainly transient respiratory symptoms.

By contrast, cattle develop severe central nervous system symptoms, which were first described by the Hungarian veterinarian Aladar Aujeszky in 1902. Eventually, the disease was named after him. Even carnivores (dogs and cats) develop a severe, invariably fatal disease, which is known as pseudorabies (▶ Sect. 15.1.5), and can virtually not be distinguished from rabies.

Pathogenesis

The virus initially infects cells of the lymphoreticular tissue, usually the tonsils and Waldeyer's tonsillar ring at the entry site. From there the virus migrates along the nerves into the ganglia, and spreads within the central nervous system. There, it causes damage that is reflected in the pronounced clinical symptoms. The virus establishes a latent infection in the ganglia. Swine that survive the infection are lifelong virus carriers which often continuously excrete viruses.

Immune Response and Diagnosis

The immunity against herpesviruses is not pronounced, and porcine herpesvirus 1 is no exception. However, the life span of porkers is about 7 months, so the duration of immunity plays a minor role for most pigs. Diagnosis of porcine herpesvirus 1 infection is usually very simple by clinical examination of animals and the progression of the disease within the livestock; it can easily be corroborated by serological detection of virus-specific antibodies, or by detecting the virus or viral proteins in the serum. Moreover, detection of viral RNA is also possible by using the PCR technique.

Control and Prophylaxis

Aujeszky's disease is a notifiable viral infection. In the case of detection of one pig infected with porcine herpesvirus 1, the entire livestock must be culled.

This disease has been successfully controlled in western Europe. In this case, the strategy was similar to that employed for the control of bovine herpesvirus 1 infection: the main objective was the eradication of pigs that tested positive and the use of marker vaccines in which the gene for the viral gE protein is deleted. Because of the significantly shorter generation time of swine in comparison with cattle, pig farms that had pigs infected with porcine herpesvirus 1 were completely free of the virus within a few years. This status is checked by random blood tests for the presence of virus-specific antibodies.

19.5.8.3 Equine Herpesviruses 1 and 4

Epidemiology and Transmission

Equine herpesviruses 1 and 4 cause latent infections exclusively in horses; they are very similar, but can be clearly distinguished by serological and molecular biological criteria. Equine herpesvirus 1 causes abortion in mares, whereas equine

herpesvirus 4 induces rhinopneumonitis. However, as equine herpesvirus 1 can also cause respiratory infections in foals and yearlings, and equine herpesvirus 4 has also been isolated from aborted foals, it seems reasonable to consider both viruses in both manifestations. They are excreted through respiratory secretions, aborted foals and afterbirths. Once reactivated from latency, the viruses can rapidly spread within a livestock, leading to abortion storms in pregnant mares. Besides the classic clinical manifestations of abortion and respiratory symptoms, equine herpesvirus myeloencephalitis has also been described as a severe clinical picture. Recent studies have revealed that equine herpesvirus 1 strains isolated from diseased horses carry a specific mutation (N752) in the gene for the viral DNA-dependent DNA polymerase which is found in almost 90 % of cases of equine herpesvirus myeloencephalitis. This genotype, as well as the resulting pathological phenotype, is apparently responsible for the manifestation of equine herpesvirus myeloencephalitis. This mutation is apparently irrelevant for the ability of equine herpesvirus 1 to induce a miscarriage.

Clinical Features

Abortions induced by equine herpesviruses 1 and 4 usually occur between the seventh and eighth months of pregnancy, i.e. in the late phase of the equine gestation period, which lasts about 11 months. The foals are stillborn and exhibit signs of a generalized infection with multiple necroses in all organs.

Nevertheless, infections with equine herpesvirus 4 are usually mild or subclinical. Affected animals develop acute respiratory symptoms that disappear after a few days. However, both viruses may cause encephalitis in rare cases.

Pathogenesis

After initial replication in the lymphatic tissue of the nasopharyngeal region, the virus causes a cell-associated viraemia infecting endothelial cells of blood vessels in the central nervous system and in the uterus. The damage to the endothelium leads to hypoxia (oxygen deficiency) and dysfunctions of the central nervous system. Unlike infections with bovine herpesvirus 1 or porcine herpesvirus 1, equine herpesvirus does not replicate lytically in neurons, and the irreversible damage to neurons is caused by oxygen deprivation. The same pathogenic mechanism may induce detachment of the placenta in the gravid uterus, leading to subsequent abortion. Furthermore, the virus can be transmitted transplacentary to the fetus, damaging it irreversibly. These fetal infections are the cause of the characteristically late abortions in the last trimester of pregnancy.

Immune Response and Diagnosis

Diagnosis is easily performed by isolation of the virus from nasal swabs or from fetal tissue. Alternatively, detection of viral DNA from this material is also possible. Serological evidence can be obtained by detecting virus-specific antibodies. An ELISA that can distinguish antibodies against equine herpesviruses 1 and 4 is available. The immune response is extremely poor. The protection after natural infection is only of short duration.

Feline Herpesvirus Is a Possible Trigger of Cat Flu

Feline herpesvirus 1, feline calicivirus (► Sect. 14.6.6), and chlamydia are the causative agents of the symptom complex of "cat flu". The contribution of feline herpesvirus 1 infections to this disease has been estimated to be 10–50 %. In young cats, the infection manifests itself in the upper respiratory tract as rhinitis and conjunctivitis. In adult cats, mild or subclinical infections are commonly observed. Abortions have rarely been described. However, viruses have not been isolated from embryos or placental membranes so far. Subsequently, the virus establishes a latent stage in the trigeminal ganglia. Almost all cats have antibodies against the virus as a result of a previous infection or vaccination. The virus can be easily isolated from nose or throat swabs and detected by PCR. Vaccination with live attenuated vaccines or inactivated vaccines is possible. However, as is the case with all herpesvirus infections, the benefit is very limited.

Control and Prophylaxis

The available vaccines – live attenuated vaccines or inactivated vaccines – are not very efficient; therefore, booster vaccinations are recommended at regular intervals of 4 or 6 months.

19.5.8.4 Canine Herpesvirus 1

Epidemiology and Transmission

Canine herpesvirus 1 can induce a fatal disease in young puppies. The puppies are congenitally infected through virus-containing secretions in the birth canal or during the first few days of life.

Clinical Features

The whelps principally develop cough and other respiratory symptoms. They are associated with high neonatal mortality (whelp death). In adult dogs, the infection causes vaginitis or a balanoposthitis, but these are usually not diagnosed. Respiratory symptoms appear only occasionally. Therefore, canine herpesvirus is discussed as a possible causative agent of the symptom complex of "kennel cough".

Pathogenesis

In whelps, canine herpesvirus 1 multiplies in virtually all organs. Striking intranuclear inclusion bodies are characteristic in infected cells. Canine herpesvirus replication has a temperature optimum of 33–35 °C. The body temperature of dogs is normally about 38 °C, i.e. significantly higher. The thermoregulation of puppies is only effective from the age of 4 weeks. Before that age and even in poor housing conditions, they can fall into hypothermia, which facilitates viral replication in all organs. After primary infection, the virus establishes a latent infection in sensory ganglia.

Immune Response and Diagnosis

The animals produce virus-specific antibodies during the disease. Reactivation of the virus from latency is possible; however, the specific antibodies seem to diminish the frequency and extent of reactivation.

The virus can be isolated from diseased animals and can be cultivated in almost all canine cell lines at 35 °C. The detection of viral DNA is possible by PCR. Serological antibody detection has only limited significance owing to the ubiquitous spread of the virus.

Control and Prophylaxis

Neither a vaccine nor a therapy is available.

19.5.8.5 Gallid Herpesvirus 2

Epidemiology and Transmission

In chickens, gallid herpesvirus 2 causes Marek's disease, which is remarkable in many aspects. Before the introduction of vaccination, it led to huge enormous economic losses in poultry farms. In addition, the oncogenic potential of this herpesvirus has contributed to the fact that this infection is being investigated intensively, and many details are known about its molecular pathogenesis. The virus persists in feather follicles, and is excreted by them. Poultry are usually infected by inhalation of virus-containing feather dust. Subclinical infections are common; thus, healthy virus carriers play an important epidemiological role in the transmission of infection.

Clinical Features

Gallid herpesvirus 2 causes diverse clinical pictures. The classic Marek's disease manifests itself as a paralysis of legs and wings. This is attributed to an impairment of innervating nerves in these regions. Morphologically, the perimeter of the sciatic nerve appears substantially increased. Acute Marek's disease is different. It leads to deaths before the development of neurological symptoms. Furthermore, the infection can also induce the ocular and cutaneous forms of Marek's disease, which produce characteristic symptoms in the eyes (depigmentation of the iris, grey eye) and skin (leucocyte-infiltrated feather follicles, skin leucosis).

Pathogenesis

Gallid herpesvirus 2 infects T lymphocytes of fowl. These cells spread the virus throughout the organism, and are transformed, immortalized and functionally altered by this viral infection. The viral genome is integrated in the chromosomal DNA of host cells. This virus is extremely cell associated. Free virus particles can virtually not be isolated; they are present only in modest quantities in the quills and feather follicles. Certain chicken lines seem to possess a genetic resistance against the infection, which is probably associated with the B21 alloantigen.

The genomes of some strains of gallid herpesvirus contain sequences that are similar to v-*onc* genes of chicken retroviruses, or to the corresponding cellular

oncogenes (▶ Sect. 18.1.6). This might explain the oncogenic potential of the virus. Infected animals develop multiple lymphoid tumours in practically all organs, especially thickening of the peripheral nerves due to metaplasia of lymphoblastoid cells (plexus cervicalis, plexus brachialis, plexus sacralis; sciatic nerve).

Immune Response and Diagnosis

Usually, diagnosis is performed clinically on the basis of the characteristic lesions.

Control and Prophylaxis

Several avirulent vaccine strains are available; however, the very closely related turkey herpesvirus is usually selected as a vaccine virus as it can be distinguished from gallid herpesvirus.

Herpesviruses of Poultry

Herpesviruses cause enormous economic losses in poultry, especially in chickens and ducks. In principle, poultry herpesviruses display the classic properties of all herpesviruses, such as latency and reactivation. Gallid herpesvirus 1 is the causative agent of infectious laryngotracheitis, which occurs globally and can cause severe diseases in infected chickens with a morbidity of 100 % and a mortality of 20–70 %. The clinical picture is characterized by a massive dyspnoea (breathlessness), which is based on a diphtheroid inflammation of the mucous membranes of the nasopharynx. Diagnosis is easily done by immunofluorescence or isolation of the virus from sick chicken. The virus spreads rapidly within a flock. Immunization with attenuated live vaccines is possible, but a drastic “all in–all out” management is more reasonable.

Duck plague is an acute infection of water fowl (ducks, geese, swans, etc.) which is caused by duck herpesvirus and is associated with a mortality of up to 100 %. The clinical picture is characterized by haemorrhagic enteritis and haemorrhages in all body cavities. The virus is introduced by migratory birds, which excrete the virus in their faeces. The virus persists in surviving birds, and is found worldwide.

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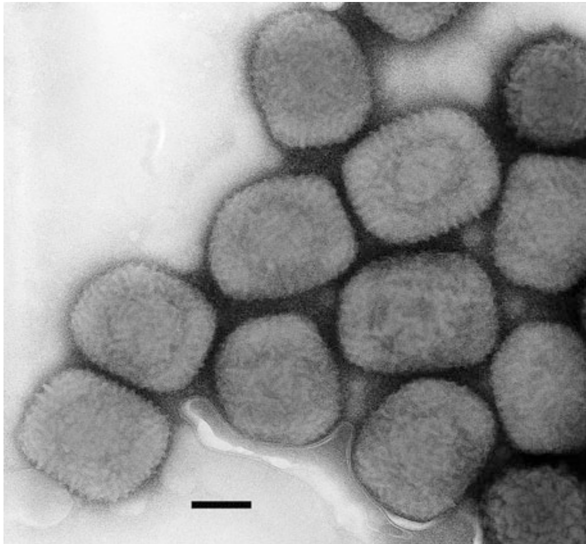
19.6 Poxviruses



The family *Poxviridae* comprises a large number of viruses which cause infections and diseases in mammals, birds and insects. Poxviruses have a very complex structure. The infection caused by the human pathogenic variola virus is the oldest known infectious disease (smallpox or variola vera) of humans (▶ Chap. 1). Important discoveries on the pathogenesis of infectious diseases were initially made with human smallpox virus without precise knowledge of the agent or the biology of infection. The first experimental vaccinations with a cowpox virus, which were performed by Edward Jenner in England at the end of the eighteenth century, became famous, and were used worldwide. Jenner named this virus vaccinia virus. However, variolation was already practised in China for prevention of infections in pre-Christian times, i.e., the cutaneous inoculation of fresh or dried smallpox pustules containing the human pathogenic smallpox virus. In 1958, the World Health Organization initiated a global vaccination programme. The aim, the eradication of variola virus and the associated smallpox disease, was achieved in 1977. Human smallpox was declared eradicated in 1979; vaccination was discontinued, and has not been required by law since then. The vaccine virus used was vaccinia virus, which was originally used by Jenner. A later molecular characterization of vaccinia viruses revealed that these viruses do not correspond to cowpox virus, which was presumably used by Jenner. Their origin remains uncertain. They are closely related neither to variola virus nor to milker's nodule virus or cowpox virus. Vaccinia virus is probably an extinct animal poxvirus which was multiplied and selected over many passages during the production of the vaccine virus.

19.6.1 Classification and Characteristic Prototypes

The family *Poxviridae* is divided into two subfamilies: *Chordopoxvirinae* comprises the poxviruses of vertebrates; *Entomopoxvirinae* comprises those of insects (Table 19.20). The subfamily *Chordopoxvirinae* contains ten genera whose members are similar in regard to morphology and host tropism. The nucleoproteins that bind to the viral DNA are also serologically related. However, the viral genomes of different genera do not hybridize to each other. The genus *Orthopoxvirus* includes the extinct variola virus, the vaccinia virus that was used as a vaccine virus and several animal pathogens with, in part, zoonotic potential. Some of them cause characteristic diseases in their hosts. Cowpox viruses, which are actually common in rodents, and are transmitted relatively frequently to cats, in which they can cause skin lesions, through which humans can be infected, are important zoonotic pathogens. Cowpox virus has recently been transmitted to humans in several cases by close contact with infected white pet rats ("cuddly rats"), which are kept as domestic animals. The virus caused localized papules, pustules and ulcers, which usually heal with scars, and are accompanied by regional lymph node swelling and general symptoms. Other orthopoxviruses or capripoxviruses cause systemic diseases in their hosts which are associated with high morbidity and sometimes high mortality, such as monkeypox, sheeppox, goatpox and lumpy skin disease in cattle.

Parapoxviruses are found worldwide and infect a variety of ruminants, such as sheep, goats, cattle and wild ruminants; they are rarely found in other animal species such as seals. They are also known as zoonotic pathogens and can occasionally cause human diseases, such as milker's nodules that are caused by orf virus along with other poxviruses. The poxviruses of insects and their larvae are classified into the subfamily *Entomopoxvirinae*, which is divided into three genera: *Alphaentomopoxvirus*, *Betaentomopoxvirus* and *Gammaentomopoxvirus*. Their molecular properties have scarcely been investigated.

Vaccinia Virus – An Expression System in Gene Engineering Today

Vaccination against smallpox was discontinued in 1979 because the risk of developing a rare side effect (postvaccinal encephalitis) was greater than that of becoming infected with smallpox. Vaccinia viruses are predominantly used as vectors for expression of heterologous genes in eukaryotic cells. Because of the long-term use of vaccinia viruses in smallpox vaccination, considerable experience has been gained in dealing with the virus as a live vaccine. Furthermore, its pathobiological properties have been very well studied. Therefore, there have been attempts to produce recombinant vaccinia viruses to generate immunological protection against certain infectious agents and tumours. For this purpose, a foreign gene which encodes a protein that is essential for the generation of a neutralizing immune response (e.g., the HIV gp120/gp41 membrane protein complex or the

G protein of rabies virus) is cloned into the vaccinia virus genome. The pertinent gene is cloned into a region of the genome that is not essential for viral genome replication. The heterologous protein is then expressed in vaccinated subjects during viral replication, leading to the generation of an immune response. In several countries such recombinant vaccinia viruses are licensed in veterinary medicine as vaccines against rabies and rinderpest.

The recombinant modified vaccinia virus Ankara (MVA) has a special status. A Turkish dermovaccinia virus strain has been attenuated so much by more than 500 passages in chicken fibroblasts that the virus is no longer capable of producing infectious progeny viruses in human cells. In humans and animals, it is not contagious, and thus has only a slight virulence. Hence, this expression vector can be applied parenterally and locally, especially by oral and intracutaneous application.

19.6.2 Structure

19.6.2.1 Virus Particle

Poxviruses belong to the largest known viruses. They have an ovoid, brick-like or pleomorphic shape and an overall size of approximately 300 × 200 × 100 nm (Fig. 19.33a, b). Four virion types are formed during infection, and they differ in regard to their structure, function, localization and quantities in which they are synthesized in the infected cell. Virions known as intracellular mature viruses (IMVs) or mature viruses are found in the cells. They have an envelope composed of a lipid bilayer in which more than 12 different non-glycosylated viral proteins are embedded. This envelope encloses a biconcave core capsid, which consists of the linear viral genome that is folded into an S-shaped structure in close association with proteins. Two lateral bodies of unknown function are accommodated in both concavities. Cryo-electron microscopy images of poxvirus particles show that these lateral bodies may be merely preparation artefacts. IMV particles are released by cell lysis and are infectious. They spread the infection in the organism as well as from one host to another. Some IMV particles are surrounded by two additional envelopes in infected cells: the outer envelope contains at least nine viral proteins: these particles are called wrapped virions or intracellular enveloped viruses (IEVs). IEV particles bind to cellular microtubules and move along them to the cell surface, where the outermost of the three envelopes fuses with the cytoplasmic membrane. This process leads to the emergence at the cell surface of cell-associated enveloped viruses (CEVs), which are surrounded by two lipid bilayer envelopes and are further transported outside the cell by growing actin filaments. The CEV particles are used for the transmission of infection from cell to cell. The extracellular virions, or extracellular enveloped viruses (EEVs), are identical to the CEVs; however, they have detached from the cell surface and are present as free virus particles, which are

Table 19.20 Characteristic prototypes of poxviruses

Subfamily	Genus	Human virus	Animal virus
***Chordopoxvirinae* (poxviruses of vertebrates)**	*Orthopoxvirus*	Variola virus Vaccinia virus	Cowpox virus
			Monkeypox virus
			Camelpox virus
			Ectromelia virus (mice)
			Raccoonpox virus
	Parapoxvirus		Pseudocowpox virus
			Orf virus
			Bovine papular stomatitis virus
	Avipoxvirus		Canarypox virus
			Pigeonpox virus
			Fowlpox virus (chicken)
			Turkeypox virus
			Quailpox virus
			Parrotpox virus
	Capripoxvirus		Goatpox virus
			Sheeppox virus
			Lumpy skin disease virus
	Leporipoxvirus		Myxoma virus
			Hare fibroma virus
			Rabbit fibroma virus
			Squirrel fibroma virus
	Suipoxvirus		Swinepox virus
	Cervidpoxvirus		Mule deerpox virus
	Crocodylidpoxvirus		Nile crocodilepox virus
	Molluscipoxvirus	Molluscum contagiosum virus	
	Yatapoxvirus	Tanapox virus	Tanapox virus
		Yaba monkey tumour virus	Yaba monkey tumour virus
***Entomopoxvirinae* (poxviruses of insects)**	*Alphaentomopoxvirus*		*Anomala cuprea* entomopoxvirus (scarab)
			Melolontha melolontha entomopoxvirus (maybug)
	Betaentomopoxvirus		*Locusta migratoria* entomopoxvirus ‘O’ (migratory locust)
			Amsacta moorei entomopoxvirus ‘L’ (fall webworm)
			Heliothis armigera entomopoxvirus ‘L’ (bollworm)

(*continued*)

Table 19.20 (continued)

Subfamily	Genus	Human virus	Animal virus
	Gammaentomopoxvirus		*Aedes aegypti* entomopoxvirus
			Chironomus luridus entomopoxvirus (non-biting midge)

important for dissemination of the pathogen in the infected host, and from host to host. Poxvirus particles have a clearly structured surface (Fig. 19.33). Most particles have channel-like, filamentous structures; however, parapoxviruses exhibit a beehive-like arrangement of filaments on their surface (Fig. 19.33c). Four glycosylated viral proteins and one non-glycosylated viral protein are embedded in the envelope of EEVs, and differ from those found in the IMV envelope. The core of EEVs is surrounded by a double envelope, of which the outer envelope originates from membrane compartments of the *trans*-Golgi network.

19.6.2.2 Genome Organization and Structure

The genome of poxviruses is composed of linear, double-stranded DNA with a length of 137–139 kpb in orf virus (a parapoxvirus). Avipoxviruses contain more than 350,000 base pairs (359,853 base pairs in canarypox virus). Both DNA ends are covalently connected by terminal loops. The genome of vaccinia virus comprises approximately 190,000 base pairs (191,738 base pairs in the strain "Copenhagen"). The ends of the genome of vaccinia virus contain many tandem repeats of 70 base pairs in length (Fig. 19.34a). The DNA sequences of both termini are identical to each other and are arranged in opposite orientation. Therefore, they are referred to as inverted terminal repeats. The size of the inverted terminal repeats is variable and depends on the virus type. Their length ranges from 725 base pairs in variola virus to 10,500 base pairs in vaccinia virus.

The genome of poxviruses accommodates a total of 150–200 genes in both DNA strands. Forty-nine of them are conserved; they have been found in all sequenced genomes of poxviruses. Members of the subfamily *Chordopoxvirinae* have about 90 conserved genes, which code for transcription factors, enzymes and structural proteins that are essential for genome replication and the viral reproduction cycle. These essential genes reside preferentially in the centre of the genome, whereas the non-conserved virulence genes are localized at the two termini (Fig. 19.34b). They code for gene products that enable the virus to evade the defence mechanisms of the hosts, and affect the induction of apoptosis, antigen presentation and recognition, the effect of interferon and signal cascades that are triggered by immune-stimulatory cytokines. Adjacent open reading frames rarely overlap. They are separated by very short non-coding intergenic sequences. The genes have no introns, and the promoters are relatively short in comparison with classic eukaryotic control elements. The early and late transcribed genes are arranged in clusters in the same orientation.

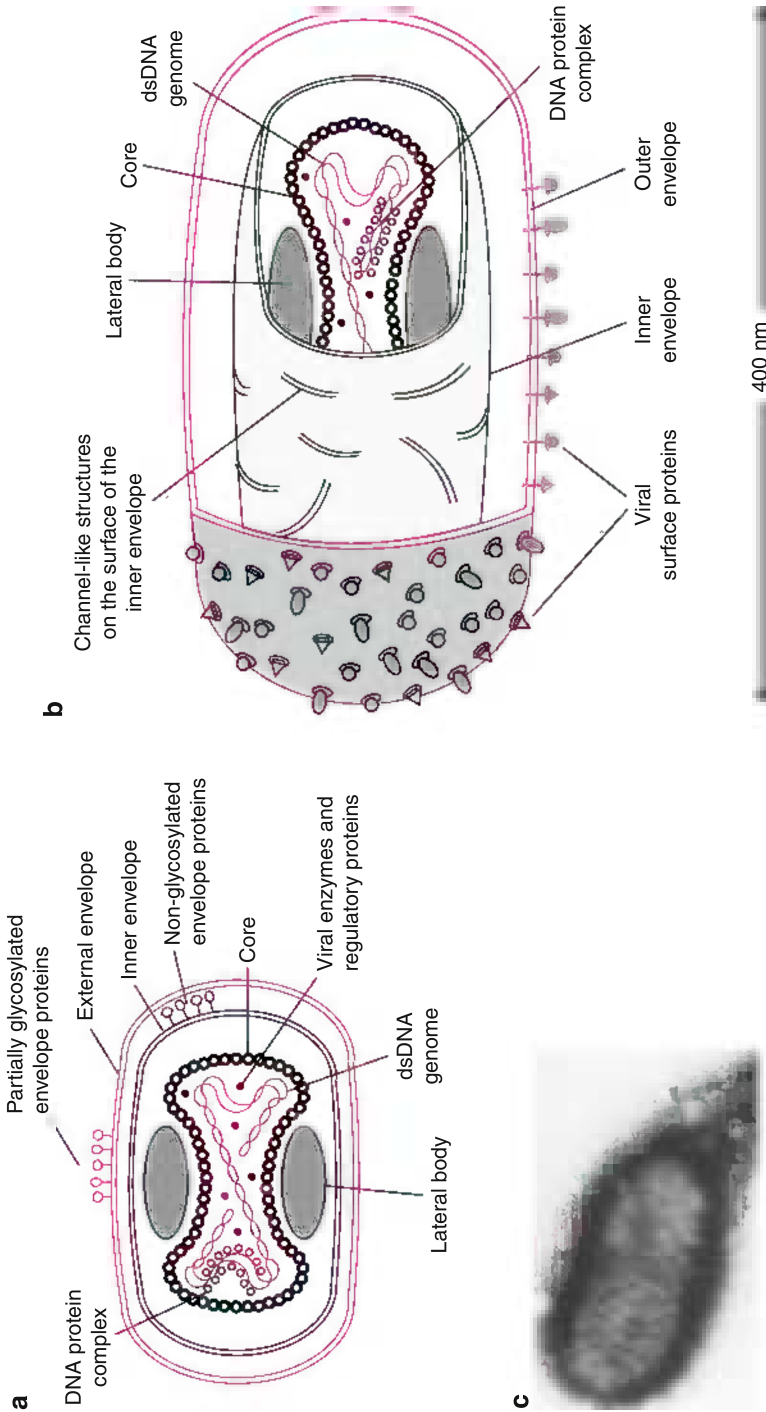


Fig. 19.33 Structure of a poxvirus particle. (**a**) Cross-section of the extracellular form of a poxvirus particle. The DNA genome resides inside the core in interaction with viral proteins and is present in an S-shaped complex. The nucleocapsid is surrounded by a core capsid shell, which in turn is enclosed by a double lipid envelope. The function and composition of the lateral bodies are uncertain; presumably, they are artefacts that arise during preparation of the

The following convention is used to denominate the different reading frames. Digestion of the vaccinia virus genome with the restriction enzyme *Hin*dIII yields 16 fragments, which are designated according to their size as A to P. Open reading frames in the individual fragments are numbered, and the direction of transcription is specified with L or R. For example, the haemagglutinin gene is called A56R, i.e., it is the 56th open reading frame within *Hin*dIII fragment A, and is directed rightwards.

19.6.3 Viral Proteins

Poxviruses have a complex structure; the virions comprise more than 70 different structural proteins, as well as enzymes and regulatory polypeptides that are not yet known in detail. Only vaccinia virus has been relatively well studied. Therefore, all data and facts that will be specified in the following sections refer to this virus.

19.6.3.1 Structural Proteins

The outer envelopes of CEVs and EEVs contain at least nine different glycosylated proteins of various molecular masses and one non-glycosylated protein (Table 19.21). Other membrane-anchored proteins are components of intracellular IEV particles and the inner envelope, which surrounds the IMV. The latter are non-glycosylated. Virus-neutralizing antibodies are formed against proteins that are embedded in the outer envelope and also against some polypeptides of the inner envelope. Several envelope proteins of IMV and EEV particles are involved in binding to the cell surface, and in the fusion between the viral envelope and the cytoplasmic membrane.

Analyses of the viral core have revealed that it is also composed of several different proteins. Some of them are synthesized as large precursor proteins (P4a, P4b, P4), which are subsequently converted by proteolytic cleavage into the mature polypeptides that are found in the infectious vaccinia virus particle (Table 19.21). Several viral proteins are associated with the DNA genome. They are responsible for the folding of the DNA molecule into a supercoiled structure.

19.6.3.2 Enzymes

Several viral enzymes are expressed during infection. In part, they are also components of the virus particle. In some cases, it is unclear whether they are needed in viral morphogenesis as enzymatically active proteins (proteases, kinases) or

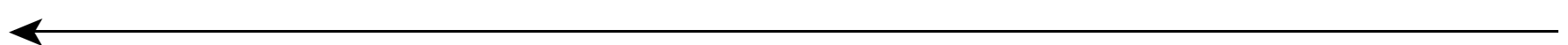


Fig. 19.33 (continued) virus particles for electron microscopy. This poxvirus particle form is infectious. (**b**) Extracellular form of a poxvirus in sagittal section. The components in the interior of the virus particle, including the inner envelope, correspond to those described in (**a**). Striking channel-like structures are present on the surface of the inner envelope, and are formed by viral membrane proteins. The virus particle is surrounded by an additional outer envelope, which also contains viral proteins. This poxvirus particle type is also infectious. (**c**) Electron micrograph of an orf virus, genus *Parapoxvirus*. *dsDNA* double-stranded DNA (Kindly provided by O.-R. Kaaden, Institute of Medical Microbiology, Ludwig Maximilian University, Munich, Germany)

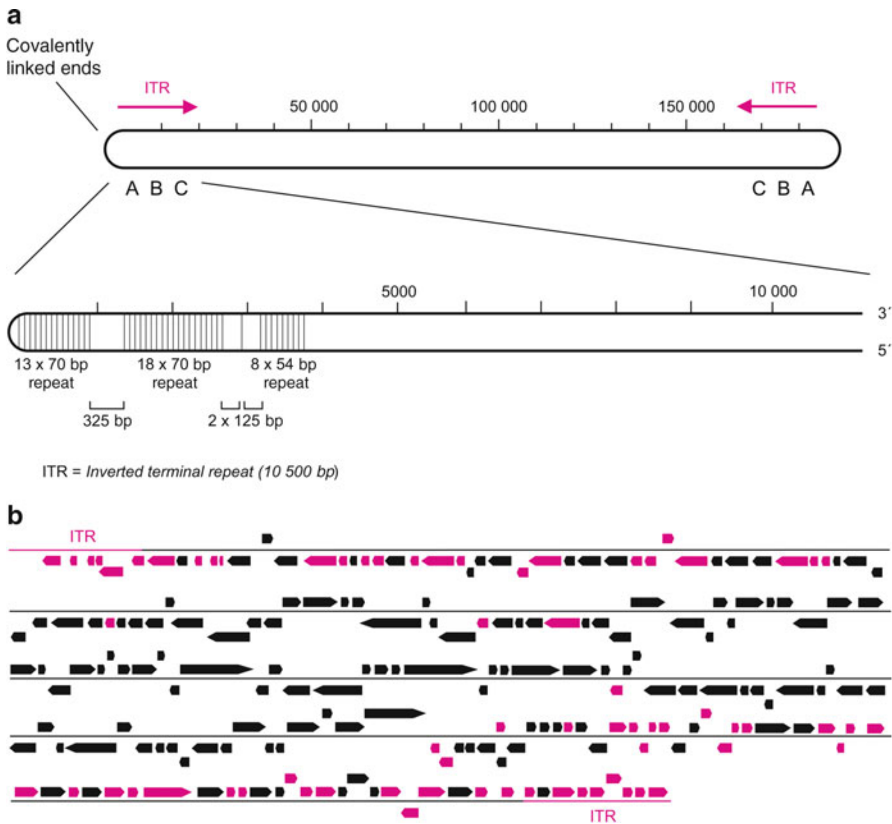


Fig. 19.34 (**a**) Genome structure of poxviruses (vaccinia virus). The genome consists of double-stranded DNA, whose termini are covalently linked by terminal loops. The ends of the genome contain inverted terminal repeats (*ITR*), which are composed of several tandem repeat units. (**b**) *Arrowheads* indicate the position and transcription direction of vaccinia virus genes. *Red* poxvirus-specific virulence genes, *black* essential genes that are conserved among the different poxviruses

chaperones, or whether they are non-specifically incorporated into the resulting virions during assembly. Since poxviruses are the only DNA viruses that are able to replicate in the cytoplasm of infected cells, they do not need to use the cellular replication machinery in the nucleus. Therefore, they harbour the genetic information for many enzymes which are involved in DNA replication, transcription, RNA modification and nucleic acid metabolism, and are introduced into the cell as components of the infectious virus particles. This applies, for instance, to all enzymes involved in the synthesis of viral mRNAs and their post-transcriptional modification (Table 19.22). Hence, the viral RNA polymerase and the capping enzymes are also components of the virions. In addition to their own RNA polymerase, which consists of several subunits, poxviruses also encode, among other things, their own polyadenylation enzymes, and enzymes that are responsible for the synthesis of cap groups at the 5′ termini of their mRNA molecules.

Table 19.21 Selected structural proteins of vaccinia virus

Localization	Locus (vaccinia virus)	Features	Molecular mass (kDa)	Function
EEV/CEV	A56R	N- and O-glycosylation	34	Envelope protein; haemagglutinin
IEV/EEV/CEV	F12L	N-glycosylation	73	Envelope protein; binding to microtubules; transport of IEV to the cell surface
EEV/CEV	F13L	Non-glycosylated, palmitoylated	42	Membrane-associated protein; member of phospholipase D superfamily
EEV/CEV	B5R	N- and O-glycosylation	35 42/glycosylated	Envelope protein; CD46 homologue; protection from complement lysis; virulence factor
EEV/CEV	A33R	Non-glycosylated	21	Envelope protein; involved in binding to actin cables
EEV/CEV	A34R	N-glycosylation	20 24–28/glycosylated	Type II membrane protein; similarity with C-type lectins, involved in binding of CEV to actin cables, causes the CEV particles to remain associated with the cell
IEV/EEV/CEV	A36R	N-glycosylation	25	Envelope protein; binding to microtubules; transport of IEV to the cell surface
EEV/CEV	K2L	?	42	Serpin (serine protease inhibitor)
EEV/CEV	E2L	?	86	Envelope protein
IMV	A26L	Non-glycosylated		Protein 4c; binding to laminin
IMV	A27L	Non-glycosylated	14	Homotrimer; attachment, binds to heparan sulphate on the cell surface; penetration, induces formation of neutralizing antibodies
IMV	A28L	Non-glycosylated	14	Constituent of the channel-like surface structures; homotrimer; induces formation of neutralizing antibodies and is involved in the fusion between IMV and the cytoplasmic membrane after attachment
IMV	L1R	Non-glycosylated, myristoylated	28	Most abundant type I membrane protein; induces formation of neutralizing antibodies; participates in the fusion between IMV and the plasma membrane after attachment
IMV	L5R	Non-glycosylated	15	Particle incorporation; fusion between IMV and the cytoplasmic membrane following attachment
IMV	D8L	Non-glycosylated	32	Attachment; sequence similarity with carbonic anhydrase; binds to

(*continued*)

Table 19.21 (continued)

Localization	Locus (vaccinia virus)	Features	Molecular mass (kDa)	Function
				chondroitin sulphate on the cell surface; induces formation of neutralizing antibodies
IMV	H3L	Non-glycosylated	37	Attachment; binds to heparan sulphate on the cell surface; induces production of neutralizing antibodies
IMV	A14L	Phosphorylated	10	Morphogenesis; dimer; interacts with A17L
IMV	A17L	Phosphorylated	33	Morphogenesis; dimer; interacts with A14L
Matrix	A30L		9	Matrix between core and envelope of IMV; morphogenesis
Core	A3L		74	Precursor protein P4b → core protein 4b
Core	A4L		39	Core protein
Core	A10L		102	Precursor protein P4a → core protein 4a
Core	A32L		35	ATPase; involved in packaging of viral genomes into the particles
Core	L4R		25	Core protein
Nucleocapsid	I1L	DNA binding	36	
Core	I7L		50	Protease
Core/ nucleocapsid	F17R	Phosphorylated, DNA binding	11	Localized between nucleocapsid and envelope

CEV cell-associated enveloped virus, *EEV* extracellular enveloped virus, *IEV* intracellular enveloped virus, *IMV* intracellular mature virus

Furthermore, they also have a uracil-DNA glycosylase that removes incorrectly incorporated uracil residues from DNA molecules. Apparently, poxviruses possess DNA polymerases with proofreading activity to enhance the accuracy of DNA synthesis, and repair systems that remove mispairing nucleotides. Similar enzymatic activities (uracil-DNA glycosylase and dUTPase) have been found only in herpesviruses so far (Sect. 19.5.3). Furthermore, poxviruses possess a protein with photolyase activity which recognizes and repairs cyclobutane pyrimidine dimers and other photoproducts that are formed between adjacent pyrimidine nucleotides in a DNA molecule by the action of ultraviolet light.

19.6.3.3 Accessory Proteins

The large coding capacity enables poxviruses to modulate their virulence in order to be optimally adapted to their host cells and organisms. Virulence genes are often similar to cellular genes, and are mainly located at the ends of the linear viral genomes.

Table 19.22 Important poxvirus enzymes (vaccinia virus)

Enzyme	Function	Molecular mass	Locus (vaccinia virus)
DNA-dependent RNA polymerase (component of the virion)	Transcription; synthesis of viral mRNAs; Mn^{2+}-dependent	>500 kDa, holoenzyme complex composed of several subunits:	
		147 kDa	J6R
		132 kDa	A24R
		36 kDa	A29L
		30 kDa	E4L
		22 kDa	J4R
		20 kDa	A5R
		18 kDa	D7R
		7 kDa	G5.5R
RNA/DNA helicase (component of the virion)	Transcription	78 kDa	I8R
		72 kDa	D11L
DNA helicase	Transcription	57 kDa	A18R
Poly(A) polymerase (component of the virion)	Polyadenylation of 3′ ends of mRNAs; primer-dependent	~80 kDa; complex of 2 subunits:	
		55 kDa	E1L
		38 kDa (identical with RNA nucleoside methyltransferase)	J3R
Capping enzyme complex (virion component)	Cap structure formation at the 5′ end of mRNAs		
	RNA guanylyltransferase	97 kDa	D1R
	RNA (guanine-7)-methyltransferase	33 kDa	D12L
	RNA (nucleoside-2′)-methyltransferase	38 kDa	J3R
Nucleoside triphosphate phosphohydrolase I (virion component)	ATPase; ATP/dATP to ADP/dADP + Pi	35 kDa	A32L
Nucleoside triphosphate phosphohydrolase II	NTPase; NTP/dNTP to NDP/NDP + Pi	37 kDa	D5R
DNA topoisomerase (part of the virion)	Replication of the double-stranded DNA genome; topoisomerase I; relaxes DNA supercoiling	117 kDa	H6R
DNA polymerase	Replication of the double-stranded DNA genome	49 kDa	E9L
Processivity factor	Replication of the double-stranded DNA genome	63 kDa	A20R

(*continued*)

Table 19.22 (continued)

Enzyme	Function	Molecular mass	Locus (vaccinia virus)
DNA ligase	Replication; formation of the covalently closed ends of the genome	20 kDa	A50R
Thymidine kinase	Phosphorylation of thymine nucleosides to TMP; nucleotide metabolism	24 kDa	J2R
Thymidylate kinase	Phosphorylation of TMP to TTP; nucleotide metabolism	24 kDa	A48R
Guanylate kinase	Phosphorylation of GMP to GTP; nucleotide metabolism	21 kDa	A57R
Ribonucleotide reductase	DNA replication; nucleotide metabolism	Complex composed of 2 subunits:	
		87 kDa	I4L
		37 kDa	F4L
Resolvase	Part of the repair system; resolves Holliday structures after recombination repair	21 kDa	A22R
Nicking-joining enzyme	Endonuclease/ligase; repair system; replication	49 kDa	K4L
Uracil-DNA glycosylase	Component of the repair system; removes uracil residues from DNA	25 kDa	D4R
dUTPase	Component of the repair system; hydrolysis of dUTP residues	16 kDa	F2L
Protease	Metalloprotease	68 kDa	G1L
Protein kinase 1	Phosphorylation of serine and threonine residues	52 kDa	F10L
Protein kinase 2	Phosphorylation of serine and threonine residues	34 kDa	B12R
Protein kinase 3	Phosphorylation of serine residues	35 kDa	B1R

P_i inorganic phosphate

The molecular processes that have led to the incorporation and integration of host genes into the viral genome are not well understood. Many of these genes are defective and are not expressed owing to the presence of stop codons that have been introduced into the sequence. It is not possible within the scope of this book to discuss all the known pertinent data. Interested readers will find appropriate descriptions in the additional literature listed in "Further Reading".

Nonetheless, some of the mechanisms that allow poxviruses to evade the immune response of the host will be briefly mentioned. A protein with a molecular mass of 38 kDa, referred to as CrmA, has been identified in various poxviruses, and is encoded by the B13R open reading frame of the vaccinia virus strain WR. This polypeptide is a protease inhibitor that blocks the activity of IL-1β-converting enzyme (ICE), a cellular cysteine protease which is not only required for proteolytic

activation of IL-1β, but is also important for induction of apoptosis. Apoptosis is one of the mechanisms through which virus-infected cells are eliminated from the host organism. Among other things, it is induced by TNFs, which are expressed during the host immune response. These factors bind to TNF receptors and Fas receptors, and induce apoptosis by triggering a signal transduction pathway. ICE seems to be one of the components that are essential for inducing apoptosis. Poxviruses block ICE activity by the specific inhibitor function of CrmA, thus evading apoptosis. This viral protease inhibitor is an important virulence factor. In addition, poxviruses also have some proteins, called serpins, which act as inhibitors of serine proteases. These include the IEV/CEV envelope-associated protein that is encoded by the K2L open reading frame of vaccinia virus and the Serp1 protein of myxoma virus. Viral serpins exert anti-inflammatory and anticoagulant effects by inhibiting serine proteases produced by thrombocytes, which should prevent infection and invasion by the virus.

Moreover, poxviruses encode several virokines. These are factors that are homologous to cytokines, and are secreted by infected cells. These proteins function as antagonists of cytokines synthesized during the host immune response. Furthermore, some viral gene products have been found to inhibit cytokine release in infected cells. Additionally, poxviruses also express secretory proteins which bind to cytokines and inhibit their functions. For example, myxoma viruses express the MT7 protein (37 kDa), which is a homologue of the IFN-γ receptor that is released into the environment, and prevents binding of this cytokine to membrane-anchored interferon receptors on the cell surface. Similarly, vaccinia viruses express a soluble receptor (B18R) for IFN-α and IFN-β which is secreted by infected cells and intercepts and neutralizes circulating interferons. Further, vaccinia viruses encode two additional gene products which also prevent the interferon response. The E3L protein binds to double-stranded RNA and prevents the activation of double-stranded-RNA-dependent enzymes, protein kinase R and 2′–5′-oligoadenylate synthetase (▶ Chap. 8). In addition, E3L prevents the IRF-dependent induction of interferon synthesis and the RNA-editing function of the interferon-induced RNA-specific adenosine deaminase ADAR1. This enzyme deaminates adenosine residues to inosine in RNA molecules and is involved, among other things, in RNA editing (Sect. 19.1.6). The viral E3L protein possesses a domain that is homologous to the RNA-binding region of ADAR1. It binds by this domain to RNA molecules, thus preventing their association with the cellular enzyme. In contrast, the K3L protein serves as a substrate for the interferon-induced protein kinase R. This prevents the phosphorylation of eIF2a and the resulting inhibition of translation. Furthermore, vaccinia virus encodes viral chemokine-binding proteins (vCKB proteins) that inhibit the action of cellular chemokines. For example, vCKBP-2 (35 kDa), also known as viral CC chemokine inhibitor, interacts with amino acid residues in the CC chemokine MCP-1 (▶ Chap. 8), which are required for interaction with the CC receptor CCR2B. Viral CC chemokine inhibitor blocks binding of MCP-1 to the receptor, thus impairing the function of the chemokine. Vaccinia and cowpox viruses possess a 33-kDa protein that resembles the type II IL-1 receptors. This polypeptide is also

secreted by infected cells; it associates with IL-1β and prevents its action. Furthermore, secreted homologues of TNF receptors have also been described. Poxviruses are also able to counteract the effect of the complement system. The C21L and B5R open reading frames of vaccinia virus encode proteins that resemble complement receptors. Components C3b and C4b bind to them, interrupting the activation of the complement cascade (▶ Chap. 7). Similar mechanisms have been found only in herpesviruses. Moreover, poxviruses have EGF-like proteins that promote proliferation of infected cells by an autocrine stimulation pathway.

19.6.4 Replication

Some poxviruses, such as orthopoxviruses and vaccinia virus are capable of infecting various cell types from different hosts, whereas avipoxviruses and leporipoxviruses have a very narrow host spectrum. The specific cellular receptor for attachment of virus particles has not been identified in poxviruses. At least five viral proteins (A27, A28, D8, H3 and L1) induce the production of neutralizing antibodies. Some surface proteins of vaccinia virus (A27, D8 and H3) have been found to mediate the interaction of particles with heparin or chondroitin sulphate. The finding that poxvirus particles can not effectively penetrate into cells with low cholesterol levels in their membranes suggests the involvement of lipid rafts in this process. Another complex of viral envelope proteins is involved in the fusion of the viral envelope with cell membranes, and in the uncoating process that releases the viral core into the cytoplasm (Table 19.21). However, the details of this process remain to be elucidated. It seems unlikely that in the case of EEV particles the fusion of the outer envelope with the plasma membrane is sufficient to ensure the entry mechanism, because the viral core is still surrounded by an envelope after having arrived in the cytoplasm. Since the proteins that mediate entry of the virus into the cell are anchored in the inner envelope, it can be assumed that the outer envelope is removed first. It seems to be relatively unstable, as electron-microscopic analyses have revealed that the outer envelope is completely intact in only a few EEV or CEV particles; accordingly, the components of the inner envelope of these particles are also surface-exposed, at least in part. However, it is unclear whether entry of the virus is performed by the fusion of the viral envelope with the cytoplasmic membrane, or via endocytosis and subsequent pH-dependent fusion of the viral envelope with the endosomal membrane.

Regardless of the uncertain identity of the penetration mechanism, the viral core, which consists of the viral genome and more than 70 different viral proteins, arrives in the cytoplasm. The transcription of early viral genes starts in the still intact viral core. The enzymes and components involved (Table 19.22) are carried as early transcription machinery together with the viral core into the infected cell. The structure of the core remains unaltered during the early transcription processes (Fig. 19.35). The cellular DNA, RNA and protein synthesis is turned off already at this early stage of the viral replication cycle. This is performed by a viral structural protein which reaches the cytoplasm during incorporation of the particle into the

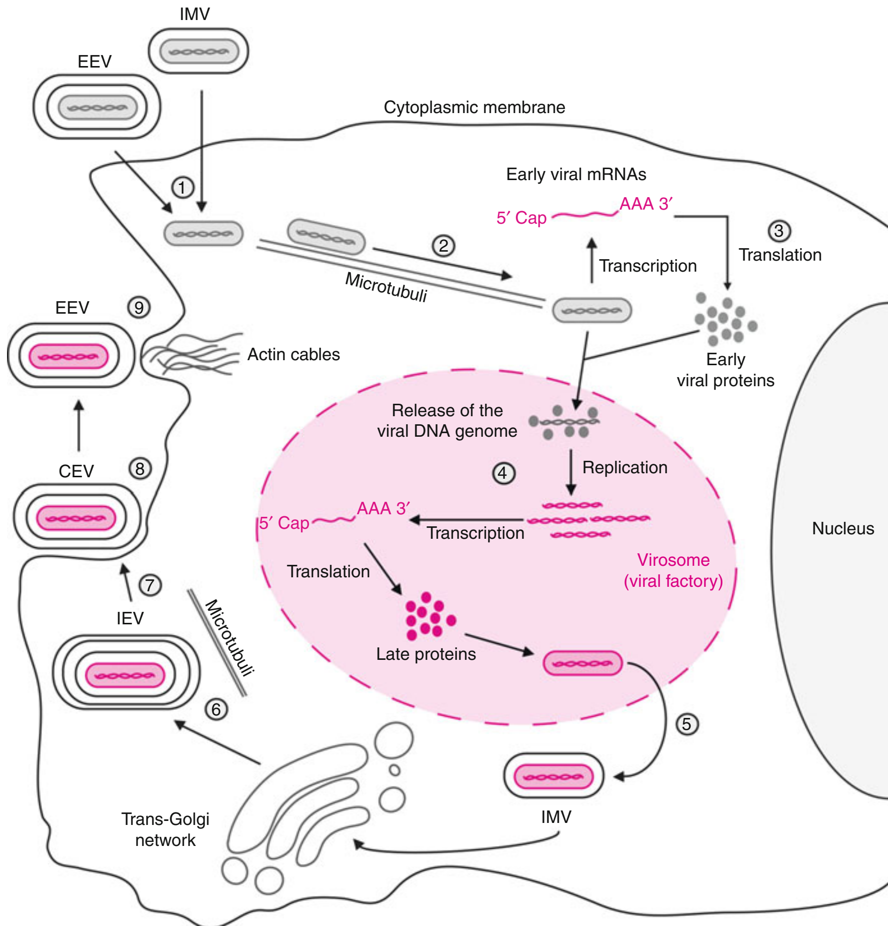


Fig. 19.35 The poxvirus infection cycle in a cell. In the first step, the poxvirus particle attaches to the cell surface and penetrates into the cell, the virion loses its envelopes, and the viral core enters the cytoplasm (*1*). It binds to microtubules, through which it is transported into the interior of the cell (*2*). Subsequently, early viral mRNAs are transcribed and translated. The newly synthesized early proteins induce the uncoating process that leads to the release of the viral genome from the core, giving rise to its replication (*3*). These processes occur in delimited regions within the cytoplasm, which are termed viral factories or virosomes. The synthesis of late mRNAs and proteins occurs after genome replication (*4*). The structural proteins assemble along with membrane fractions and viral genomes into immature virus particles, resulting in intracellular mature virus (*IMV*) particles (*5*). They are transported to the membrane compartments of the *trans*-Golgi network, and are enclosed by two additional envelopes, thus generating intracellular enveloped virus (*IEV*) particles (*6*). These particles bind to microtubules and are transported by them to the cell surface (7), where the outer envelope of IEV particles fuses with the cytoplasmic membrane (*8*). A subset of virions remain attached to the cell surface as cell-associated enveloped virus (*CEV*) particles, and translocate into the neighbouring cell via actin cables, whereas the other fraction is released into the environment in the form of extracellular enveloped virus (*EEV*) particles (*9*)

cytosol, and thus can exert its activity independently of the expression of other viral genes. However, the molecular mechanism is still unknown.

Approximately half of the viral genes are transcribed in this early phase of infection. They have similar promoter sequences, which have a length of about 28 nucleotides and possess the conserved nucleotide sequence 5′-$(A)_6$TG $(A)_9$TTTATA$(T)_5$(A)5 G-3′. The actual initiation site for mRNA synthesis is situated within the last seven nucleotides at the 3′ end of this sequence. Another viral protein is required for binding of the viral RNA polymerase complex to the promoter and for transcription initiation; this protein is also introduced into the cytoplasm of the cell as a component of the particle (Table 19.22). It is a heterodimeric early transcription factor (vaccinia virus early transcription factor, VETF), which is constituted of two different viral proteins (A7L, 82 kDa, and D6R, 77 kDa). VETF seems to be functionally similar to the cellular transcription factor TFIID, which binds to the TATA box in the promoter region of cellular genes. The termination site of transcription of early mRNA species is determined by the consensus sequence TTTTTNT, which is located about 50 nucleotides before the actual termination site. Thus, the 3′ termini of early transcripts are relatively uniform, with the exception of their last 50 nucleotides. Another viral encoded protein is involved in transcription termination, and is identical to the viral capping enzyme. Early mRNA species are cotranscriptionally modified with a cap structure at the 5′ terminus, and the 3′ ends are subsequently polyadenylated. Early mRNA molecules translocate from the viral core into the cytoplasm, where they are translated. The synthesized proteins are necessary for the release of the viral genome from the core particle into the cytoplasm. This step occurs only after early transcription, and is a prerequisite for genome replication. Possibly, the action of a viral protease with trypsin-like activity generates holes or pores in the core through which the genome in complex with proteins translocates into the cytoplasm.

The DNA polymerase and all other enzymes necessary for genome replication are synthesized during translation of early mRNAs. Apart from them, two factors which are necessary for the induction of transcription of intermediate and delayed early genes are also translated. Since these mRNAs are transcribed from the newly synthesized viral genomes, replication of viral DNA must occur previously. These processes suggest that a complex, cascade-like mechanism governs poxvirus replication. Up to 10,000 new genome copies per cell are synthesized during DNA replication in vaccinia virus. The molecular details have not been completely elucidated. DNA replication seems to be initiated at both ends of the genome, which contain highly AT-rich sequences and 12 non-pairing nucleotides to which the viral proteins I1L and I6L bind. A viral endonuclease, K4L, catalyses the formation of single-strand nicks at both termini of the double-stranded DNA. This mechanism probably generates the 3′-OH ends that are required as primers for new strand synthesis. A replication origin, as known from other DNA viruses, has not been identified in poxviruses so far. After initiation of replication and polymerization of the first nucleotide sequences, hairpin structures are formed at the ends of newly synthesized DNA strands, and the other strand is displaced.

DNA replication can be continuously reinitiated at the ends of newly synthesized genomes, resulting in concatemeric, multiply branched DNA molecules, which can be isolated from infected cells. This DNA replication mode is similar to that of parvoviruses (▶ Sect. 20.1).

The cytoplasmic region in which the DNA replication and late transcription occur is enclosed by a membrane which is probably derived from that of the ER. These membrane vesicles remain present throughout the entire viral replication cycle and during late gene transcription, forming so-called virus factories, or virosomes (Fig. 19.35), which are resolved only at the beginning of viral morphogenesis. A viral membrane protein (32 kDa) which is encoded by the E8R open reading frame is anchored in these membranes and appears to be oriented into the lumen. These virosomes constitute the cytoplasmic Guarnieri inclusion bodies which were formerly detected in poxvirus-infected cells.

The synthesis of new viral genomes is a prerequisite for the synthesis of the intermediate early and late transcripts. Intermediate genes encode, inter alia, three regulatory proteins that induce the transcription of late genes. Some of the late transcribed genes are scattered throughout the entire genome. However, many are 3located side by side in the central region of the genome. These mRNA species have heterogeneous, polyadenylated 3′ termini which are frequently far from the end of the respective reading frames. This feature distinguishes them from early transcripts. The transcriptional termination signal of early transcripts is then no longer active and is read through. A viral endoribonuclease can appear in the late phase of the replication cycle. It catalyses the sequence-specific cleavage of late mRNAs at so-called AX elements in vaccinia viruses ("AX" stands for the recognition sites of the restriction enzymes *Ala*I/*Xba*I). In this way, a 3′ terminus is generated, and is subsequently polyadenylated. Hence, poxviruses use different mechanisms for transcriptional termination of early and late RNA molecules. The promoter sequences of late genes are different from those of early genes. The actual initiation site is highly conserved. It is made up by the nucleotide sequence TAAAT, which is accompanied by an A/T-rich upstream region of about 30 nucleotides. Late mRNA molecules predominantly encode viral structural proteins, but also some enzymes that are components of the infectious virus particles. These include two different protein kinases, and the protease that is required for the release of the DNA genome (Table 19.22).

The assembly of the various viral structural components into infectious virus particles is a sophisticated process. It occurs in the cytoplasm of the infected cell in defined, delimited regions, which exhibit a granular appearance in electron micrographs. The first structures that can be observed are dome-shaped membrane fragments containing needle-like processes or projections. It has not been conclusively elucidated from which cellular compartments this viral envelope is derived; even its de novo synthesis has been discussed in some studies. Electron micrographs suggest that the membrane is derived from the smooth ER, in which the nucleoprotein complex accumulates as an electron-dense mass before the membrane completely surrounds it to form a spherical vesicle, giving rise to

IMV particles. Viral proteins, including those that are anchored in the IMV membrane, must be actively transported to the developing complexes, since no polyribosomal structures are observed. Therefore, there is also no protein modification with sugar groups, and the formation of disulphide bonds in envelope-anchored proteins of IMVs is catalysed by specific chaperones in the non-reducing environment of the cytoplasm which are encoded by the O2L, G4L and A2.5L genes of the vaccinia virus genome. Subsequently, the biconcave core structure with the two lateral bodies inside the IMV particle becomes visible. Viral proteases cleave, among other precursor proteins, P4a and P4b into the core proteins, which are found in infectious virus particles. A subset of IMV particles attach to microtubules, and are transported to the cell surface. During this process, IMV particles are surrounded by an additional double envelope, which originates from the compartments of the *trans*-Golgi network. This gives rise to the emergence of IEV particles, which finally reach the cytoplasmic membrane. In the final step, actin cables transport the IEV particles further to the cytoplasmic membrane, whereby the outer envelope of IEVs fuses with the plasma membrane, and the viral particles reach the outside of the cell membrane. Some of them (CEVs) remain associated with it, whereas the others are released from the cells into the environment in form of EEVs.

19.6.5 Human Pathogenic Poxviruses

19.6.5.1 Variola Viruses

Epidemiology and Transmission

Smallpox (variola or variola vera/variola major), which is caused by variola virus, was originally spread in the Eastern Hemisphere, the Indian subcontinent and China. In the Middle Ages, the virus was spread through the Silk Road and other major trade routes into the Near East, and from there to Africa and Europe. Emigrants carried the infection to America. The disease appeared as an epidemic disease, which affected the population in endemic form approximately every 4–7 years. The virus was transmitted particularly by droplet infection from smallpox-infected people in early stages of infection, but also by virus-containing smallpox pustules, dried crusts and textiles that had come into contact with contaminated skin. About 10–40 % of infected individuals died of smallpox. In addition to variola vera, there is another form of smallpox, which is referred to as variola minor or alastrim; it appeared primarily epidemically and endemically in South America, Africa and Australia, and was caused by a less virulent strain of variola virus (alastrim virus). The mortality rate was only about 1–2 %.

The global vaccination programme with vaccinia viruses initiated by the World Health Organization resulted in the eradication of smallpox and alastrim viruses. The last case of smallpox was reported in Somalia in 1977. In rare cases, people can still contract a smallpox-like disease, which may be acquired through contact with animals infected with animal poxviruses.

Sporadically, the so-called monkeypox virus can be transmitted to humans from wild animals (especially rodents such as rats and squirrels, and rarely non-human primates) that are hunted and eaten as bush meat by the indigenous peoples of Africa. From this reservoir, it can be transmitted to monkeys and humans, especially to children who capture infected animals. However, transmission from human to human seems to be significantly less frequent than in the case of variola virus. For example, about 300 people were infected with monkeypox virus in the Democratic Republic of the Congo between 1980 and 1985. There are also reports indicating that 88 people were infected with monkeypox virus in the Democratic Republic of the Congo in 1996–1997. An outbreak of monkeypox caused sensation in some states of the USA in 2003 in which 81 infected people were reported, and the diagnosis "monkeypox" was confirmed serologically in about 40 % of cases. The disease was predominantly mild, and infected people developed superficial skin lesions and a pronounced lymphadenopathy. A shipment of African rodents from Ghana turned out to be the cause; particularly rope squirrels (*Funisciurus* spp.) seem to be endemically infected with monkeypox virus. Sequence analysis revealed that 96.3 % of the central region of the genome of monkeypox virus is identical to that of variola virus. However, monkeypox virus is not a variant of variola virus, but is a separate species of virus. Immunization with vaccinia virus largely protects against monkeypox because of the close relationship between monkeypox and variola viruses.

Besides this form of monkeypox, another zoonotic disease is known in which tanapox virus or Yaba monkey tumour virus (the causative agent of yabapox) can be transmitted to humans by close contact with different African and Asian monkey species (including rhesus monkeys, baboons and macaques). Yaba monkey tumour virus is oncogenic in monkeys. In humans, the infection causes mainly harmless, slightly febrile diseases. It may be accompanied by the emergence of fibroids (benign tumours made of fibre- or cell-rich connective tissue), which disappear spontaneously after a while.

Rare smallpox diseases in humans which especially occurred after bite and scratch injuries caused by cats have caused a stir in recent years. The causative agent has been identified as an orthopoxvirus which is identical to cowpox virus. The reservoirs of this poxvirus are small rodents, from which the disease is transmitted to cats. This poxvirus can also be transmitted to cattle (cowpox) and other animals such as camels and lions.

Variola Viruses Exist only in Freezers Today

Following eradication of smallpox, the international committee responsible for research into smallpox infection demanded that all remaining laboratory strains and reserves be destroyed, and that research with variola viruses be stopped. However, the destruction of the stocks did not occur. The viruses are still stored in two high-security laboratories at the Centers for Disease Control

and Prevention in Atlanta, USA, and the State Research Center of Virology and Biotechnology VECTOR in Koltsovo, Russia. In addition, a lively debate has begun whether replication-proficient variola viruses also exist in other laboratories, and whether they may be accessible to bioterrorists. This issue has not been definitively resolved. Moreover, the genetic information of the virus is still present in fragments that have been cloned in various bacterial plasmids.

Clinical Features

The incubation period of the classic smallpox infection (variola major) lasted 10–14 days. An acute outbreak was characterized by high temperature, severe headache, joint and testicular pain, bleeding of the conjunctiva and mucous membranes and swallowing difficulties caused by rash in the mouth and oropharyngeal mucosa. About 2 days later, these initial symptoms were followed by typical back pain and characteristic skin rash (papules, pustules, vesicles, haemorrhagic ulcers), which could spread to the whole body and mucous membrane regions. The pustules dried and crusted after 2–3 weeks, but often left scars behind. Ulcers in the nasopharyngeal region and bronchia were observed in some patients. In some cases, infections were associated with severe, often fatal haemorrhages ("black smallpox"). The infection with variola minor virus (alastrim) was similar, but considerably milder.

Pathogenesis

Following transmission into the mucous membranes of the mouth, nose and throat, variola virus replicated in the upper respiratory tract, and spread from there into the lower respiratory tract, including pulmonary alveoli. Originating from mucous membranes, the virus infected macrophages and lymphocytes, which transported it to local lymph nodes, where it multiplied. A first minor viraemia started from these lymph nodes, which spread the virus throughout the entire reticulohistiocytic system. Further proliferation gave rise to a second major viraemia, through which the virus spread into the whole organism. The virus was detectable in many internal organs, which, however, rarely exhibited histopathological changes or lesions. At that time, the symptoms of rush and blister eruptions began to develop. The infected subject was contagious and capable of transmitting viruses. Initially, small patches appeared on the skin, followed by the emergence of papules, which developed into blisters that finally healed with crusting and scarring. The latter is an indication that apart from the skin epithelium, also the basement membrane was affected by the infection. Local cell proliferation occurred in the infected areas, which was triggered by an epithelial-cell-specific viral growth factor. Another viral protein with homology to vascular endothelial growth factor might have been crucial for the extent of skin lesions that resulted from the infection. This protein was discovered in

parapoxviruses, which infect sheep and occasionally even humans. It induced a pronounced proliferation of the vascular endothelium in the region of smallpox pustules, which seems to have been based on the effect of the vascular endothelial growth factor homologue viral protein. It is unclear what led to the severe and frequently fatal diseases. On the one hand, they might have been determined by infection of smallpox blisters with bacterial pathogens, which caused a toxic shock syndrome. On the other hand, it is suspected that deaths might have been caused by massive cytokine release (TNF-α, TGF-β and other cytokines; ▶ Chap. 8), which impaired cardiac and renal function. Black smallpox was caused by massive bleeding in the skin and mucous membranes as well as in internal organs.

Immune Response

Early after infection, cytotoxic T lymphocytes were active, and eliminated infected cells after recognition of HLA class I protein complexes containing peptides from different viral proteins. They were responsible for elimination of the virus from the organism and survived for a long time. The fact that only patients with an impaired cellular immune response exhibited vaccination complications in the form of necrotizing vaccinia virus infections indicates that cytotoxic T cells played a decisive role in the early coping with the infection. Neutralizing IgG antibodies were produced later and were directed against viral surface proteins. This combination of cellular and humoral immune responses protected against reinfections, but did not last a lifetime. Thus, inapparent and mild diseases, known as varioloid, were observed.

Therapy and Prophylaxis

The occurrence of smallpox was prevented by intradermal vaccination with the live vaccinia virus in the first and 12th years of life (scarification). The absolute immunity persisted for 1 or 2 years. Therefore, people were revaccinated when travelling to smallpox endemic or epidemic areas. Four to 5 days after vaccination, a swelling formed owing to viral replication in the cells at the inoculation site, into which monocytes, macrophages and T lymphocytes migrated in the following days. The pustules enlarged until the tenth day after inoculation. A swelling of lymph nodes and mild fever were also observed during this period. The pustules healed 3 weeks after vaccination, but left a scar indicating a lifelong successful vaccination with vaccinia viruses. Severe complications occurred relatively seldom. A generalized skin rash was commonest, and occurred in about one in 100,000 vaccinated individuals, and usually healed without problems. In rare cases (1:1,000,000), a progressive vaccinia virus infection was observed in immunocompromised patients. Furthermore, postvaccinal encephalitis was observed in approximately one case per 50,000 vaccinations, which was manifested as perivenous inflammation and demyelination. Smallpox cases have recently been observed in cattle and milkers in Brazil. They were caused by a variant of vaccinia virus (cantegalovirus) which has emerged from a still unknown reservoir, and is mutated in comparison with the original vaccine virus.

During the period in which people were vaccinated worldwide with vaccinia viruses to eradicate smallpox, it was found out that horses and swine are also very susceptible to infection with these attenuated human viruses. The formerly frequently observed clinical pictures of horsepox and swinepox (not be mistaken for the disease caused by swinepox virus of the genus *Suipoxvirus*) disappeared concomitantly with the cessation of smallpox vaccination in humans.

Today, it is being considered to reintroduce a smallpox vaccine based on a highly attenuated variant of vaccinia virus. This is the virus strain MVA, which was isolated after continuous passages of vaccinia virus in chicken fibroblast cultures. The genome of this virus comprises only 178,000 base pairs; six major regions are deleted and others are rearranged. The attenuating mutations affect the genes for the viral IL-1β receptor and other proteins that are important for virus–host interactions. The MVA virus replicates lytically only in chicken fibroblasts; in mammalian cells there is no morphogenesis of infectious particles, and thus no production of the virus. However, viral genes are expressed in mammalian cells, leading to the development of humoral and cellular immune responses.

A semicarbazone derivate has been successfully used for treatment of smallpox in field experiments. Since smallpox viruses are now considered as pathogens entailing a potential risk for bioterrorist attacks, intensive research is being done to develop new therapeutics for the treatment of smallpox infections. Cidofovir seems to be effective. Immunoglobulin preparations containing antibodies against vaccinia viruses are generally recommended to treat poxvirus infections, both natural infections with orthopoxviruses and laboratory contaminations. They are available from the Centers for Disease Control and Prevention in Atlanta, USA, and can successfully contain smallpox virus infections when applied at the appropriate time.

Waning Immunity Causes Occasional Problems

There is no longer a licensed vaccinia virus vaccine. This situation leads to some problems. Recombinant vaccinia viruses are increasingly being used for genetically engineered production of different proteins in eukaryotic cells. Since vaccinia viruses may lead to symptomatic infections in particular when transmitted to the conjunctiva of non-vaccinated adults, a new approval of the vaccine is urgently needed for laboratory personnel. Moreover, the vaccinia virus vaccine also confers protection against most animal poxviruses because of cross-immunity. Since the protection against smallpox is continuously decreasing in the population, there is an increased risk of infection for people (such as veterinarians) who have frequent contact with rodents and small animals. The increasing concern that variola viruses might be used for bioterrorist attacks, which would trigger epidemics in populations without immunity, makes it clear that the issue of new licensing of smallpox vaccination must be urgently addressed. For this reason, 260 million vaccine doses have been stored in the USA to protect large parts of the population from possible bioterrorist attacks with smallpox viruses.

19.6.5.2 Molluscum Contagiosum Virus

Epidemiology and Transmission

Molluscum contagiosum was first described in the late eighteenth century, and it was defined as a clinical disease in 1817. In 1905, it was shown that it is caused by a virus, which was classified into the family of poxviruses in 1930. To date, four genotypes have been identified (MCV-1, MCV-1a, MCV-2 and MCV-3). The MCV-1 genotype is the commonest, and causes skin infections (benign epidermal tumours) that particularly affect children and young adults. MCV-2 is usually transmitted by sexual intercourse, causing lesions in the genital mucosa of adults. Viruses of genotype MCV-3 are very rare. Molluscum contagiosum is usually a localized disease of the skin, and is observed more frequently in immunodeficient individuals. The prevalence in the population increases with age, and can reach up to 40 % in immunologically healthy people and up to 90 % in HIV-infected patients. The virus is transmitted by direct skin contact, through contaminated household items (e.g., towels) or, like papillomaviruses, it spreads autonomously from infected skin areas to other regions. The virus may occasionally be transmitted to animals such as horses, in which it may cause symptoms.

Clinical Features

The incubation period until the onset of skin lesions is variable. The benign skin tumours, which can occur anywhere, are characterized by flesh-coloured nodules. The virus is released by destroyed epithelial cells from slowly emerging nodules. They regress spontaneously in immunocompetent individuals in 3–12 months.

Pathogenesis

The virus penetrates into the stratum basale through small skin injuries. Neoplastic growths proceed from infected areas. Virus-containing inclusion bodies can be found in infected cells. The central depression in the nodules is caused by disintegration of epithelial cells. Like all poxviruses, molluscum contagiosum virus also expresses a number of proteins which enable it to evade the host immune response. MC54L encodes a protein which exhibits homology to the human IL-18-binding protein and acts in an anti-inflammatory manner. The MC148 gene product is an antagonist protein that binds to chemokine receptor 8, and impedes the migration of macrophages into the infected skin areas. The MC159 protein inhibits the induction of apoptosis by binding to Fas proteins, TNF and TNF-related apoptosis-inducing ligand (TRAIL). The product encoded by open reading frame MC80R is a homologue of MHC class I proteins that is no longer able to bind and present peptides because the respective amino acids have been altered by mutations.

Immune Response and Diagnosis

Infected individuals develop virus-specific antibodies and cytotoxic T lymphocytes; however, the details are not known. The high incidence of molluscum contagiosum in HIV-infected patients indicates that the cellular immune response is crucial for the control of infection. The diagnosis is usually made clinically; detection of the virus can be performed by PCR analysis.

Therapy and Prophylaxis

The nodules of molluscum contagiosum can be removed surgically or by colour laser therapy. Clinical studies have shown that the use of the chemotherapeutic drugs imiquimod and cidofovir is effective (▶ Chap. 9).

19.6.6 Animal Pathogenic Poxviruses

Many poxviruses are economically important pathogens in livestock. Some of these viruses have a zoonotic potential. They may be transmitted from infected animals to humans, and may even lead to fatal diseases in exceptional cases; e.g., monkeypox and cowpox viruses as well as orf virus. Most animal pathogenic poxviruses play only a minor role in Europe owing to successfully conducted control programmes. These include swinepox virus, which has been classified as the sole member of the genus *Suipoxvirus*. They are transmitted mechanically by pig lice (*Haematopinus* spp.). The rare disease is always mild and transient.

19.6.6.1 Cowpox Virus

Epidemiology and Transmission

Cowpox virus is a member of the genus *Orthopoxvirus*, which includes the extinct variola virus, monkeypox virus (Sect. 19.6.5) and mousepox virus (ectromelia virus). All these viruses have zoonotic potential, and they can be transmitted to other species, including humans. The designation “cowpox” is misleading because cattle are rarely infected by this virus. The principal hosts are rather mice and other rodents, which can transmit the virus to many animal species, including cattle, cats and various zoo animals, especially elephants and rhinos. Humans also are susceptible to cowpox virus infections, and these may have a fatal outcome.

Clinical Features

Rodent hosts are usually infected asymptomatically. Besides the afore-mentioned species, cats are infected relatively frequently. The cats develop symptoms after an incubation period of about 10 days which are usually limited to sporadic skin lesions. However, cats that are simultaneously infected with immunosuppressive pathogens such as feline leukaemia virus and feline immunodeficiency virus (▶ Sect. 18.1.6), or feline infectious peritonitis virus (▶ Sect. 14.8.6), can develop severe diseases. They frequently develop pneumonia as well as extensive lesions, and secrete large amounts of the virus. Especially, these cats constitute a serious threat for humans.

Mousepox Virus Causes Ectromelia

Mousepox virus belongs to the orthopoxviruses. It causes a systemic infection in certain mice strains, murine ectromelia, which is associated with a high mortality rate. BALB/c and C3H mice are highly susceptible and rapidly develop a fatal disease during which particularly the liver and spleen are

destroyed. By contrast, C57BL/6 mice develop only subclinical symptoms, or establish a slowly emerging chronic infection with ulcerative dermatitis of the muzzle, paws and tail. They can easily introduce the virus undetected into mice populations, in which it is spread further by contaminated litter or the hands of the keeper. Mousepox virus does not cause disease in humans.

Pathogenesis

The infection begins with the formation of a local primary lesion on the skin. The virus invades the bloodstream, spreads viraemically and causes secondary lesions, which are visible as prominent papules on the skin. Scabby lesions arise from the papules, and usually heal within a few weeks. However, a generalized infection can be established in exceptional cases, which may be associated with viral replication in a variety of organs, thereby causing various symptoms.

Immune Response and Diagnosis

The diagnosis is made by isolation of the virus and/or by PCR. An initial diagnosis is usually done by electron-microscopic depiction of poxvirus particles from the lesions.

Control and Prophylaxis

A specific immune prophylaxis is not available, but immunization with the vaccinia virus vaccine protects completely against infection with cowpox virus. Therefore, elephants that are kept as zoo animals are routinely vaccinated with the highly attenuated vaccinia virus strain MVA in several countries (Sect. 19.6.5).

19.6.6.2 Goatpox Virus, Sheeppox Virus and Lumpy Skin Disease Virus

Epidemiology and Transmission

Various poxviruses are grouped in the genus *Capripoxvirus* which mainly infect small ruminants (sheep, goats) and cattle. Their distribution is limited almost exclusively to Africa. Outside this continent, infections with capripoxviruses have been found only in India, affecting goats and sheep. Goatpox virus can be distinguished neither serologically nor molecular biologically from sheeppox virus. However, both species show a specific tropism for infecting their respective hosts. Another poxvirus which can also not be distinguished from sheeppox virus or goatpox virus causes lumpy skin disease in cattle. This disease is also confined to Africa, and the reservoirs are wild ruminants, possibly African buffalos. It has been described only once outside the African continent, namely in Israel.

Epidemiologically important is the great stability of all these types of viruses, which remain infectious in desquamated pox lesions (scabs) for weeks and months. They are transmitted by direct contact between animals or with the scabs, but possibly also by biting insects. Transmission to humans has not yet been observed.

Clinical Features

Sheeppox virus and goatpox virus cause a systemic infection that may be associated with a high mortality of up to 50 %. Lumpy skin disease is a systemic infection of cattle which is associated with a generalized lymphadenopathy and node formation in the skin of the entire body. The morbidity is high, but the mortality is low. The economic relevance of the disease is based not on acute symptoms, but on the long recovery period and the decline in performance associated with the disease.

Pathogenesis

Infection occurs by mechanical injuries by stinging insects or through the respiratory tract. After an initial reproduction at the infection site, the virus multiplies in the lymph nodes, from where it spread through the blood into many organs, including the skin, in which the formation of the characteristic papules and nodules occurs.

Control and Prophylaxis

Successful vaccinations with an attenuated live vaccine or a vaccine based on sheeppox and goatpox viruses are performed in areas where lumpy skin disease virus is endemic. Capripoxvirus infections are notifiable in most developed countries, and possible introductions are prevented by strict quarantine measures. Europe has long been free of sheeppox and goatpox.

19.6.6.3 Myxoma Virus

Epidemiology and Transmission

Myxoma virus is assigned to the genus *Leporipoxvirus*. It is found worldwide, and constitutes the most important viral pathogen in rabbits. The virus causes only local, benign tumours in wild rabbits on the American continent, whereas it provokes a severe disease associated with high mortality in European rabbits (*Oryctolagus cunniulus*). The virus is transmitted by flies, fleas, lice, ticks and other insects, which mechanically transmit the virus from animal to animal. Direct transmission through secretion and excretion products of infected rabbits is also possible. Humans are not susceptible to myxoma virus.

About 60 years ago, around 1950, myxoma virus was the first pathogen used for biological control of a pest, namely rabbits that were imported from Europe to the Australian continent, where rabbits did not exist. Retrospectively, this measure was initially considered a resounding success. However, the rabbits could not be exterminated in the long term, and this led to an accidental adaptation of the virus to the host (attenuation) and to selection of resistant rabbit variants. The result was a recovery of the rabbit population. In 1996, a second attempt was made with the introduction of rabbit haemorrhagic disease virus, a calicivirus (▶ Sect. 14.3.6). However, this virus was also not able to solve the rabbit problem satisfactorily.

Clinical Features

The incubation period lasts a few days. The classic symptoms of rabbit myxomatosis are generalized oedema, especially in the head – whence the name lion's

head – and in the anogenital region. Most rabbits die within a few days. Surviving animals frequently exhibit subcutaneous oedema with gelatinous deposits in the tissue, which give the disease its name.

Pathogenesis

The pathogenesis is typical of a systemic poxvirus infection. The virus enters the mucous membranes by contact with insects, replicates in the lymphoid tissues and spreads systemically. The disease is usually acute, and the animals die within a few days.

Diagnosis and Immune Response

The clinical picture is usually clear. Diagnosis of the virus can be done easily by the electron-microscopic imaging of virus particles from the lesions.

Control and Prophylaxis

Available vaccines are based on attenuated virus strains that induce a reliable protective immunity. An annual booster vaccination is required.

19.6.6.4 Orf Virus

Epidemiology and Transmission

Orf virus is ubiquitous and is the causative agent of the contagious pustular dermatitis in sheep and goats. A similar disease is papular stomatitis in cattle (pseudocowpox). However, pseudocowpox virus plays only a minor role in comparison with orf virus. The name "orf" stems from Old English, and means rough. Orf virus causes zoonotic infections in humans, and these are invariably mild and locally delimited. The virus is transmitted by direct contact or by insects; the infection arises through small skin lesions.

Clinical Features

The incubation period lasts a few days. Subsequently, the animals develop the symptoms of pustular dermatitis, which is characterized by local lesions in the mucous membranes of the muzzle and nose. In severe cases, diseased sheep starve because the painful lesions hinder intake of feed. More rarely, lesions are also found on the hooves or on the udder.

Pathogenesis

Orf virus infections are locally delimited, and there is no systemic spread of the virus.

Immune Response and Diagnosis

The diagnosis is made by electron-microscopic evidence of virus particles from the lesions (skin scrapings) or by PCR analysis.

Control and Prophylaxis

A vaccine based on attenuated virus strains is available, but it induces only a short-term immunity. If ewes are vaccinated before lambing, then the lambs are protected by maternal antibodies in the milk during the first few weeks of life.

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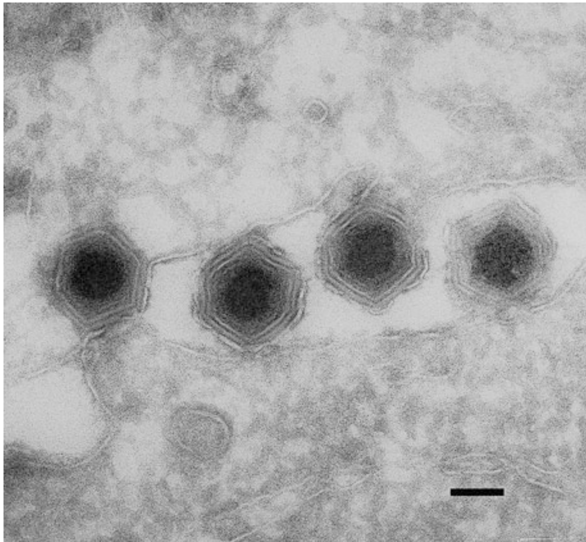
19.7 Asfarviruses



19.7.1 Classification and Characteristic Prototypes

The family *Asfarviridae* comprises only one genus (*Asfivirus*), with a single member, namely African swine fever virus. It is also the only known DNA virus that is

transmitted by tick bites. African swine fever virus was first described in East Africa in 1921. Today, it is also endemic in southern Africa and Sardinia. However, several disease outbreaks have been observed in other countries, e.g., in France and Italy in 1960, in South America and the Caribbean in 1970 and in Holland in 1986. In 2007, the virus was transported to Georgia. From there, outbreaks were also reported in neighbouring countries.

Compared with other animal diseases, the biology of asfarviruses is poorly investigated, not least because no human pathogenic virus type is known. African swine fever virus was long classified as an iridovirus; this virus family includes many pathogens of insects, fish and amphibians. However, new insights into the structure of its genome made it necessary to create a separate family for African swine fever virus.

19.7.2 Structure

19.7.2.1 Virus Particle

The enveloped virions of asfarviruses are approximately 200 nm in diameter, and have a very complex structure (Fig. 19.36). The outer envelope is derived from the cytoplasmic membrane of infected cells. The icosahedral capsids are composed of 1,892–2,172 capsomers, which have a diameter of 13 nm and consist of different proteins. To date, 17 open reading frames encoding structural proteins have been identified in the genome of African swine fever virus. Two of the translated proteins (pp220 and pp60) are further processed by a viral protease. A cylindrical structure, the core, is enclosed in the capsid, which contains the viral genome associated with proteins. This core is surrounded by an inner envelope, which is derived from the membrane of the ER. There is evidence that this inner envelope is present as a single lipid layer.

19.7.2.2 Genome Organization and Structure

The asfarvirus genome is a linear, double-stranded DNA molecule 170–190 kb long. Inverted terminal repeats are located at the ends of the genome, and are similar to the inverted terminal repeats of poxviruses. The inverted terminal repeats length of 2,100–2,500 base pairs; they are covalently closed at the ends of the genome by hairpin structures with a length of 37 nucleotides. The genomes of ten isolates of African swine fever virus have been completely sequenced. Both DNA strands are coding, in which 160–175 open reading frames have been identified. Approximately a third of the genome is constituted by six multigene families whose function is unclear: they are regions containing open reading frames with very similar sequences and differing copy numbers, which are specific for each isolate. These gene families have probably arisen by duplication events from a common evolutionary ancestor. The regions at the centre and the left end of the genome are conserved in the different virus isolates. The region at the left end of the genome encodes multigene family 110.

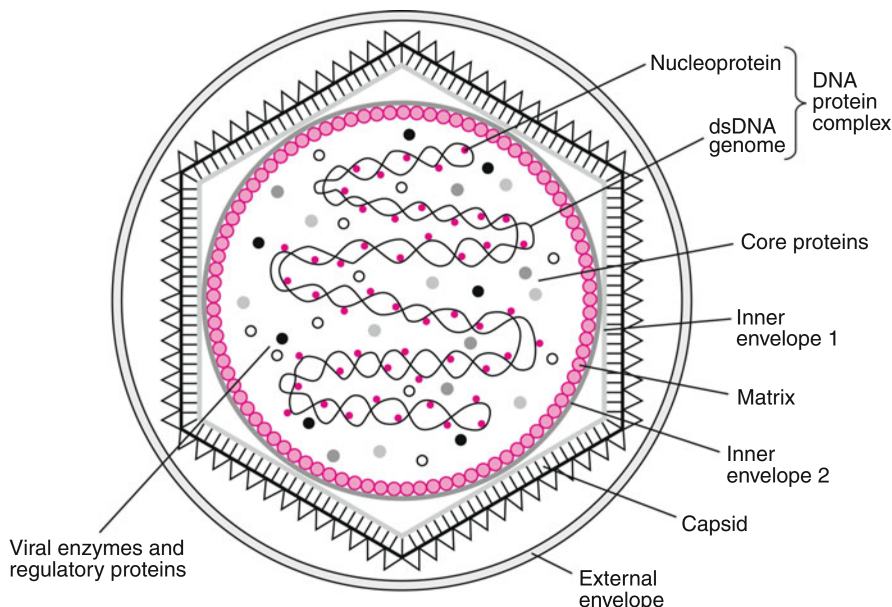


Fig. 19.36 Structure of an asfarvirus particle. The virus has a complex icosahedral structure. The genome nucleoprotein complex is enclosed by an inner envelope (*inner envelope 2*), which is associated with a layer of matrix proteins at the inner side. Another envelope (*inner envelope 1*) and the capsid surround these particles, which are finally enwrapped by the outer envelope. *dsDNA* double-stranded DNA

19.7.3 Viral Proteins

The asfarvirus genome contains the genetic information for the synthesis of almost 200 proteins, which have hardly been characterized in terms of their functions and properties. These include the enzymes required for viral reproduction, viral transcription and DNA replication. During these processes, asfarviruses are largely independent of the enzymatic machinery of the host cells. A similar autonomy is found only in poxviruses. About 70–85 proteins are not essential for viral replication; some of them have functions that enable the virus to evade the immune defence mechanisms of the host. Owing to a highly conserved cysteine-rich domain, the products of multigene family 110 seem to play important functions in an oxidizing environment – as in the ER. In addition, these proteins possess signal peptides indicating their localization in this cell compartment.

19.7.4 Replication

Although it is assumed that African swine fever virus encodes all the enzymes necessary for DNA replication, genome replication is dependent on some nuclear

functions of the host cells which have not yet been characterized. In this regard, asfarviruses are similar to iridoviruses, whose DNA replication also starts in the nucleus. All subsequent steps occur perinuclearly within the cytoplasm of the infected cell. It is believed that the DNA genome of asfarviruses is replicated in a rolling-circle-like mechanism, resulting in concatemeric molecules that contain multiple genome copies, which are subsequently resolved into individual genomes that are packaged into new virus particles, as during replication of herpesviruses and poxviruses (Sects. 19.5 and 19.6).

The morphogenesis occurs in viral factories whose structures are similar to aggresomes and are detectable as paracrystalline inclusion bodies in the cytoplasm near the nuclear membrane. This process involves the incorporation of membrane structures which are taken from the adjacent ER. The final step during particle maturation is the condensation of the core with the packaging of the viral genome into the empty capsid. The newly formed viruses are released by budding from the plasma membrane.

Aggresomes

Aggresomes are protein-containing inclusion bodies that arise in the cytoplasm when the cellular degradation pathways are impaired or overloaded. They arise by active transport around the microtubule organizing centre near the nucleus when the cell accumulates excessive amounts of abnormal, damaged or misfolded proteins, which can no longer be coped with by the ubiquitin-mediated proteasomal degradation pathway. They are frequently surrounded by a "cage" of vimentin. There seems to be a connection to cellular autophagy. Therefore, aggresomes are a kind of intracellular depot for protein waste.

19.7.5 Animal Pathogenic Asfarviruses

19.7.5.1 African Swine Fever Virus

Epidemiology and Transmission

African swine fever virus is transmitted by ticks of the genus *Ornithodoros* in areas where African swine fever is endemic (southern Africa and Sardinia). The species involved are *O. moubata* in Africa and *O. erraticus* in Sardinia. The virus infects only swine. In Africa, warthogs are subclinical carriers in which the virus persists lifelong. In contrast to warthogs, the disease develops acutely in domestic swine, which excrete the virus in high titres with all secretions and excretions. It is estimated that most of the warthog population in Africa is infected, and that even 25 % of *Ornithodoros* ticks are virus-positive, i.e. the infection is widespread. The viruses multiply in the ticks, also infecting the gonads, so a transovarial transmission to the next generation is possible. The ticks excrete the virus in the faeces and the secretion of the salivary glands. In recent years, African swine fever has spread from Africa to some countries that were previously regarded as free from infection, including the islands of Mauritius and Madagascar.

The virus has also been introduced into the USA and several European countries by feeding domestic pigs with leftover containing meat from infected swine. A major outbreak was recorded in 2007 in Georgia, from where the virus spread to several neighbouring countries. Since then, the infection has continued to spread, and new outbreaks in Russia were reported at the end of 2009. There is great concern regarding possible introduction into the member states of the European Union.

Clinical Features

African swine fever typically manifests itself as an acute or a peracute general infection, the symptoms of which are indistinguishable from those of classical swine fever (▶ Sect. 14.5) Pathologically, petechial haemorrhages (in combination with thrombopenia and lymphopenia) are detectable in nearly all tissues as a result of disseminated intravascular coagulation. After an incubation period of about 4–5 days, the animals show high temperature, diarrhoea, respiratory symptoms and cyanosis. They die within a few days or recover and remain lifelong virus carriers and excreters.

Pathogenesis

After absorption through the upper respiratory tract, the virus initially replicates in the tonsils and lymphatic tissue of the nasopharynx. During an initial viraemia, it replicates in monocytes and macrophages, and is excreted in high concentrations in almost all secretions and excretions. Lesions in the blood and lymph vessels can be found at an early stage in infected animals. In hyperacute or acute infections, the disease is manifested by bleeding and haemorrhagic infiltration of lymph nodes and petechial haemorrhages in the skin and mucosa. In protracted cases, the animals show skin ulcers, gastroenteritis and pneumonia. The lesions are not directly caused by the lytic viral replication in endothelial cells: they are caused immunopathogenetically by cytokines, particularly IL-1, IL-6 and TNF-α, which are released by infected macrophages and monocytes. This immunopathogenetic cause of disseminated intravascular coagulation is also found in other viral infections, such as the haemorrhagic fevers caused by Rift Valley fever virus and dengue virus.

African swine fever virus possesses many proteins that enable it to evade the host immune response. These evasion mechanisms lead to an inhibition of synthesis of neutralizing antibodies in animals infected with African swine fever virus. Some of the proteins that are not essential for viral replication interfere with the immune system via three different pathways: (1) downregulation of the NFκB-mediated signalling cascade, which is essential for the transcription of various cytokines; (2) hindrance of apoptosis by some viral proteins which have a great similarity to apoptosis inhibitors of the inhibitor of apoptosis protein (IAP) family; (3) synthesis of the viral EP402R protein, which is a homologue of adhesion molecule CD2. CD2 is responsible for binding of the virus to erythrocytes. The binding of EP402R causes a masking of the virus, protecting it from immunological attack.

Immune Response and Diagnosis

No neutralizing antibodies are synthesized during infection; thus, the animals do not develop a protective response based on the humoral immune response. This has

decisively impeded the development of good vaccines. It remains to be seen whether a reliable cellular immunity can be induced by appropriate vector vaccines.

Isolation and cultivation of the virus is possible in primary swine cells. After adaptation, the virus is capable of replicating in numerous cell lines. The pathogen can subsequently be detected by the haemadsorption test. Alternatively (or for verification), viral proteins can be detected in mononuclear cells from lymph nodes or spleen. Furthermore, the viral genome can also be detected by PCR analysis.

Control and Prophylaxis

African swine fever is a World Organisation for Animal Health listed disease and is thus notifiable. In the case of an outbreak, extensive quarantine and culling measures are stipulated. No vaccine is available.

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Viruses with a Single-Stranded DNA Genome

20

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Only a few human and animal pathogenic viruses are known that have a single-stranded DNA genome. The members of the family *Parvoviridae* have a linear genome, whereas the genome of the members of the family *Circoviridae* and that of the recently created family *Anelloviridae* have a circular structure. The members of the family *Geminiviridae* are also characterized by a circular, single-stranded DNA genome, but they infect only plants. Circoviruses are pathogens in both plants and various animal species (monkeys, swine, poultry, etc.). A circular, single-stranded DNA virus, torque teno virus, was first isolated from humans in 1997. Several types of torque teno virus have been detected and classified into the family *Anelloviridae*; all persist, like the later discovered torque teno mini viruses and the torque teno midi viruses, in most people and various animals without causing any apparent disease.

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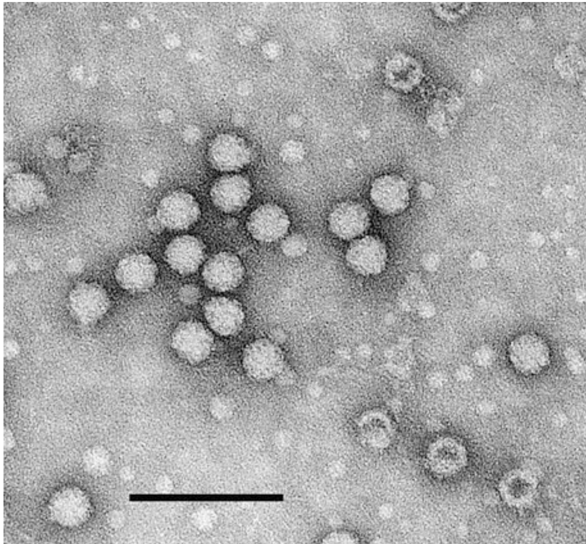
20.1 Parvoviruses



Parvoviruses (from Latin *parvus*, meaning “small”) belong to the smallest viruses known to date. They are extremely resistant to external influences and detergents, have a narrow host range and a strong tropism for infection of dividing cells. Today, three human pathogenic species are known among parvoviruses, namely human parvovirus B19, human bocavirus and parvovirus 4. In addition, there are a large number of animal pathogenic parvoviruses, which are of great importance in both domestic and livestock husbandry. The reproduction capability of adeno-associated viruses is dependent on the presence of helper viruses, but their infections seem to be asymptomatic.

20.1.1 Classification and Characteristic Prototypes

The family *Parvoviridae* encompasses two subfamilies (Table 20.1): *Parvovirinae* and *Densovirinae*. The subfamily *Parvovirinae* comprises five genera:

1. The genus *Dependovirus* includes the adeno-associated viruses and its members also infect humans. They are only able to replicate and to induce a productive infection cycle when the cells are concomitantly infected with a helper virus (adenovirus, vaccinia virus or herpesvirus). Thus, their productive multiplication is dependent on co-infection with these viruses. Alternatively, dependoviruses are capable of establishing a latent infection by integrating their genomes into the genome of the host cell.
2. The genus *Erythrovirus* comprises autonomous parvoviruses that do not require a helper virus, and have a pronounced tropism for erythroid progenitor cells. Human pathogenic parvovirus B19 causes fifth disease (erythema infectiosum) and belongs to this genus.

Table 20.1 Characteristic prototypes of parvoviruses

Subfamily	Genus	Human virus	Animal virus
Parvovirinae	*Parvovirus*		Feline panleucopenia virus[b]
			Canine parvovirus (canine parvovirus type 2)[b]
			Mink enteritis virus[b]
			Porcine parvovirus
			Minute virus of mice
	Erythrovirus	Parvovirus B19	Cynomolgus monkey parvovirus Rhesus macaque parvovirus Pig-tailed macaque parvovirus Chipmunk parvovirus
	Partetravirus[a]	Human parvovirus 4	Hong Kong virus *Eidolon helvum* parvovirus Parvovirus 4 like viruses from chimpanzee, gorilla
	Bocavirus	Human bocavirus	Bovine parvovirus (type 1) Canine minute virus (type1: Bat bocavirus)
	Amdovirus		Aleutian mink disease virus
	Dependovirus	Adeno-associated viruses 2, 3 and 5	Bovine adeno-associated virus
			Monkey adeno-associated viruses 1 and 4
			Goose parvovirus
Densovirinae	*Densovirus*		Galleria mellonella densovirus
	Iteravirus		*Bombyx mori* densovirus
	Brevidensovirus		*Aedes aegypti* densovirus *Culex pipiens* densovirus
	Pefudensovirus		*Periplaneta fuliginosa* densovirus

[a]Proposed genus
[b]Feline panleucopenia virus, canine parvovirus and mink enteritis virus are frequently summarized by the collective term "feline parvoviruses"

3. The genus *Parvovirus* contains animal pathogenic viruses, which can cause serious diseases in domestic and farm animals. They are usually very host-specific, and transmissions to humans are not observed. In contrast to the erythroviruses, they principally cause enteritis and myocarditis. In addition to canine parvovirus, which causes the most important infectious disease of dogs – and apart from the pathogen of feline panleucopenia – porcine parvovirus warrants special mention. It is the cause of economically important fertility disorders in swine. Other members of the genus *Parvovirus* are of minor significance, even though in individual cases they can induce a clinically apparent infection in the corresponding animal species. Minute virus of mice is a well-studied prototype. Most molecular biological processes concerning replication and transcription have been elucidated in this model virus, which does not play

a role as an animal pathogen. This viral species comprises several strains which predominantly cause asymptomatic infections in mice.

4. The genus *Amdovirus* also comprises solely animal pathogenic viruses: the prototype is Aleutian mink disease virus, which infects minks, causing Aleutian mink disease. It is considered a serious threat in regions where fur farming is of economic importance.
5. Infections with viruses belonging to the genus *Bocavirus* cause respiratory and gastrointestinal disorder in animals and humans. In addition to some animal pathogenic species, such as bovine parvovirus and canine minute virus, human bocavirus has also been discovered recently, and its infections in humans may be associated with disease, especially in young children.

The genus *Partetetravirus* has been proposed for human parvovirus 4, which has been discovered in human blood and blood products. Symptoms or diseases have not been correlated with the acute infection. Similar viruses have been isolated from several mammals, e.g. chimpanzees, gorillas, pigs, wild boars (Hong Kong virus) and sheep.

Integration of viral DNA into the genome of the host cell has not been found in any members of the subfamily *Parvovirinae* except the dependoviruses; all are able to replicate independently of helper viruses.

The subfamily *Densovirinae* is divided into four genera, which include the parvoviruses of insects.

20.1.2 Structure

20.1.2.1 Virus Particle

Viral capsids have an icosahedral structure and a diameter of 18–26 nm (Fig. 20.1). In some cases, the capsid structure has been determined by X-ray crystallography. With the exception of parvovirus B19, they possess protein projections at their vertices, which are about 7 nm long. The particles of autonomous parvoviruses consist of 60 capsomers composed of proteins VP2 and VP1 in a ratio of 95 % and 5 %, respectively. VP2 is identical to the carboxy-terminal region of VP1. Some parvoviruses contain a third capsid protein (VP3) in differing amounts. It is generated by proteolytic cleavage of VP2 during maturation of minute virus of mice. In adeno-associated viruses, VP3 is an amino-terminal truncated version of VP1, which is synthesized by using the rare ACG codon as a translation start site (Fig. 20.3c). The virions are non-enveloped. The viral genome resides inside the capsid. It is associated through 11 nucleotides with the internal moiety of VP2 proteins.

20.1.2.2 Genome Organization and Structure

Parvoviruses have a single-stranded, linear DNA genome with a length of 4,860, 5,217 and 5,594 nucleotides in adeno-associated virus 2 (AAV-2), human bocavirus and parvovirus B19, respectively. Whereas the capsids of dependoviruses and erythroviruses (parvovirus B19) can contain DNA strands with both polarities in about equal ratio, members of the genus *Parvovirus* preferentially package genomes,

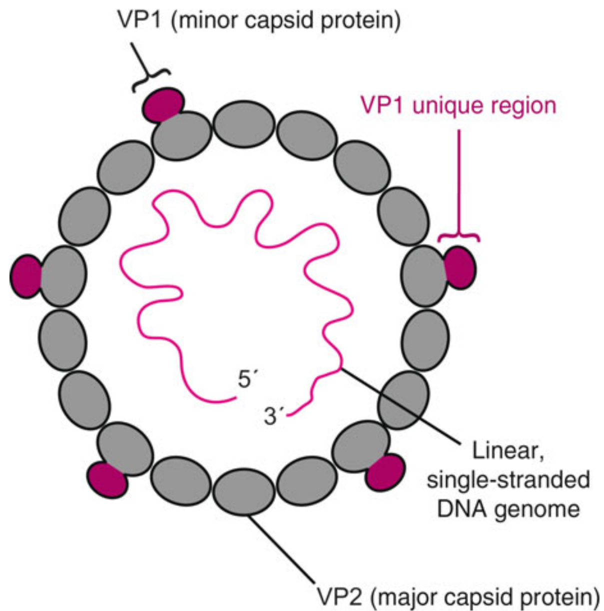


Fig. 20.1 Structure of a parvovirus particle (parvovirus B19)

which are complementary to the messenger RNAs (mRNAs) that are synthesized during infection. It is still unknown whether bocavirus and amdovirus particles contain single-stranded genomes with two polarities or only one type. Palindromic sequences are located at both termini, they flank the genome and are referred to as inverted terminal repeats (ITR). These sequences can fold into T- or Y-shaped structures, which facilitate the formation of double-stranded hairpin loop structures at the ends of the genome (Fig. 20.2a). In parvovirus B19 and adeno-associated viruses, both ITR sequences are complementary to each other, and have a length of 383 and 145 nucleotides in parvovirus B19 and AAV-2, respectively. They can hybridize with each other and keep the genome in a quasi-circular structure containing a panhandle-like double-stranded region (Fig. 20.2b). The ITR regions of members of the genera *Parvovirus* and Bocavirus have a length of 115–300 nucleotides, and their 3′- and 5′-terminal sequences are not complementary. For example, the 5′ ITR of minute virus of mice contains 207 nucleotides, whereas the 3′ ITR contains only 115 nucleotides.

The nucleotide sequence of parvoviruses is less variable than that of other viruses. Sequence differences found in different parvovirus B19 isolates are clustered in the regions encoding the carboxy-terminal end of the NS1 protein and the amino-terminal domain of VP1. The genomic arrangement is similar in all parvoviruses (Fig. 20.3): Two large open reading frames are located in the coding DNA strand, which is complementary to the mRNA. The *rep* gene locus, which is located in the 3′ half of the viral genome, encodes non-structural proteins involved in both viral DNA replication and regulation of gene expression. These proteins are the non-structural NS1 protein in parvovirus B19, two differently sized forms of a non-structural protein, NS1 and NS2, in animal pathogenic parvoviruses and a family of

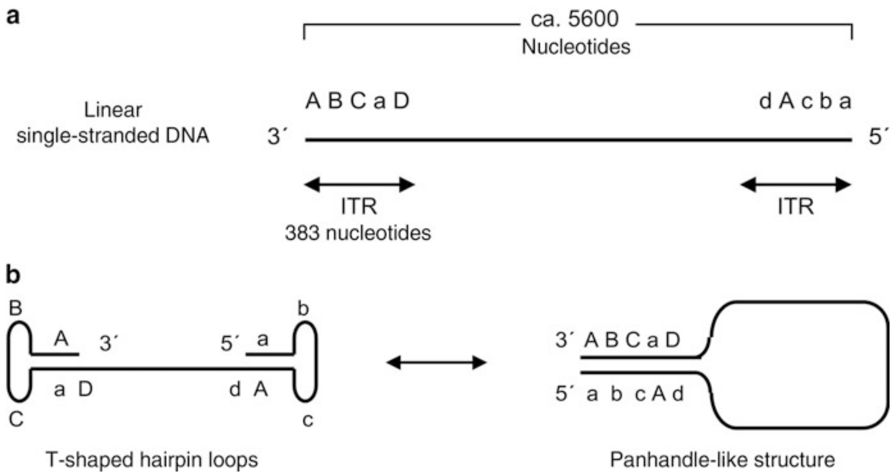


Fig. 20.2 The genome of parvovirus B19. (**a**) The single-stranded, linear DNA genomes are flanked by inverted terminal repeats (*ITR*) of 383 nucleotides in length (they are 145 nucleotides long in adeno-associated virus 2). The *upper-case letters* and *lower-case letters* represent DNA sequences that are complementary to each other. (**b**) Inasmuch as not only the sequences of the terminal 3′ and 5′ ITRs are complementary to each other, but also short regions within the repeats, T-shaped hairpin loops can be formed by the generation of double-stranded regions. Alternatively, the genomes can also adopt panhandle-like structures

four Rep proteins in adeno-associated viruses. Apart from the gene encoding the non-structural protein NS1, bocaviruses contain additional reading frames that direct the synthesis of NS2 and NP1 proteins using alternative start codons. The genes encoding structural proteins are situated in the 5′ half of the viral genome. In parvovirus B19, there are two additional, short open reading frames in the central region and at the 5′ end of the genome which code for small non-structural proteins (7.5 kDa, 11 kDa/NS2).

The promoters that control gene transcription are arranged differently in the various viruses: in erythroviruses, bocaviruses and amdoviruses, all mRNA species are initiated from a common promoter at the 3′ end of the genome. The mRNA molecules, from which the various structural and non-structural proteins are translated, are formed by alternative splicing (Fig. 20.3a, d). There are two polyadenylation sites in the genome of these viruses, one of which is located at the 5′ terminus and the other is situated in the middle of the genome. In addition, splicing is regulated by cell-specific influences: e.g. non-permissive cells synthesize a bicistronic mRNA, which contains sequences upstream of the genes encoding structural proteins; it is generated by using unusual splice sites. This leads to the synthesis of a product in which the amino-terminal end of the NS1 protein is linked to the 11-kDa NS2 polypeptide. In contrast, the autonomous viruses of the genus *Parvovirus* (minute virus of mice) possess two promoters: one promoter is also located at the 3′ end of the genome, and controls the transcription of non-structural genes (*NS* or *rep*); the second, centrally located promoter (genome position 38) is responsible for

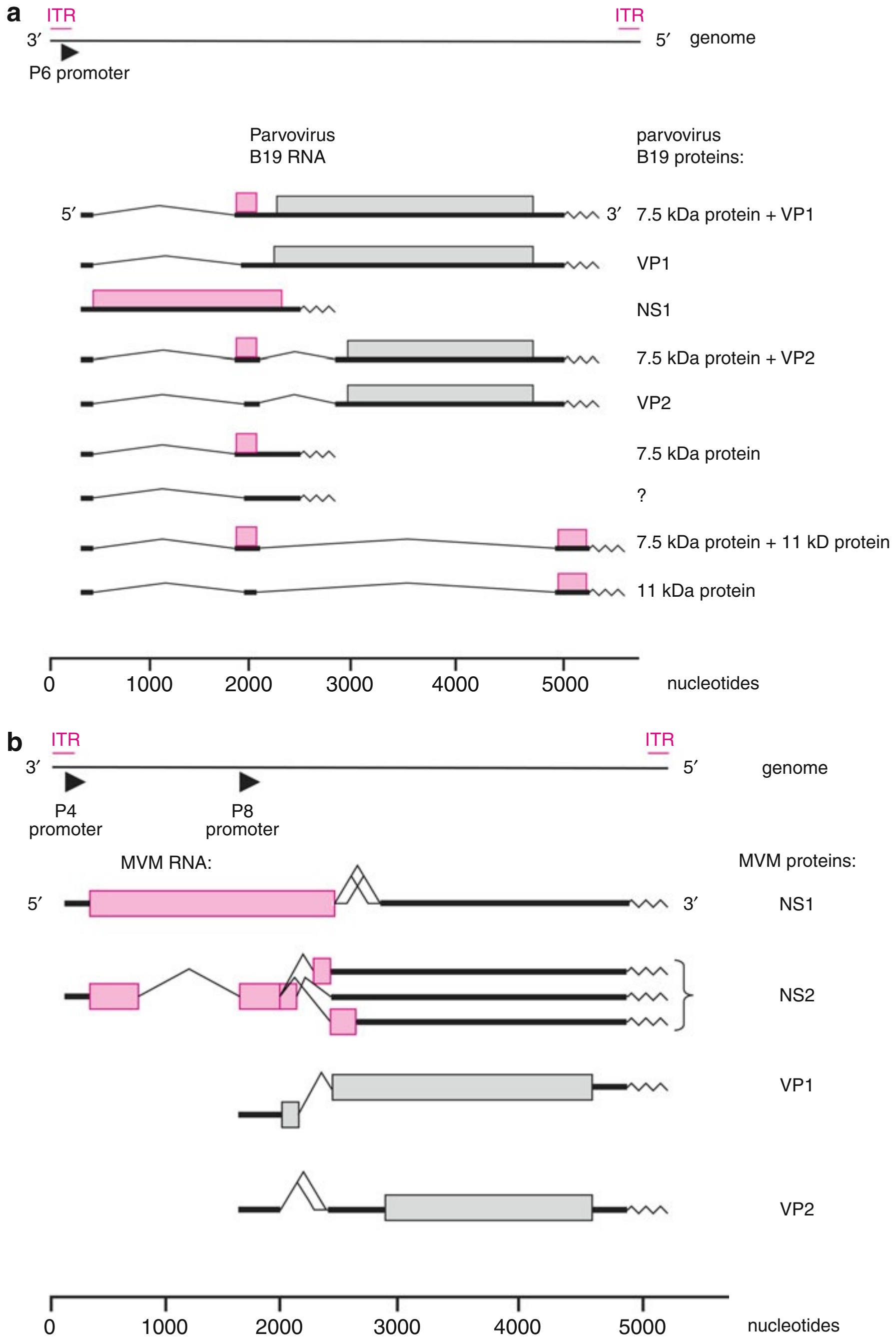


Fig. 20.3 (continued)

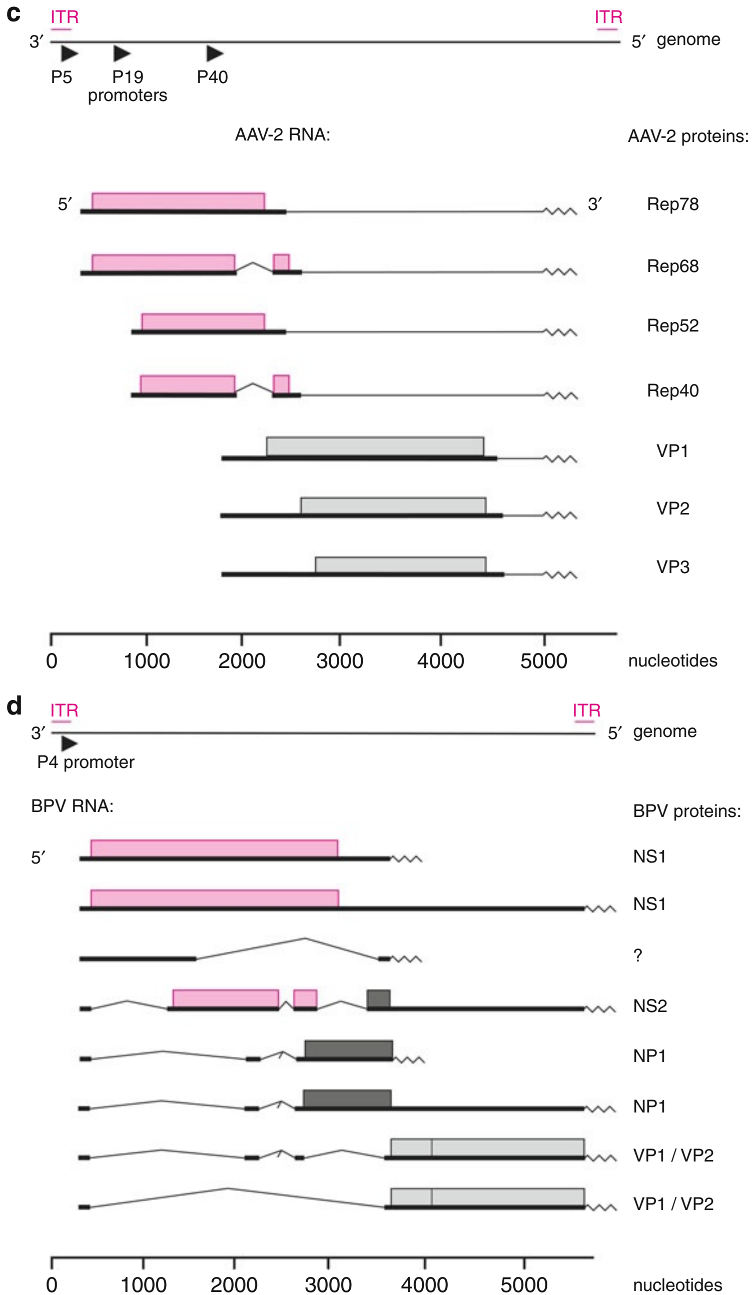


Fig. 20.3 Genome organization transcription and translation in parvoviruses. The genomes, including the positions of ITR elements and promoters that regulate transcription, are shown in

transcribing the genes that encode structural proteins. These viruses have only one polyadenylation site at the end of the genome, and it is used for termination of all transcripts (Fig. 20.3b).

Adeno-associated viruses also use a common polyadenylation site at the end of the genome for all mRNA species. Their genome contains three promoters. The promoter at the 3′ end of the genome (position 5) regulates the synthesis of the mRNA for the Rep78 and Rep52 proteins. The latter is translated from a spliced mRNA. The promoter at position 19 controls the transcription of genes that encode the Rep68 and Rep40 proteins. Rep40 is also translated from a spliced mRNA. In both cases, the splicing process leads to excision of the same intron sequence in Rep52 and Rep40. The synthesis of mRNA species coding for capsid proteins is under the control of the third promoter, which is located at genome position 40 (Fig. 20.3c). Hence, gene expression in the individual virus types is regulated differentially and specifically by the different localization of transcriptional control elements in combination with alternative splicing, despite the similar gene arrangement found in all parvoviruses.

20.1.3 Viral Proteins

20.1.3.1 Structural Proteins

Two structural proteins compose the capsids of parvoviruses: VP1 (molecular mass 80–86 kDa) and VP2 (58–62 kDa). VP2 is identical to the carboxy-terminal region of VP1; the VP1 proteins of parvovirus B19 and human bocavirus have 227 and 129 additional amino acids at the amino terminus, respectively (VP1 unique region). For the synthesis of VP2, the VP1-specific mRNA is spliced. The excision of the several-hundred-nucleotide-long intron leads to removal of the start codon of the VP1 protein. Therefore, the translation of VP2 starts at another AUG codon in the same reading frame (Fig. 20.3a, d). The regions necessary for particle formation are located in the VP2 protein, and in the corresponding VP2-specific part of VP1. Therefore, both proteins are present as capsomers of the virions. The VP1 unique region of parvovirus B19 is exposed on the capsid surface. Amino acid sequence variations are frequently found in the amino-terminal region. Most neutralizing antibodies are directed against epitopes in this protein domain. However, part of the neutralizing immune response is also directed against the VP2

Fig. 20.3 (continued) the *upper part* of (**a**–**d**). *Underneath*, the different transcripts from which the viral proteins are translated are depicted (*red* structural proteins, *grey* non-structural proteins). The exons are indicated by thick lines, the excised introns by *thin lines* and the 3′ poly (A) tail by *serrated lines*. The regions of messenger RNAs coding for proteins are shown as *bars*. The 7.5- and 11-kDa proteins of parvovirus B19 have not yet been definitively demonstrated. (**a**) *Erythrovirus* – parvovirus B19. (**b**) *Parvovirus* – minute virus of mice (*MVM*), (**c**) *Dependovirus* – adeno-associated virus 2 (*AAV-2*), (**d**) *Bocavirus* – bovine parvovirus type 1 (*BPV*)

Table 20.2 Properties and functions of parvovirus proteins

	Molecular mass				
Protein	**Parvovirus B19**	**Adeno-associated virus 2**	**Minute virus of mice**	**Human bocavirus**	**Function**
VP1	83 kDa	87 kDa	85 kDa	72 kDa	Capsid protein, PLA2-like activity in the VP1 unique region
VP2	58 kDa	62 kDa	68 kDa	~60 kDa	Capsid protein, main component
VP3	–	73 kDa	65 kD	Synthesis not demonstrated[a]	Capsid protein
NS1	71 kDa (phosphorylated, NTP-binding)	–	80 kDa (phosphorylated, NTP-binding)	90–95 kDa[b]	Helicase, ATPase, endonuclease, transactivator, initiation of genome replication
NS2	11 kDa	–	20–28 kDa	Synthesis not demonstrated[a]	Several isoforms, required for viral infectivity
NP1	–	–	–	28–30 kDa[b]	?
7.5 kDa	7.5 kDa	–	–	–	?
Rep78	–	78 kDa	–	–	Transactivator, helicase, endonuclease, tumour suppressor, genome integration
Rep52	–	52 kDa	–	–	?
Rep68	–	68 kDa	–	–	Transactivator, helicase, endonuclease, tumor suppressor, genome integration
Rep40	–	40 kDa	–	–	?

PLA2 phospholipase A2
[a]The synthesis of this protein has not been demonstrated in bocaviruses so far.
[b]Molecular masses of the non-structural proteins of human bocaviruses are not known; the data shown were taken from bovine parvovirus, whose reading frames have a similar size.

moiety. The VP2 protein of parvovirus B19 binds to globoside (erythrocyte P antigen), which is used as a cellular receptor. The carboxy-terminal domain of the VP1 unique region has a phospholipase A2 like enzyme activity. Table 20.2 summarizes the various functions and properties of parvovirus proteins.

The VP3 protein of minute virus of mice is a degradation product of VP2 and is generated by proteolytic cleavage of about 20 amino acids at the amino terminus. In the case of adeno-associated viruses, in the same reading frame there is a rare

upstream start codon of the VP1-specific mRNA which is used for the synthesis of VP3 (73 kDa). Thus, its amino terminus is located between those of the VP1 and VP2 proteins.

20.1.3.2 Non-Structural Proteins

Minute Virus of Mice

The non-structural NS1 and NS2 proteins of parvoviruses are encoded by the *rep* gene cluster. The NS2 protein constitutes a heterogeneous protein group that is translated from spliced mRNA molecules. Starting from the splice donor site, these mRNAs are translated in all three possible reading frames (Fig. 20.3b). NS proteins have essential functions in viral DNA replication. NS1 is a sequence-specific endonuclease that is active during genome replication. It cleaves the double-stranded DNA within ITR elements, which serve as the origin of replication, thus generating a nick that provides a free 3′-OH end that serves as a primer for DNA synthesis. NS1 remains covalently linked to the 5′ termini of newly synthesized genome intermediates during replication. Furthermore, it has been shown that this protein acts as an ATP-dependent helicase in vitro. This enzyme activity is necessary for unwinding of DNA duplexes at the origin of replication. Moreover, the NS1 protein acts as transactivator of viral promoters, especially of the central promoter that controls the expression of capsid genes, e.g. in members of the genus *Parvovirus*. In addition, it also influences cellular promoters, whereas it does not bind to the sequence elements, but it activates or represses them by interacting with cellular factors. Both non-structural proteins possess nuclear transport signals, and the larger NS1 protein is phosphorylated at several positions by cellular protein kinase C. Point mutations in the non-structural genes altering the phosphorylation pattern lead to replication-defective viruses, decreased helicase activity and a reduction of the cytotoxic effect of NS1. The cytotoxic effect of the NS1 protein is probably associated with the ability to arrest the cell cycle at the G_1, S or G_2 phase. It has been demonstrated that NS1 interacts with the catalytic α subunit of casein kinase 2. This process interferes with intracellular signal transduction pathways, and gives rise to the development of the typical cytopathic effect. In particular, interruption of the cycle in the G_2 phase seems to be caused by the ability of the NS1 protein to induce the expression of cellular protein p21 (Waf1/Cip1), which functions as an inhibitor of cyclin A dependent kinase. Furthermore, the NS1–casein kinase 2 complex is responsible for phosphorylation of capsid proteins.

The function of NS2 proteins is less well explored: they interact with the nuclear export protein Crm1, and have a very short half-life; furthermore, they are degraded by a ubiquitin-independent pathway through the proteasome. Probably, they act as helicases, and are involved in strand displacement during genome replication.

Parvovirus B19

Parvovirus B19 expresses only one non-structural protein, NS1 (71 kDa). It is translated from an unspliced mRNA that is terminated at the central polyadenylation signal. NS1 is a phosphorylated, multifunctional protein that exerts its activities in

the nucleus. Accordingly, the NS1 protein carries a nuclear localization signal andis essential for genome replication. It is presumably responsible for the formation of the initiation complex at the origin of replication, which is composed of the cellular DNA polymerase and other additional host factors. Furthermore, the ATP-dependent helicase and endonuclease activities of NS1 are also involved in viral replication. In addition, NS1 is a transcriptional activating protein; it induces the parvovirus B19 specific promoter p6 by interacting with the cellular transcription factors Sp1, Sp3 and YY1 and possibly other cellular transcription factors. It is also capable of influencing cellular promoters such as the IL-6 promoter. It induces cell cycle arrest in the G_1 phase, a process that is associated with an increased expression of p53 as well as the cyclin-dependent kinase inhibitors p21/Waf1 and p16Ink4. The NS1 protein also interacts with the cellular transcription factor Sp1 to upregulate the respective promoter activities. NS1 protein expression is toxic in eukaryotic cells. The toxic effect is probably associated with its ability to induce the synthesis of the proapoptotic factors Bax and Bad (members of the Bcl-2 protein family) as well as that of a variety of cellular caspases (caspases 3, 6, 8 and 9), and its ability to induce the TNF- and Fas-dependent apoptosis pathway in infected cells.

The function of the 11-kDa NS2 protein of parvovirus B19, which codes at the 5′ end of the genome and is translated from a twofold spliced mRNA, has not been finally resolved. It appears to be required in the production of infectious progeny viruses, and to influence both the synthesis and the intracellular transport of capsid proteins. When the synthesis of the 7.5-kDa protein, which is encoded by a short reading frame located upstream of the genes coding for structural proteins, is prevented, there is no reduced viral production. Thus, the function of the 7.5-kDa protein in the infection cycle remains mysterious.

Adeno-Associated Viruses

Adeno-associated viruses synthesize four different non-structural proteins. Rep78 and Rep52 differ by an internal deletion, which is based on the use of a spliced mRNA for translation (Fig. 20.3c). The same is true for Rep68 and Rep40, which are translated from transcripts whose synthesis is controlled by the promoter at genome position 19. They are amino-terminally truncated protein variants of Rep78 or Rep52, respectively. Therefore, all Rep proteins share large homology regions. It is believed that further splicing events may contribute to the generation of additional Rep protein variants.

Rep78 and Rep 68 are localized in the nucleus. Their functions in replication correspond in many respects to those of NS1 proteins of animal parvoviruses. The amino-terminal domains of Rep78 and Rep68 are essential for genome replication of adeno-associated viruses: the 208 amino-terminal residues harbour the endonuclease domain, which cleaves double-stranded DNA at the terminal resolution site (nucleotide 125), within ITR elements during replication. In contrast to the NS1 protein of minute virus of mice, Rep proteins of adeno-associated viruses do not remain associated with the nascent 5′ termini. In vitro, an ATP-dependent helicase activity has been demonstrated to be associated with the large Rep proteins, and is capable of unwinding not only DNA–DNA double strands,

but also DNA–RNA heteroduplexes. Rep78 and Rep68 also transactivate viral promoters by interacting with cellular transcription factors – such as the cellular protein PC4 – and factors encoded by helper viruses, especially the immediate early proteins of herpesviruses or adenoviruses. In addition, Rep68 and Rep78 also recruit the single-strand-binding ICP8 protein of herpesviruses to the centres of adeno-associated virus replication, and thus presumably inhibit herpesvirus replication. Apart from adenoviruses and herpesviruses, human papillomaviruses can also provide helper functions: The E1 protein of human papillomavirus 16 – also a helicase – binds to Rep78, resulting in modulation of enzyme function. During latency of adeno-associated viruses, i.e. during non-productive infection in the absence of helper viruses, in which the viral genome is integrated in the chromosomal DNA of the cell, a repressing effect has been attributed to Rep proteins, especially on the promoter of capsid protein genes. The expression of cellular and other viral promoters is also negatively influenced by Rep78 and Rep68, which repress the activity of the long terminal repeat promoter of human immunodeficiency virus 1 and that of the c-H-*ras* promoter.

To what extent the functions of the large Rep proteins are related to the property of adeno-associated viruses to suppress uncontrolled cell proliferation of various origins, and thus act as tumour suppressors, has not yet been conclusively resolved. Independently of the presence of p53 in infected cells, Rep78 induces apoptosis by activating caspase 3. Recently, it has also been discovered that Rep78 prevents phosphorylation of retinoblastoma protein by a still unknown pathway, thereby stabilizing the RB105/E2F complex. This leads to an inhibition of E2F protein function, thereby blocking the induction of specific cellular proliferation genes. Moreover, Rep78 binds to EF2-dependent promoters, thus additionally impeding E2F binding to the promoters. As a result, the cells are arrested in the S phase of the cell cycle. In this regard, Rep78 acts as an antioncogene, functioning conversely to the oncogenic proteins of hepatitis B, polyomaviruses, papillomaviruses, adenoviruses and herpesviruses (▶ Sects. 19.1–19.5).

In vitro, during establishment of the latent state in the absence of helper viruses, the genome of adeno-associated viruses integrates specifically into the 19q13.3-qter locus of human chromosome 19. The proteins Rep78 and Rep68 are involved in this site-specific integration. They interact concomitantly with Rep-binding sites (RBS) located in the ITR elements at the ends of the genome, and with RBS-like sequences at the integration site (AAVS1) on chromosome 19 via a second protein domain. They bring the viral genome close to the chromosomal DNA of the cell, and cleave one DNA strand by their endonuclease activity.

The two smaller Rep52 and Rep40 proteins are found predominantly in the cytoplasm. They also possess helicase activity, which is required during morphogenesis. They bind to the preassembled capsids of adeno-associated viruses, and bring about the introduction of the single-stranded DNA genomes in 3′ to 5′ direction. Hence, Rep proteins are versatile and multifunctional. Since these activities are performed in interaction with cellular and helper-virus-specific proteins, they are dependent on the type and differentiation state of the infected cell and the presence of certain functional activities of helper viruses.

20.1.4 Replication

20.1.4.1 Parvovirus B19 and Other Autonomous Parvoviruses

Parvoviruses can replicate only in cells that are in the S phase of the cell cycle. Unlike papillomaviruses, polyomaviruses or adenovirus (▶ Sects. 19.2–19.4), they can not stimulate resting cells to divide. Expression and replication of the viral genome are highly dependent on host cell factors. This explains the narrow cell and host tropism of the virus. The various parvoviruses bind to different receptors on the cell surface of the respective target cells: parvovirus B19 infects erythroid progenitor cells in the bone marrow, and interacts with the erythrocyte P antigen, a glycosphingolipid that is exposed on the cell surface. This interaction is mediated by surface structures of the capsid protein VP2. Integrin $\alpha_5\beta_1$ and the autoantigen Ku80 are being discussed as possible co-receptors. Animal pathogenic feline parvoviruses (feline panleucopenia virus and canine parvovirus) use the transferrin receptor for attachment. By contrast, glycophorin A has been identified as a receptor for bovine parvoviruses, which bind to terminal sialic acid residues that are present as a modification on this surface component of erythrocytes. Minute virus of mice also uses *N*-acetylneuraminic acid residues as a cellular interaction partner.

After attachment, bovine parvovirus particles enter the cells probably by receptor-mediated endocytosis. In feline parvovirus, it is considered as proven that the bound virus particles accumulate in areas containing clathrin-coated pits and clathrin-coated vesicles, and that dynamin, a component of intracellular filaments, is involved in the particle internalization process. The phospholipase A2 like activity of parvovirus VP1 proteins, which is necessary for their infectivity, facilitates the release of virus particles from the endosomes. There is only little information concerning the further transport of virus particles or viral genomes into the nucleus, where viral gene expression and genome replication occur.

In the nucleus, the single-stranded DNA genome is converted into a double-stranded molecule using the OH group at the 3′ ITR as a primer, and is transcribed by the cellular RNA polymerase II. In minute virus of mice, it has been shown that the promoter of *NS* genes is supported by binding of a viral structural component. It is postulated that this component, similar to the α-transinducing factor, a tegument protein of herpes simplex virus, acts in combination with cellular factors to facilitate transcription of immediate early genes (▶ Sect. 19.5). The resulting mRNAs are capped, partially spliced and polyadenylated. After translation, NS proteins, as well as capsid proteins, are transported into the nucleus using their transport signals, where the NS proteins are required for both DNA replication and induction of transcription of genes encoding structural proteins. Thus, they are an essential prerequisite for the further replication process, which occurs in delimited nuclear areas in autonomous parvoviruses (parvovirus-associated replication bodies).

In contrast to cellular DNA synthesis, no RNA primers are needed for viral genome replication. This function is performed by the 3′-OH group of the ITR region at the 3′ terminus of the single-stranded DNA genome. A lagging-strand synthesis does not occur. The model described here has been developed for

replication of the genome of minute virus of mice. Whether this model can be applied in all aspects to parvovirus B19 is not known. Because of its pronounced tropism for erythroid progenitor cells, it has not been possible to cultivate this virus, and therefore its genome replication mechanisms have not been investigated. The ITR sequences, which form double-stranded hairpin loop structures at the ends of the genome, are essential for initiation of DNA replication (Fig. 20.2). The 3′-OH group of the 3′ ITR serves as a primer for the first polymerization reactions, which are probably catalysed by the cellular DNA polymerase δ complex. The result is an intermediate product which is present as a nearly completely double-stranded DNA molecule, with the exception of a short single-stranded region at the T-shaped ITR structure that remains covalently closed. In the next step, the sequence-specific endonuclease activity of the NS1 protein generates a nick at the terminal resolution site within the 5′ palindrome. The NS1 protein of minute virus of mice remains covalently linked to the newly generated 5′ end of the nick. The secondary structures at the end of the genome are resolved by unwinding by the ATP-dependent helicase, and the 3′-OH end of the nick provides the necessary primer to start the synthesis of the complementary strand by a single-strand-displacement DNA replication mechanism (Fig. 20.4). The replication fork progresses along the template strand, thus generating a replication intermediate that comprises two single-stranded genomes in double-stranded configuration. The nuclease activity of the NS1 protein cleaves the dimeric genomes at the respective terminal resolution site, and the resulting 3′-OH ends are used for initiation of new polymerization and strand-displacement reactions. The displaced DNA strands interact with the structural proteins to shape precursor capsid forms.

Viral morphogenesis has also been scarcely examined. It is not known whether the DNA is introduced into a preformed capsid structure or whether the proteins attach to a condensed form of the genome. The newly assembled capsids are detectable as nuclear inclusion bodies only a few hours after infection. Later, they are also found in the cytoplasm and in evaginations of the cell membrane through which the particles are partially released by the cell. In these vesicles, viruses are protected from attack by the immune system and can be easily spread throughout the body. However, most virions are released by apoptosis of infected cells.

20.1.4.2 Adeno-Associated Viruses

Productive Viral Replication

Adeno-associated viruses infect various human epithelial cells. The receptor of adeno-associated virus 5 is, like for certain influenza A viruses, *N*-acetylneuraminic acid (sialic acid), which is linked with oligosaccharides as a modification of cell surface proteins by α-2,3 glycosidic bonds (▶ Sect. 16.3.3). Capsids of AAV-2 have been found to bind to heparan sulphate. There is evidence that $\alpha_v\beta_5$ integrins and/or the human fibroblast growth factor receptor act as co-receptors during attachment. Furthermore, an interaction of AAV-2 capsids with nucleolin has also been described. This protein (110 kDa) functions as a transport protein between the cytoplasm and the nucleolus during cellular processes such as ribosomal RNA synthesis and ribosome assembly. Therefore, nucleolin may be involved in the

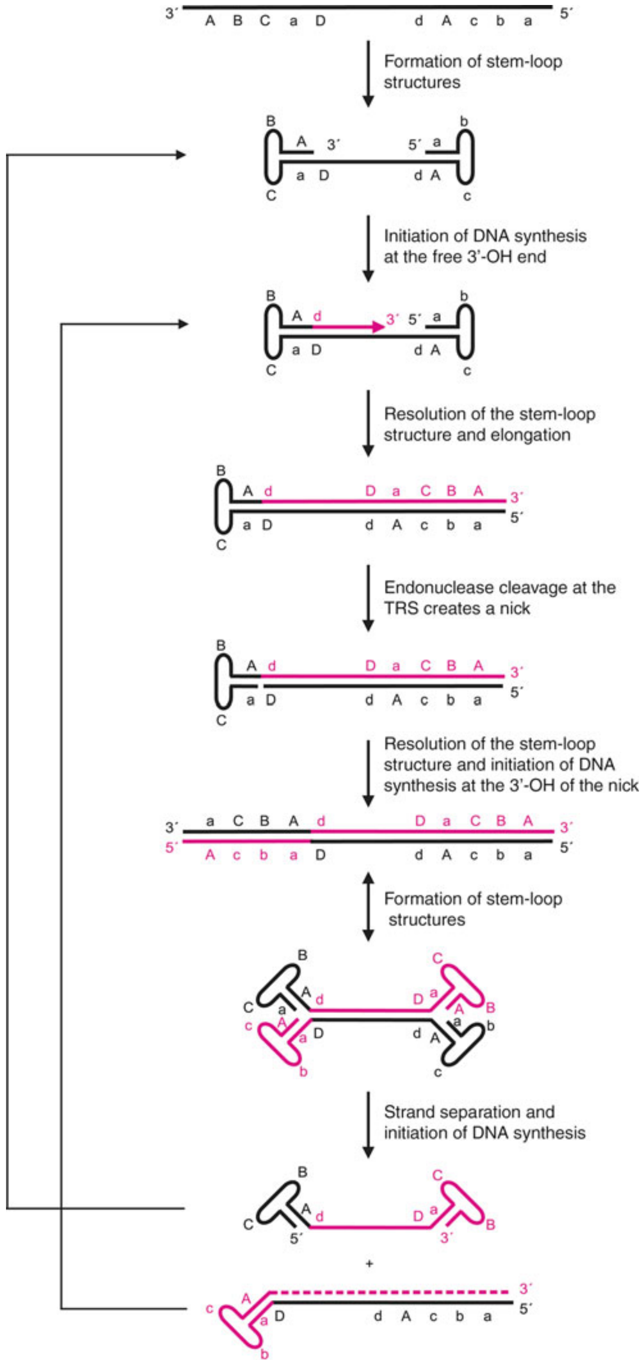


Fig. 20.4 (continued)

transport of virus particles into the nucleus. A similar interaction has also been described for capsid proteins of coxsackievirus B5 (▶ Sect. 14.1). The productive replication process is very similar to that of autonomous parvoviruses. The $3'$-OH ends of the terminal palindromes are also used as primers in adeno-associated viruses. Further steps are DNA polymerization, strand displacement and synthesis of a double-stranded replication intermediate.

The major difference from the replication of autonomous parvoviruses is the fact that adeno-associated virus replication is dependent on the simultaneous infection with a helper virus (adenovirus or herpesvirus). The presence of certain proteins, especially the immediate early gene products of helper viruses, is a prerequisite for DNA replication of adeno-associated viruses, and thus for production of infectious progeny viruses. Particularly, adenoviral E1A proteins are involved in transactivation of adeno-associated virus promoters. Possibly, the 35-kDa E4 protein is also involved, in complex with the E1B protein, in the process that regulates viral mRNA export from the nucleus to the cytoplasm (▶ Sect. 19.4). This assistance seems to impair adenovirus replication. Even the oncogenic properties of E1A and E1B proteins are neutralized.

Latent Infection

In the absence of helper viruses, adeno-associated viruses can establish persistent infections and remain in the organism for life. In vitro, it has been found that the genome of adeno-associated viruses is integrated in human chromosome 19 by a site-specific recombination mechanism during latency. Two genome copies remain at the integration site and are present in tandem. Intact ITR elements and the large non-structural proteins Rep78 and Rep 68 are required for the integration process. The integrated genome can be reactivated from latency by infection with an appropriate helper virus. A similar site-specific integration of the adeno-associated virus genome does not seem to occur in vivo.

Gene Therapy Vectors Are Derived from Adeno-Associated Viruses

Since adeno-associated viruses possess the unique ability to integrate stably into the human genome, infection with them does not cause severe genetic consequences and their reactivation from latency is dependent on the

Fig. 20.4 Parvovirus genome replication model. DNA synthesis is catalysed by cellular enzymes and is initiated at the $3'$-OH end of the ITR element, which is folded into a stem-loop structure. With progressive elongation, the secondary structure of the $5'$-terminal palindrome is resolved and DNA synthesis continues until the end of the genome. The recognition site for the endonuclease activity of the NS1 protein resides at the transition of the double-stranded $3'$ ITR to the single genome sequences; it generates a single-strand nick. This creates a $3'$-OH end, which serves as a primer to initiate DNA synthesis, which continues until the other end of the genome. The result is a completely double-stranded DNA genome. Subsequently, the ITRs fold into stem-loop structures, thus providing new initiation sites for DNA replication. Newly synthesized sequences are depicted in *red*. *TRS* terminal resolution site

presence of helper viruses, they appear to be suitable vectors for gene therapy approaches. In such cases, the genes for structural proteins or the entire coding region is replaced by the desired heterologous gene to be expressed. The chimeric viral genome is packaged into capsids in so-called helper cells, which constitutively synthesize the deleted viral functions, and are infected with a helper virus. These recombinant capsids are able to transduce the chimeric genome into cells, and it can be stably integrated into the human genome via ITR elements.

20.1.5 Human Pathogenic Parvoviruses

20.1.5.1 Parvovirus B19

Epidemiology and Transmission

In 1975, Yvonne Cossart and co-workers serendipitously discovered parvovirus B19 in blood samples. A few years later, the group of Sergeant found that parvovirus B19 is the causative agent of fifth disease (erythema infectiosum), a classic childhood disease that is associated with rash. Nowadays, three genotypes of this virus are known which cause the same disease, but exhibit a different regional distribution: genotype 1 predominates in central and southern Europe, Asia and the Americas, genotype 2 is frequently found in northern Europe, particularly in elderly people, whereas genotype 3 occasionally prevails in West Africa and France. The prevalence of parvovirus B19 is high in the human population. In Western countries, antibodies against the structural proteins VP1 and VP2 have been found in 40–70 % of adults examined; the seroprevalence is approximately 65–75 % among adults In the first few days of infection, before the onset of symptoms, very large amounts of virus particles are detectable in the blood of infected individuals (up to 10^{13} particles per millilitre). In this phase, the virus is excreted in the saliva. It is transmitted by droplet infection or through contaminated blood and blood products. Because of its high stability, the virus is difficult to inactivate. It can be detected as infectious virus even in purified factor VIII and factor IX preparations for blood clotting and in highly pure samples of human serum albumin. If parvovirus B19 infects pregnant women, it can be transmitted diaplacentally to the fetus, causing hydrops fetalis, with lethal consequences.

Clinical Features

The incubation period of parvovirus infection lasts on average 1–2 weeks. In this phase, the patient is already viraemic, and can transmit the virus. The infection is frequently asymptomatic in children. The commonest clinical picture is erythema infectiosum, also known as fifth disease. It is found mainly in childhood, and is characterized by flu-like symptoms with mild fever. It is associated with a rash, which initially appears on the cheeks concurrently with the first virus-specific antibodies, it spreads further to the inner sides of the arms and legs, and usually lasts 1–2 days. Because of the viral destruction of erythrocyte progenitor cells,

all infected individuals develop a transient anaemia. Occasionally, acute parvovirus B19 infection may lead to the development of papular purpuric gloves and socks syndrome, thrombocytopenia and neutropenia or hepatitis and myocarditis; encephalitis has also been reported in individual cases. If individuals with impaired formation and maturation of red blood cells are infected (e.g. sickle cell anaemia, or thalassaemia), a severe life-threatening, aplastic crisis may be caused by the virus-induced destruction of erythrocyte progenitor cells.

Acute infections are frequently associated with arthralgia and severe inflammation of the joints. The forms of acute arthritis usually last a few weeks after infection. However, they may also cause problems for years and are often similar to rheumatoid arthritis. In these cases, a persistent parvovirus infection is the basis of protracted, parvovirus-associated arthritis, in which the viruses are present in low concentrations in the synovial fluid of inflamed joints for prolonged periods. In addition, it is found that other autoimmune diseases can be established in some patients after infection. These include various forms of vasculitis, Hashimoto's thyroiditis and autoimmune anaemia, neutropenia and thrombocytopenia. Chronic persistent infections occur especially in immunocompromised patients: they usually develop long-lasting severe diseases, particularly chronic anaemia or thrombocytopenia. The viral DNA can be detected permanently in relatively large quantities in the blood.

Other complications are caused by parvovirus B19 infection during pregnancy. An acute parvovirus B19 infection can cause spontaneous abortions during the first trimester of pregnancy. As compared with non-infected pregnant women, in pregnant women with acute parvovirus B19 infection, the spontaneous abortion rate is increased by 5–6 %. Especially in the second trimester, the virus is transmitted diaplacentally to the embryo in about one third of infections, where it infects proerythroblasts in the liver. Acute infection of the expectant mother up to and including the 20th week of pregnancy can lead to the development of hydrops fetalis (an accumulation of large amounts of fluid in the embryo, which is associated with oedema, anaemia, hydraemia and liver failure). In the case of early diagnosis, blood transfusion through the umbilical vein can prevent abortion. If the fetus develops an additional myocarditis, it usually dies. Parvovirus B19 infections are possibly also associated with intrauterine fetal death in late pregnancy. There is no evidence that parvovirus infection causes malformations of embryos.

Pathogenesis

During transmission, parvovirus B19 reaches the mucous membranes of the mouth and throat area. What cells are primarily infected is unclear. The cellular receptor for parvovirus B19, the blood group P antigen, is expressed on many cells, including endothelial cells and megakaryocytes. However, a productive infection cycle cannot occur in them. It is also unknown in which way the virus reaches its target cells, the erythroid progenitor cells of the differentiation stages erythrocyte burst-forming unit, erythrocyte colony-forming unit and erythroblasts in the bone marrow. During the early phase of infection, in which the virus multiplies in erythrocyte progenitor cells, it is present in high concentrations in the peripheral

blood (10^{11}–10^{13} particles per millilitre). This viraemic phase is attenuated by two factors: first, through the production of neutralizing antibodies that prevent further spread of the virus, and second, by the virus-induced elimination of erythroid progenitor cells, which are permissive for viral infection. Patients are temporarily anaemic in this phase. The damage to this cell population can cause severe aplastic anaemia in individuals with genetic erythropoietic disorders because these patients cannot adequately regenerate erythrocytes.

The exanthem develops concomitantly with the virus-specific antibodies. Therefore, it is assumed that immune complexes attach to the endothelium of blood capillaries and cause inflammatory reactions that cause the manifestation of rash symptoms. Alternatively, there are also hypotheses suggesting that the rash is generated by non-permissive infection of endothelial cells.

About 20 % of immunocompetent patients cannot effectively control the virus: they produce viruses for months and occasionally even for years, and these can be detected in blood. Protracted arthritis is associated with such persistent parvovirus B19 infection. Viral DNA and virus particles complexed with antibodies can be detected in the synovial fluid of inflamed joints, which can cause immunological reactions and contribute to inflammation. The occurrence of NS1-specific antibodies that are increasingly found in these patients indicates a chronic, persistent infection with a delayed and incomplete control of virus production. It is believed that the continued presence of the virus results in infection of non-permissive cells, inducing an abortive infection cycle in them. These cells do not generate infectious progeny viruses, but may produce NS1 proteins. The cytotoxic effect of NS1 protein can be manifested as can its capability of transactivating cellular promoters which regulate the expression of inflammatory proteins such as IL-6 and TNF-α. Transgenic mice carrying the parvoviral NS1 gene in their genome are more susceptible to the development of arthritis than control mice. A central role in the induction of chronic inflammatory diseases can also be ascribed to the phospholipase A2 like activity in the VP1 unique region of viral capsids, which may contribute to the continuous production of prostaglandins, leukotrienes and inflammatory mediators in the joints, and thus to the maintenance of arthritis. The enzymatically active VP1 unique region can activate synoviocytes in vitro, leading to an increased production of prostaglandin E in them. On the other hand, the involvement of autoimmune mechanisms is also conceivable as a cause of prolonged inflammation: patients frequently produce antiphospholipid antibodies, whose development may be induced and triggered by the VP1 unique region and its similarity to cellular protein–lipid complexes.

Apart from the blood of patients with chronic persistent infectious, viral genomes are also present in various tissues and organs (liver, myocardium, skin, muscle, tonsils, bone marrow, synovial tissue) of healthy people; probably, low amounts of viral genomes remain latent in endothelial cells of all individuals with a past parvovirus B19 infection. However, the underlying mechanism of this DNA latency is unclear. Whether the latent parvovirus B19 genomes can be reactivated for renewed production of viruses by other diseases or immunosuppression is also unclear.

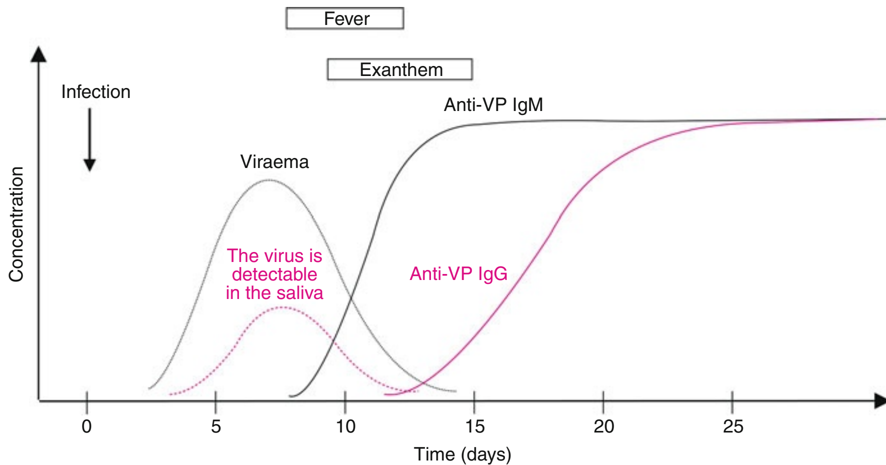


Fig. 20.5 Time course of fifth disease (erythema infectiosum) by parvovirus B19 infection including the phases in which viral proteins and IgM and IgG antibodies are detectable. *VP* viral protein

Immune Response and Diagnosis

The diagnosis is performed by detecting specific antibodies against viral structural proteins by means of ELISA or Western blot analyses (Fig. 20.5). The presence of IgG indicates a past infection. IgM antibodies are usually detectable only in fresh parvovirus B19 infections, and are preferentially directed against particulate forms of structural proteins. A renewed increase in IgM levels is occasionally found in patients with persistent infections. Viral DNA in the serum of patients with fresh or persistent infections can be detected by using polymerase chain reaction (PCR). Antibodies against the NS1 protein of parvovirus B19 can be found particularly in patients with persistent infections (protracted granulocytopenia, thrombocytopenia) and in individuals with parvovirus B19 associated persistent arthritis.

Therapy and Prophylaxis

There is no vaccine against parvovirus B19. In acutely infected pregnant women, the therapeutic focus is on the treatment of fetuses with intrauterine transfusions, which are applied when the fetal haemoglobin level decreases below 8–10 g/dl. This can generally prevent the development of hydrops fetalis in the embryo. The use of immunoglobulin products has yielded successful results in the treatment of immunocompromised patients (transplant patients) with chronic anaemia and erythroblastopenia caused by persistent parvovirus B19 infections.

20.1.5.2 Human Bocavirus

Epidemiology and Transmission

Human bocavirus was discovered by Tobias Allander in airway aspirates of children with acute respiratory disease in 2005. Sequence analysis revealed that this

virus is a member of the parvoviruses, and that it exhibits similarities to bovine parvovirus type 1 and canine minute virus, which are members of the genus *Bocavirus*, which was been named after the bovine and canine viruses that it includes. The new virus has been denominated human bocavirus, and has been assigned to the genus *Bocavirus*; four genotypes have been characterized to date (genotypes 1–4); their nucleic acid sequences coding for the non-structural proteins are conserved to 70 %. Epidemiological studies have shown that human bocavirus has a worldwide distribution, and causes diseases of the respiratory and gastrointestinal tract primarily in infants aged less than 3 years. A seroprevalence of 95 % is found in children older than 6 years and in adults. The virus is probably transmitted by droplet infection.

Clinical Features

Little is known about the incubation period until the onset of symptoms. Probably, most human bocavirus infections are asymptomatic. Genotype 1 can be detected particularly in children with acute illnesses of the lower respiratory tract (bronchitis, pneumonia). By contrast, genotypes 2–4 are preferentially found in young children with gastrointestinal disorders (diarrhoea). It has not been conclusively clarified whether human bocavirus is causally associated with these disorders because other viruses (respiratory syncytial virus, human metapneumovirus, norovirus) and bacteria (streptococci) have also been found in many of the affected children. Nevertheless, many findings suggest that human bocavirus infections are associated with diseases of the lower respiratory tract in infants.

Pathogenesis

There are no data concerning the pathogenesis of human bocavirus infection; the target cells are also unknown. The virus is found in the sputum and pharyngeal lavage as well as in the blood of children with respiratory diseases; the latter is an indication that this is not a local, but a systemic infection. The pathogen is also detectable in the stool of children with diarrhoea.

Immune Response and Diagnosis

In individuals with past infection, IgG antibodies against the capsid VP2 protein can be detected by ELISA; furthermore, $CD4^+$ cell-mediated cellular immune responses against the VP2 protein are also observable. VP2-specific IgM and viral DNA in blood or respiratory tract aspirates are indicative of acute infection.

Prophylaxis and Therapy

There is neither a vaccine nor an effective antiviral therapy.

Human Parvovirus 4

The virus was discovered in 2005. It was found in a patient who had symptoms of acute infection, and was hospitalized in a clinic in San Francisco. It was also detected by PCR in human blood plasma, especially in plasma pools of blood donors from the west coast of the USA. Analysis of the genome revealed that this virus is a parvovirus, and it received the provisional designation parvovirus 4 because it is the fourth member of this virus family which is capable of infecting humans. It has not yet been conclusively elucidated whether parvovirus 4 can be classified into the genus *Erythrovirus*. Two genotypes of this new virus have been described to date. It is unclear whether parvovirus 4 infection is associated with symptoms. Parvovirus 4 has been found sporadically in plasma pools and blood products (clotting factors) as well as in drug addicts and patients who are infected with hepatitis C virus. These data indicate a transmission by human blood.

20.1.5.3 Adeno-Associated Viruses

Epidemiology and Transmission

Adeno-associated virus 2, adeno-associated virus 3 and adeno-associated virus 5 can infect humans. They were found as a contaminant in adenovirus preparations, with the exception of adeno-associated virus 5, which was isolated from a flat penile condyloma. Adeno-associated viruses are likely transmitted along with adenoviruses by droplet infection during childhood. More than 90 % of adults are infected with these viruses, which are present in a latent stage after primary infection.

Clinical Features

Despite the high prevalence rate, no disease has been associated with adeno-associated virus infections so far.

Pathogenesis

In vivo, it is unknown what cells are infected by adeno-associated viruses, and in which cells they integrate their DNA into the genome of the host cell. In vitro, they can replicate in various epithelial cells with the assistance of a helper virus. Adeno-associated virus 2 has been detected by PCR in endometrial biopsies.

It is unclear how adeno-associated viruses inhibit tumour cell proliferation. This antioncogenic function is closely related to the activity of the proteins Rep78 and Rep68, and is probably associated with their repression effect on cellular promoters. There is evidence that adeno-associated viruses can also be reactivated by human papillomavirus, and that both virus types infect the same cells. Whether the antioncogenic effect of adeno-associated viruses plays a role in preventing

papillomavirus-associated tumours is unclear. Overexpression of Rep proteins is toxic to cells, and it cannot be excluded that some of their oncolytic properties may be based on this phenomenon.

Immune Response and Diagnosis

IgG antibodies against structural proteins can be detected by ELISA in infected individuals. IgM antibodies are found after reactivation of the virus during pregnancy. Acute infections are rarely diagnosed because they are asymptomatic.

Prophylaxis and Therapy

There is neither a vaccine nor an effective antiviral drug. They seem to be unnecessary because infections are not associated with disorders.

20.1.6 Animal Pathogenic Parvoviruses

20.1.6.1 Feline and Canine Parvoviruses

Epidemiology and Transmission

The prototypes of feline parvoviruses – feline panleucopenia virus, mink enteritis virus and canine parvovirus – are very closely related to each other: their genome sequences exhibit a homology of more than 98 %. They cause almost identical diseases in their respective hosts, mainly a haemorrhagic gastroenteritis. The evolution of these viruses can be traced in detail because canine parvovirus emerged suddenly in 1978–1979, and all data suggest that it has developed directly or indirectly from the long-known feline panleucopenia virus, possibly via infections of foxes (Fig. 20.6, ► Chap. 12).

Feline parvoviruses are transmitted by direct animal contact or indirectly through contact with virus-contaminated materials (bedding, bowls, litter boxes, etc.). Humans are considered to be a frequent source of transmission, e.g. breeders or whelp buyers, who can introduce them via contaminated clothing or the soles of shoes into other animal populations. This indirect dissemination is facilitated by the high physical stability of parvoviruses. They are capable of remaining infectious in dried faeces for months. This property was responsible for the rapid global spread of canine parvovirus in 1979: it was introduced to continents such as Australia without delay, although strict quarantine requirements were imposed for importation of dogs. The virus is also excreted in very large quantities by infected animals (up to 10^9 infectious particles per gram of faeces). Approximately 10^2–10^3 infectious viruses are sufficient to elicit an infection. Therefore, a high infection pressure can be rapidly developed in infected farms or animal shelters. Feline panleucopenia virus and mink enteritis virus infect cats and raccoon-like animals. Both viruses are considered virulent variants of the same virus because they cannot be distinguished at the level of the DNA sequence. Dogs and other canids (wolves, jackals, foxes, raccoon dogs, etc.) do not seem to be susceptible to feline parvoviruses under natural conditions. Canine parvovirus type 2 was first isolated in 1978, and triggered a devastating worldwide pandemic with millions of deaths in the dog

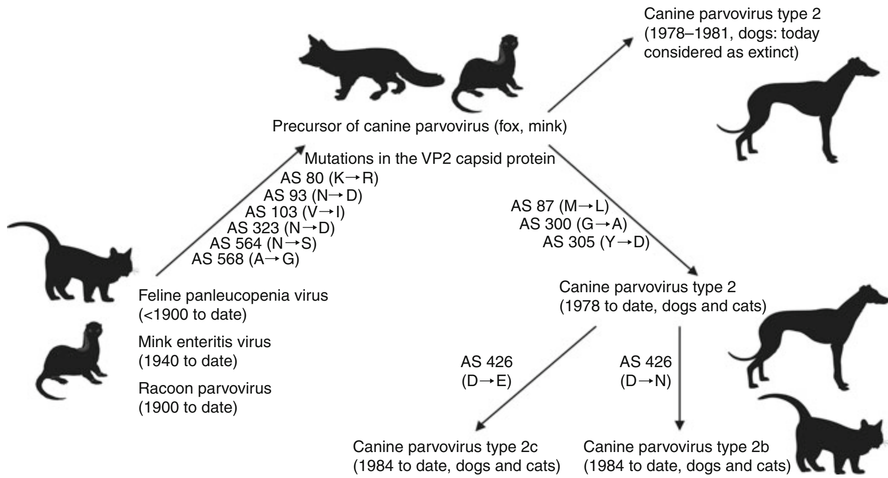


Fig. 20.6 Evolution of animal pathogenic parvoviruses. The mutations in the amino acid sequence in the structural VP2 protein which have led to the host tropism change from feline panleucopenia virus to canine parvovirus type 2 are indicated. Foxes have been postulated as hypothetical intermediate hosts. Infections with canine parvovirus type 2 were associated with lethal diseases in dogs only. Additional mutations changed the amino acid sequence of this virus, leading to canine parvovirus types 2a and 2b, which infect both cats and dogs, replacing the highly virulent type 2

population. However, it seems to be limited to dogs and other canids. The emergence of the so-called new antigenic types CPV-2a, CPV-2b and CPV-2c changed this clear picture, as these viruses can infect dogs and cats and cause a disease in both types of animals.

Clinical Features

The symptoms of feline parvovirus infections are largely identical in cats and dogs; however, there are some differences. The main symptoms are acute haemorrhagic diarrhoea, lymphopenia and a generalized leucopenia, especially in cats. In addition to these classic manifestations, young kittens which are infected with feline parvovirus during the first few days of life develop an infection of Purkinje cells of the cerebellum, which leads to cerebellar hypoplasia. The result of this injury is the clinical picture of feline ataxia: it manifests itself in an age of 3–4 weeks, when the kittens begin to run. However, dogs infected with canine parvovirus develop no cerebellar infection. If dogs are infected during the first few days after birth, they develop a myocarditis, which can lead to a complete malfunction of the heart, with a fatal outcome.

Pathogenesis

After entering the body, the virus infects the epithelial cells of the nasopharyx and regional lymphoid organs, in which the virus initially replicates, spreading then to the lymphatic organs and bone marrow via lymphocyte-associated viraemia.

The virus infects the cells of the Lieberkühn glands through the Peyer patches, i.e. the regeneration site of the intestinal epithelium. These cells are damaged by direct cell lysis, which leads to atrophy of the villi and impairment of intestinal function. The associated clinical symptom is acute haemorrhagic diarrhoea. Infected animals develop a generalized leucopenia owing to infection of the bone marrow, which is particularly pronounced in feline parvovirus infections of cats. The generalized infection of lymphoid organs leads to a marked relative lymphopenia.

Immune Response and Diagnosis

The diagnosis can be made with certainty because of the histological changes in the Lieberkühn glands. In living animals, the high-grade leucopenia and haemorrhagic diarrhoea provide clear clinical evidence. The virus can be detected by immunofluorescence of biopsy material from the infected organs, through isolation of the virus or by detecting viral DNA by PCR. Detection of the virus in living animals is easily performed by means of electron-microscopic or immunochromatographic analysis of samples of faeces. The different antigenic variants can be distinguished by haemagglutination-inhibition tests using type-specific monoclonal antibodies.

Neutralizing antibodies are the main component of the protective immunity; they function for all virus types. A prerequisite for infection of young animals is that pups originate from parvovirus-seronegative mother animals. Seropositive mothers pass on neutralizing antibodies to the pups via colostrum, and these protect the pups from infection in the first few weeks of life. A cellular immunity is proven, but the extant of its contribution to protection is unknown.

Control and Prophylaxis

Parvovirus infection in dogs and cats is controllable by vaccination. Different, highly effective attenuated live vaccines are available. Inactivated vaccines have proved to be less effective and are not used any more. The parvovirus component is contained in every standard combination vaccine.

Just a Few Amino Acid Residues of the Capsid Protein Determine the Host Range of Feline Parvoviruses

The parvovirus capsid consists of 60 proteins molecules, in which VP1 is represented by approximately six molecules and VP2 by about 54 molecules. The spatial capsid structure of both feline panleucopenia virus and canine parvovirus has been determined by X-ray crystallography. This allows the exact assessment of the location of individual amino acids in the capsid, and the determination of which amino acids are located on the surface of the virus, and what contacts individual residues have with amino acids of the same protein or with those of neighbouring proteins.

Experiments with recombinant viruses which were derived from infectious DNA clones of feline and canine parvoviruses have revealed that three amino acids are important in determining the feline host range, namely lysine at position 80, asparagine at position 564 and alanine at position 568.

They reside in a region of the capsid protein which mediates the interaction of four neighbouring molecules. In contrast, the canine host range is defined by the asparagine at position 93, alanine at position 103 and asparagine at position 323. The new virus types CPV-2a and CPV-2b differ from the originally isolated canine parvovirus by the amino acid residues at positions 87, 300 and 305 (Fig. 20.6). The differences between CPV-2a, CPV-2b and CPV-2c concern the amino acid residue at position 426, which is an aspartic acid, an asparagine or a glutamic acid. The mutated amino acids are also located in the region responsible for the intermolecular interaction, and apparently also determine the feline host range. The mechanisms that underlie this phenomenon are unclear. On the one hand, a change in the capsid stability may be associated with them, which is especially important during the release of the DNA genomes after infection of the cell. On the other hand, they might cause a slightly altered binding to the feline or canine transferrin receptors, thus determining the host spectrum.

20.1.6.2 Porcine Parvovirus

Epidemiology and Transmission

Porcine parvovirus causes clinical pictures in swine which are quite different from those induced by feline parvoviruses. The commonest manifestations of porcine parvovirus infections are fertility disorders and fetal infections, which are summarized by the term “stillbirth, mummification, embryonic death and infertility” (SMEDI). The infection of seronegative, receptive sows is economically important. The virus is transmitted by direct contact among animals or through contaminated equipment and stall accessories. It is excreted by infected asymptomatic adult pigs in the faeces. The high physical stability of the parvovirus capsid is of epidemiological importance also in this case. However, the long persistence of maternal antibodies in young animals is significant as well. These immunoglobulins can be detected up to the age of 6 months, and effectively protect animals from infection during this period. However, they can interfere with vaccination, thus hindering its success.

Clinical Features

Adult pigs do not become sick by porcine parvovirus infection. The symptoms affect exclusively the fetuses of acutely infected seronegative pregnant sows. Depending on the gestation stage at the time of infection, different consequences can be observed: in the first trimester of pregnancy (up to about the 40th day), the infection leads to fetal death and resorption of the fetus. In the sow, this is clinically manifested by a return to heat (oestrous cycle). In the second trimester (until the 70th day), infected fetuses also die; however, they are usually not aborted, but mummified, and remain in the sow, and are finally farrowed at the expected date. Infection in the last trimester of gestation eventually leads to the generation of an immune response in the fetus, resulting in elimination of the virus. These piglets are born healthy and virus-free, but they are serologically positive before intake of colostrum.

Pathogenesis

After oral incorporation, the virus proliferates in the regional lymphoid organs, and reaches virtually almost all organs of the animal during a first viraemia, even the uterus of pregnant females and the accessory glands of the boar. As a result of infection of the uterus, the fetuses are infected. However, all fetuses of a litter are rarely affected.

Immune Response and Diagnosis

Induction of an immune tolerance in the fetuses has not been described for porcine parvovirus infections. Virological diagnosis is not easy because isolation of the virus from mummified piglets is rarely successful. Therefore, porcine parvovirus infections can be better diagnosed by detection of the viral genome using PCR technology.

Control and Prophylaxis

Effective inactivated vaccines are available; hence, vaccination management is crucial to prevent porcine parvovirus infection. Therefore, all gilts have to be immunized before insemination. However, the already mentioned long persistence of maternal antibodies in young animals can impede successful vaccination in these cases.

20.1.6.3 Aleutian Mink Disease Virus

Epidemiology and Transmission

A completely different clinical picture is caused by Aleutian mink disease virus. It principally causes immune-complex-induced glomerulonephritis and arteritis in minks. The development of symptoms depends mainly on the virulence of the pathogen, but also on the genotype of the minks. Mink carrying the Aleutian genotype, which are characterized by a specially desired blue-grey coat colour (sapphire minks), have Chediak–Higashi syndrome, a malfunction of mononuclear phagocytes, which affects the induction of both the specific and the non-specific immune response. The virus is primarily transmitted by direct contact among animals. However, because of the high physical stability of all parvoviruses, Aleutian mink disease virus can also be spread by contaminated equipment, stables, animal keepers and animal breeders.

Clinical Features

Overall, Aleutian mink disease is a very complex process. The fulminant disease develops only after infection of Aleutian minks with virulent strains of Aleutian mink disease virus. In such cases, they cause the development of a hypergammaglobulinaemia and the formation and deposition of immune complexes in the kidneys and arteries. The resulting glomerulonephritis is invariably fatal. If non-Aleutian-genotype minks are infected, then the disease is milder, and, under certain conditions, the minks can even eliminate the pathogen.

In young pups of all genetic variants, the virus infects type II pneumocytes, which are responsible for the synthesis of surfactant factors, and thus for the surface tension in the alveoli. This provokes an acute interstitial pneumonia, which also has an invariably fatal outcome.

Pathogenesis

The pathogenesis of Aleutian mink disease virus infection is unclear; it is currently assumed that the virus induces a dysregulation of cytokine synthesis. This leads to an increased synthesis of IL-6 in virus-infected macrophages, which secrete the cytokine. These elevated IL-6 concentrations lead to a hypergammaglobulinaemia and to a proliferative glomerulonephritis, the main symptoms of Aleutian mink disease.

Immune Response and Diagnosis

Even though virus-specific antibodies are produced during Aleutian mink disease virus infection, they have no neutralizing effect. The diagnosis can be performed by detecting these antibodies.

Control and Prophylaxis

Vaccination is not possible; therefore, the disease is controlled by elimination of infected animals or infected flocks.

20.1.6.4 Animal Bocaviruses: Bovine Parvovirus and Canine Minute Virus

Epidemiology and Transmission

Bovine parvovirus has a global distribution. It was first isolated from calves with diarrhoea in 1961. Infected animals excrete the virus in high titres in the faeces, and the virus persists for long periods in the environment owing to the strong physical stability of the capsid. Canine minute virus is also known as canine parvovirus type 1 because it was isolated before canine parvovirus, which it is also referred to as canine parvovirus type 2. It is widespread, and its transmission routes are similar to those of bovine parvovirus.

Clinical Features

Gastrointestinal symptoms are the primary manifestation of bovine parvovirus and canine minute virus infections. Animals infected experimentally with bovine parvovirus develop, besides diarrhoea, also respiratory symptoms and especially abortions. Infections during the first two trimesters of pregnancy can lead to abortion of fetuses, mummifications, malformations of the central nervous system and stunted growth; contrarily, infections at later embryonic development stages are subclinical. The clinical picture of canine minute virus infections is different: in adult dogs, infections are usually either subclinical or accompanied by mild diarrhoea. Even in this case, the main manifestation is infection of the fetus. Depending on the stage of gestation, it results in abortion, resorption, mummification and live birth of weak pups. Myocarditis and malformations (anasarca) have also been described.

Pathogenesis

There are only a few data regarding the pathogenesis of animal bocavirus infections. Following oronasal infection, bovine parvovirus, like all autonomous

parvoviruses, initially replicates in the nasopharyngeal region, leading to a viraemia. Thenceforth, the virus can be detected in epithelial cells of the small intestine (Lieberkühn glands or intestinal follicles) and immune cells.

Immune Response and Diagnosis

Haemagglutinating antibodies with virus-neutralizing activity are detectable within a few days after bovine parvovirus infections. Since infected fetuses produce antibodies as early as the 140th day of gestation, and the pathogens are widespread, many commercial bovine fetal calf serum batches contain antibodies against bovine parvoviruses. Since fetal calf sera are routinely used for cell culture applications, it is difficult to isolate and cultivate the virus from biopsy samples. Diagnostic detection of the viral genome can be easily performed by PCR.

So far, canine minute virus can be grown only on a specific canine cell line (Walter Reed canine cell line). The reason for this restriction is not known. The serological detection is performed by haemagglutination-inhibition tests using erythrocytes from rhesus monkeys or by detection of viral proteins by immunofluorescence in infected cell cultures.

Prophylaxis and Therapy

There are neither vaccines nor effective antiviral drugs.

20.2 Circoviruses and Anelloviruses

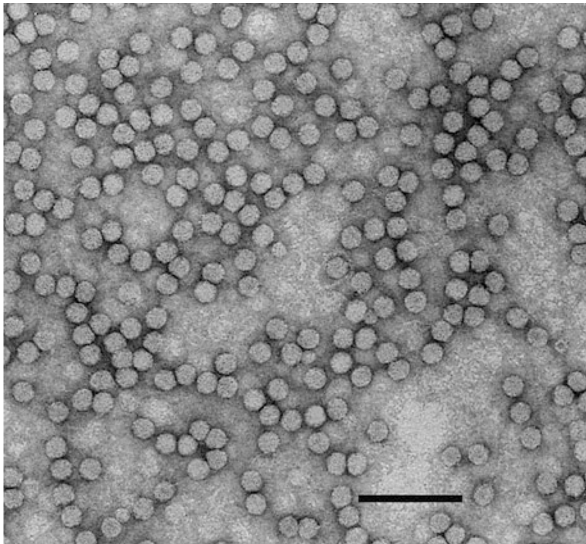


Table 20.3 Characteristic prototypes of circoviruses

Genus	Human virus	Animal virus
Gyrovirus		Chicken anaemia virus
Circovirus		Porcine circovirus 1
		Porcine circovirus 2
		Beak and feather disease virus
		Duck circovirus
		Goose circovirus

20.2.1 Classification and Characteristic Prototypes

The circoviruses and anelloviruses comprise viruses containing a single-stranded DNA genome. However, the DNA genome is not linear like in parvoviruses, but it forms a covalently closed circular molecule. They are possibly a transition to geminiviruses: these plant viruses have circular, partially twofold segmented single-stranded DNA genomes, and show a significant homology with some circoviruses.

The family *Circoviridae* is divided into the genera *Circovirus* and *Gyrovirus* (Table 20.3). The genera *Circovirus* and *Gyrovirus* contain only animal pathogenic viruses. Whereas chicken anaemia virus, the characteristic member of the genus *Gyrovirus*, can be isolated only from chickens, circoviruses infect pigs – porcine circovirus 1 (PCV-1) and porcine circovirus 2 (PCV-2) – and various species of birds, e.g. parrots, canaries, finches, pigeons, ducks and geese. Sequence homologies have been found only in the genome sequences of beak and feather disease virus and porcine circovirus. None of them exhibit sequence similarity to chicken anaemia virus, which, not only for this reason, has been classified into the separate genus *Gyrovirus*. Despite their similar structure, circoviruses differ substantially in their respective replication strategy.

In 1997, torque teno virus was isolated from a Japanese patient with the initials TT who developed post-transfusion hepatitis, and was originally called TT virus. The name “torque teno” (Latin for “twisted ring”, “twisted cord”) is an allusion to the single-stranded, circular DNA genome of this virus, which is present in a pronounced secondary structure. Since the molecular characteristics are similar to those of circoviruses, torque teno viruses were classified initially into the family *Circoviridae*. Recently, a separate virus family *Anelloviridae* (Latin *anellus*, “ring”) was created for the multifarious types of torque teno virus. Twenty-nine types of torque teno virus have been isolated from humans and are classified into the genus *Alphatorquevirus*. They are related to the various types of human torque teno midi virus (genus *Gammatorquevirus*) and human torque teno mini virus (genus *Betatorquevirus*); related virus types can also be found in many animal species and have been classified into various additionally created genera (Table 20.4). All human virus types are present worldwide in most healthy people. So far, no illnesses have been associated with their infections. Perhaps, torque teno viruses, torque teno midi viruses and torque teno mini viruses are infectious agents which

Table 20.4 Characteristic prototypes of anelloviruses

Genus	Human virus	Animal virus
Alphatorquevirus	Torque teno viruses 1–29	
Betatorquevirus	Torque teno mini viruses 1–9	
Gammatorquevirus	Torque teno midi viruses 1–2	
Deltatorquevirus		Torque teno tupaia virus
Epsilontorquevirus		Torque teno tamarin virus
Etatorquevirus		Torque teno felis virus
Iotatorquevirus		Torque teno sus virus 1a, 1b
Kappatorquevirus		Torque teno sus virus k2
Lambdatorquevirus		Torque teno zalophus virus
Thetatorquevirus		Torque teno canis virus
Zetatorquevirus		Torque teno douroucouli virus

have adapted well to cohabit with their hosts without damaging them. Since the molecular characteristics of anelloviruses and circoviruses share many similarities, they are discussed together.

20.2.2 Structure

20.2.2.1 Virus Particle

The particles of circoviruses and anelloviruses are not surrounded by an envelope; they are probably composed of just one capsid protein (VP1), and have an icosahedral structure (Fig. 20.7). Their diameter ranges from 17 nm (porcine circovirus, beak and feather disease virus), to 22 nm (chicken anaemia virus) to 30 nm (torque teno virus). Therefore they are the smallest known viruses. Details of the particle structure are not known.

20.2.2.2 Genome Organization and Structure

The genomes of circoviruses and anelloviruses consist of a closed circular, single-stranded DNA with a length of 1,759 (porcine circovirus), 2,319 (chicken anaemia virus), 2,700–2,900 (torque teno mini virus) and 3,100–3,200 (torque teno midi virus) nucleotides. The genome of torque teno virus is significantly longer (3,500–3,900 nucleotides. The torque teno virus isolate TA278 comprises 3,825 nucleotides. The genome contains three (beak and feather disease virus, chicken anaemia virus) to four (porcine circovirus) open reading frames. Whereas all three reading frames of chicken anaemia virus are oriented in the same direction (Fig. 20.8a), they are arranged in opposite directions in the porcine circovirus and in beak and feather disease virus (Fig. 20.8b). Here, a hairpin loop of nine nucleotides is located at the origin of replication within the untranslated region, which does not occur in the genomes of gyroviruses and anelloviruses. These viruses follow an ambisense transcription strategy, which is usually only seen in arenaviruses and some bunyaviruses (▶ Sects. 16.1 and ▶ 16.2). Like the animal

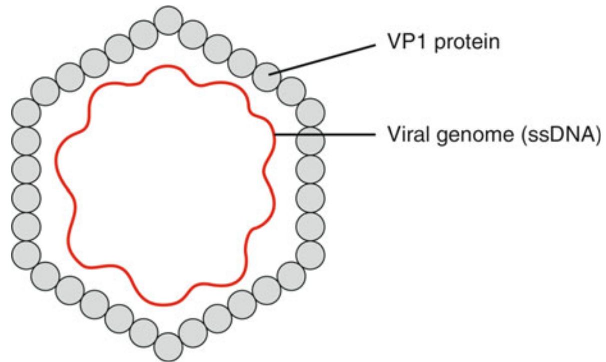


Fig. 20.7 Structure of a circovirus particle. The icosahedral capsids are composed of VP1 proteins and enclose the single-stranded, circular DNA genome. *ssDNA* single-stranded DNA

pathogenic chicken anaemia virus, the genomes of torque teno viruses, torque teno midi viruses and torque teno mini viruses have negative polarity. Despite their divergence in length, their genome organization is similar. Their sequences comprise two large (ORF1, ORF2) and three small (ORF3, ORF4, ORF5) open reading frames, which partially overlap with each other and with ORF1. In torque teno viruses, a non-coding, untranslated region is located between ORF4 and ORF2, and encompasses approximately 1,200 nucleotides and has a high GC content. It probably contains the regulatory elements necessary for genome transcription and replication. Whereas the nucleotide sequence is conserved in animal pathogenic circoviruses, a high variability can be observed in torque teno viruses: Twenty-nine types of human torque teno virus have been identified so far, and their DNA sequences of ORF1 differ from each other by more than 20 %.

20.2.3 Viral Proteins

20.2.3.1 Structural Proteins

The capsids are made of the VP1 protein. The largest open reading frame, ORF2, of chicken anaemia virus encodes a precursor protein from which the capsid protein VP1 is cleaved. In contrast, the capsid protein of porcine circovirus is encoded by ORF2. In ORF1 of torque teno viruses it is believed that the capsid protein is encoded together with a polypeptide that is implicated in the replication of the viral DNA. Although other proteins are also packaged into the virion, their function and origin are unknown. Table 20.5 provides an overview of the proteins of circoviruses and anelloviruses.

20.2.3.2 Non-Structural Proteins

The largest reading frame (ORF1) of porcine circovirus is responsible for the synthesis of the Rep and Rep’ proteins. These are important for replication of the viral genome by a rolling-circle mechanism. By use of alternative splice sites,

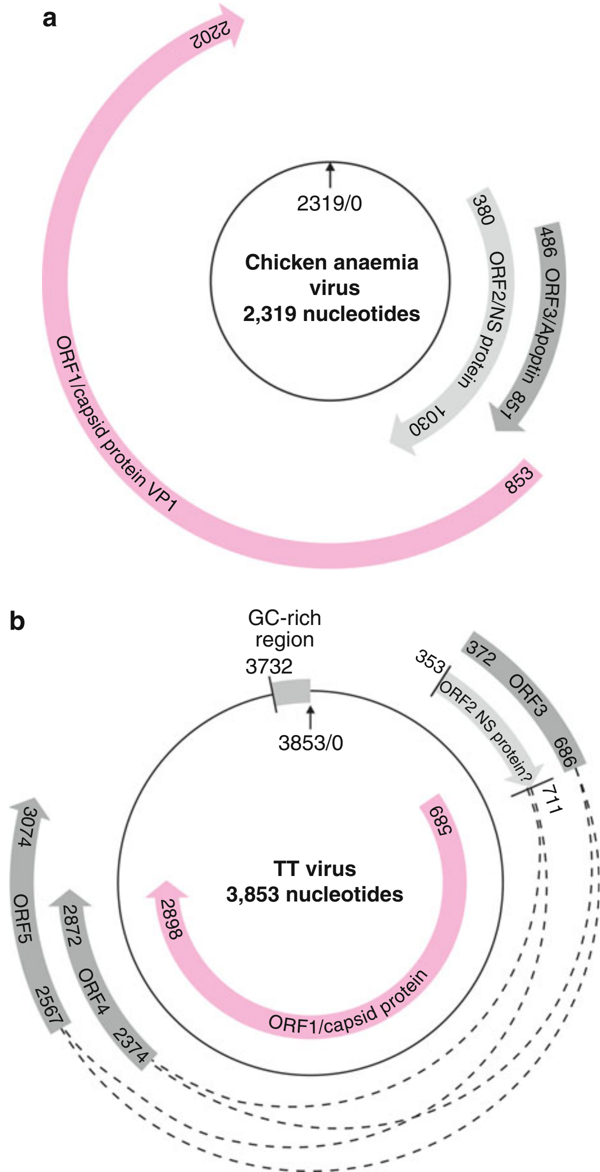


Fig. 20.8 Genome organization of circoviruses. (**a**) Chicken anaemia virus. (**b**) Torque teno virus. The transcriptional regulatory sequences are located within the non-coding regions (*at the top*). In both viruses, only the genomic negative-sense DNA is transcribed, even after the postulated synthesis of the complementary DNA strand. The genome of chicken anaemia virus encodes up to three proteins, whose functions have hardly been explored. The same applies for torque teno virus. Under the assumption of alternative splicing, the sequences of ORF2 and/or ORF3 may be fused with those of ORF4 and ORF5. However, the corresponding non-structural protein versions have not yet been experimentally detected during infection, or such findings have not been reproduced

a shortened version of the Rep' protein is synthesized, and this is also involved in replication of the single-stranded DNA genome. The function of the protein encoded by ORF3 of porcine circovirus is unknown.

ORF2 of both chicken anaemia virus and torque teno virus probably also encodes a non-structural protein involved in viral genome replication; Rep-protein-like functions have been attributed to this gene product. Considering genotype-specific

Table 20.5 Properties and functions of circovirus and anellovirus proteins

Protein	Torque teno virus	Chicken anaemia virus	Porcine circoviruses	Function
VP1	770 aa (precursor protein/ ORF1)	449 aa (precursor protein/ORF1)	30 kDa (ORF2)	Capsid protein
NS (VP2)	120–202 aa (ORF2)	216 aa (ORF2)		Non-structural protein replication, protein phosphatase
Rep			312 aa 36.5 kDa (ORF1)[a]	Non-structural protein replication; homology to Rep proteins in plant circoviruses and geminiviruses
Rep′			19.2 kDa (ORF1)[a]	Truncated Rep protein
VP3	57-105 aa (ORF3)		? (ORF3)	?
VP3		13 kDa (ORF3)		Apoptin, non-structural protein

aa amino acids
[a]In some publications, ORF1 of porcine circoviruses is termed ORF4

differences, it has a length ranging from 120 to over 200 amino acids. In vitro, it has been shown that the proteins encoded in ORF2 of chicken anaemia virus and of torque teno viruses possess the activity of a dual-specificity protein phosphatase. In addition to phosphotyrosines and phosphoserines, this protein tyrosine phosphatase subfamily also dephosphorylates non-protein substrates. In torque teno viruses there may be additional variants of this non-structural protein which are generated by using alternative splice donor and acceptor sites. This alternative splicing may lead to fusion of ORF4 and ORF5 with ORF2.

Chicken anaemia virus directs the synthesis of a 13-kDa non-structural protein, apoptin (VP3). It is probably encoded by ORF3 (Fig. 20.8a), and is able to trigger a p53-independent apoptosis mechanism in a variety of transformed cells or tumour cells. The simultaneous synthesis of the cellular Bcl-2 protein seems to accelerate this process. The physiological function of apoptin is unknown. It is possible that the ORF3 gene product of torque teno virus has a similar function. Depending on the virus isolate, this protein comprises 57–105 amino acids. Expression of ORF3 induces apoptosis in hepatocellular carcinoma cell lines.

20.2.4 Replication

Torque teno viruses, torque teno midi viruses and torque teno mini viruses cannot be propagated in cell culture systems. There are virtually no data regarding their genome replication. It is believed to occur in the nucleus, presumably by a rolling-circle mechanism involving DNA-dependent DNA polymerase encoded by the host cell. A GC-rich sequence is located in the non-coding genome region, and is

believed to be involved in regulating transcription and replication. Three transcripts have been detected in cells transfected with the genomic DNA of torque teno virus; there lengths are 3,000, 1,200 and 1,000 nucleotides.

Only little is known about the replication mechanism of animal pathogenic circoviruses. Viral genome replication occurs in the nucleus of infected cells, whereby circoviruses, like parvoviruses (Sect. 20.1), are dependent on the presence of cellular proteins, which are synthesized during the S phase of the cell cycle, e.g. DNA polymerases. Presumably, the single-stranded DNA genome is initially converted into double-stranded DNA. Chicken anaemia virus synthesizes a probably polycistronic transcript of 2,000 nucleotides in length. In contrast, porcine circovirus generates two mRNAs from the negative DNA strand (i.e. the genomic DNA strand) and a spliced mRNA from ORF1 by using the positive DNA strand as a template. There is evidence that the viral genome is replicated by a rolling-circle mechanism. The origin of replication of porcine circovirus is located in the untranslated region of the viral genome, whereby a hairpin loop forms the initiation structure. The genome of chicken anaemia virus lacks such a structure. Assembly of the various components into new virus particles occurs in the cytoplasm. They are probably released by cell lysis as a result of apoptotic processes.

Apoptin: A Novel Antitumour Therapeutic Approach?
The apoptin of chicken anaemia virus is able to induce apoptosis exclusively in tumour cells. It forms multimeric complexes that bind to DNA. In tumour cells, apoptin is present as a phosphoprotein mainly in the nucleus, whereas it is present as an unphosphorylated protein in the cytoplasm of normal untransformed cells, where it is rapidly degraded. The activity of apoptin, its modification and its nuclear transport can be induced temporarily by enabling the cells to express the T antigen of simian virus 40 (▶ Sect. 19.2). In tumour cells, apoptin seems to identify “survival signals” such as DEDAF, Nur77, NMI, Hippi and APC1, leading to the development of its activity. In transgenic mice and in animal models, apoptin has proven to have safe and effective antitumour activity. Whether it can be used as a general apoptotic agent in tumour cells remains to be elucidated.

20.2.5 Human Pathogenic Anelloviruses

20.2.5.1 Torque Teno Virus

Epidemiology and Transmission

Torque teno virus was first isolated from a patient with post-transfusion hepatitis in 1997. Therefore, it was initially considered as a possible member of non-A, non-B hepatitis viruses. However, it has turned out that torque teno viruses, like torque teno mini viruses and torque teno midi viruses, are widespread in the human population throughout the world, and their infections do not appear to be associated with diseases. Both viruses persist in more than 90 % of examined subjects,

and their genomes are found in blood, saliva and various tissues. The virus isolates are genetically highly heterogeneous, whereby the highly variable regions are concentrated in the central regions of ORF1. The protein domains encoded by this region exhibit differences of up to 70 % at the amino acid sequence level. To date, more than 20 genotypes have been discovered, and these are classified into five groups: these include SANBAN and SEN viruses. Phylogenetic analyses suggest that frequent genetic recombination additionally contributes to variability. Up to five different genotypes of torque teno virus and torque teno mini virus have been found concurrently in one individual. Torque teno virus sequences which are not distinguishable from human isolates have also been detected in the blood of different primates (chimpanzees, gorillas) and other animals (pigs, cattle, sheep and chickens),. Therefore, it is assumed that these viruses are transmitted as zoonotic pathogens from domestic animals to humans.

In humans and animals, these viruses establish persistent infections, and the viruses are excreted through saliva and faeces. Viral DNA has been found in blood and semen, as well as in lachrymal fluid and bile. Besides the transmission by droplet infection and smear infections, the physically very stable virus can also be transmitted by blood and blood products. This notion is additionally supported by the disproportionately high prevalence of torque teno virus in haemophilia and transplant patients as well as in drug addicts who use intravenous drugs.

Clinical Features

According to current knowledge, torque teno virus, torque teno midi and torque teno mini virus infections are asymptomatic. There is some evidence that increased genome copy numbers can be detected in patients with severe idiopathic myopathies, cancer and lupus erythematosus. However, it is likely that the increased viral DNA load is a consequence of immunosuppression and is not the cause. Signs of viral replication have been observed in children with acute respiratory infections.

Pathogenesis

Little is known about the pathogenesis of infection. The target cells of torque teno virus are unknown, and there is no cell culture system for cultivation of the virus in vitro. The viruses seem to infect mononuclear cells of the peripheral blood as well as certain cells in the liver and bone marrow. Intermediates of genome replication can be detected in the liver by PCR. In addition, this organ exhibits higher amounts of viral genomes than other tissues. However, an increase in transaminase levels or histological liver alterations have not been documented.

Immune Response and Diagnosis

Nothing is known about immunological responses to viral proteins. However, it has been reported that acutely and chronically infected individuals may spontaneously excrete torque teno virus. There is neither an ELISA nor a Western blot test for detection of the virus. The diagnostic detection of infection is performed using PCR, whereby the high genetic variability of the virus has to be taken into account.

Therapy and Prophylaxis

Owing to the asymptomatic course of infection, there is no need for the development of vaccines or antiviral drugs. Blood donors are not tested.

20.2.6 Animal Pathogenic Circoviruses

20.2.6.1 Chicken Anaemia Virus

Epidemiology and Transmission

Chicken anaemia virus has a worldwide distribution. It causes an economically important infectious disease in chickens. After experimental infection, it has been found that chickens excrete the virus for up to 30 days in faeces and saliva. The infection of adult animals is usually asymptomatic, although co-infections with other viruses (retroviruses, herpesviruses) may cause severe clinical courses. The classic clinical picture is found in the infection of 1-day-old chicks, which can be infected by virus-contaminated bedding or direct contact. Chicks from seropositive hens are infected, but the maternal antibodies usually prevent a disease. A vertical transmission is also possible when laying hens are in the viraemic phase. This explains why even specific-pathogen-free chickens initially excrete chicken anaemia virus.

Clinical Features

The infection leads to immunosuppression in 1-day-old chickens, and is characterized by anorexia, lethargy, anaemia and systemic bleeding. The mortality rate can be up to 50 %.

Pathogenesis

The virus infects the erythroid and lymphoid progenitor cells in the bone marrow and thymus of chickens. B lymphocytes are not infected by chicken anaemia virus. The infection results in destruction of erythroid progenitor cells, whereby the animals develop severe anaemia, thrombocytopenia and granulocytopenia. The destruction of T progenitor cells in the cortex of the thymus may be associated with the apoptotic effect of the VP3 protein (apoptin) and determines the depletion of mature cytotoxic T lymphocytes and T-helper cells, which leads to severe immunosuppression. Pathologically, the atrophy of all lymphoid organs, including bone marrow, is striking.

Immune Response and Diagnosis

Diagnosis can be performed by isolation of the virus in susceptible chicken cell lines. Diagnosis is verified by immunoflorescence or PCR. A past infection confers protective immunity.

Control and Prophylaxis

Oral vaccination based on live attenuated chicken anaemia virus is available and used in many countries.

Beak and Feather Disease Virus

Another scarcely understood avian circovirus infection affects parrot-like birds such as cockatoos, parrots and parakeets in Australia and more recently also in South America. The disease is characterized by a massive immunosuppression, feather loss, regrowth of deformed feathers in terms of size and structure, and less frequent disorders of the horn growth on the beak. The virus is transmitted by various secretions and excretions from infected birds. The disease is controlled by eliminating infected animals and strict hygiene measures.

20.2.6.2 Porcine Circoviruses

Epidemiology and Transmission

In 1974, a small non-enveloped virus with a circular single-stranded DNA genome was described as a contaminant of a porcine kidney cell line. It was named porcine circovirus (PCV-1). Further virological and serological studies have revealed that this virus is widespread in the swine population; however, it could not be associated with any disease. Around 1995, another circovirus was occasionally found in swine, and has been found more frequently since that time. It causes a non-specific disease that is called porcine postweaning multisystemic wasting syndrome (PMWS). The genome of this ubiquitous virus (PCV-2) is only 70 % homologous with that of PCV-1 from the 1970s. The host range seems to be restricted to swine. In recent years, extensive investigations have revealed that PCV-2 alone is not able to cause diseases, but other factors (including housing conditions, co-infection with porcine parvovirus; Sect. 20.1.6) must be involved in the development of PCV-2-associated diseases.

Circoviruses are physically very stable, and are excreted in the faeces of infected animals. Infection occurs through direct oral contact or via contaminated bedding or equipment. Vertical transmission from the mother to the piglets is possible. Whether piglets born with a persistent infection exhibit an immune tolerance is still unclear.

Clinical Features

Infections with PCV-2 cause two main diseases: PMWS and porcine dermatitis and nephropathy syndrome. The former is characterized by runtiness of individual piglets of a litter. The animals frequently show interstitial pneumonia and lymphadenopathy, but rarely diarrhoea or jaundice. Porcine dermatitis and nephropathy syndrome is a dramatic disease that resembles classical swine fever. The principal symptoms are petechial haemorrhages on the skin, which can develop into necrotizing lesions. The adrenal cortex also shows petechial haemorrhages and interstitial nephritis.

Pathogenesis

The pathogenesis of this disease is largely unknown. The virus is regularly detected in macrophages, and PCV-2 infection may lead to a generalized immunosuppression, in which just a few pathogens may cause an apparent disease. In particular,

co-infections with porcine parvovirus (Sect. 20.1.6) or porcine reproductive and respiratory syndrome virus (an arterivirus; ► Sect. 14.5.5) are a matter of discussion.

Immune Response and Diagnosis

The infection usually leads to seroconversion and elimination of the virus. Infections with PCV-2 can be verified by detecting the viral genome in altered tissues. This can be performed by in situ hybridization or by detecting the viral DNA by PCR. PCV-1 infections can be reliably distinguished from PCV-2 infections by selection of appropriate primers.

Control and Prophylaxis

Recently, recombinant vaccines which are composed of VP1 proteins that are produced in different expression systems have become available. Although there were obvious doubts about their real effectiveness because of the multifactorial origin of the disease, the experience with the vaccines shows, however, that they are extremely effective. Immunization of piglets in experimental infections has revealed a significantly reduced viraemic phase and weakened PMWS-associated disease symptoms. However, particularly noteworthy is that both the frequency and the severity of other infections are also weakened and the animals exhibit better growth and increase in weight. In addition to vaccination, hygiene measures are of great importance for preventing viral transmission in livestock.

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21 Prions

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Prion diseases, also referred to as transmissible spongiform encephalopathies (TSEs), are caused not by viruses, but by prions, which are infectious protein particles without coding nucleic acid in the infectious agent. Prions cause fatal neurodegenerative disorders in humans and animals. They use an epigenetic mechanism for transmission of infection which is based on profound changes in the spatial arrangement and biophysical properties of the underlying protein. Hence, they constitute the prototype of protein conformational or protein misfolding diseases.

The history of prion diseases goes back a long way. As long as 250 years ago, a fatal disease was described in sheep which was manifested by hyperexcitability and pruritus (scrapie). As early as 1936, Jean Cuille and Paul-Louis Chelle demonstrated that scrapie could be transmitted by inoculating healthy sheep and goats with spinal cord material from diseased animals. Almost concurrently, slowly progressive degenerative central nervous system diseases in humans were described: Creutzfeldt–Jakob disease (CJD) in 1920–1921, Gerstmann–Sträussler–Scheinker (GSS) syndrome in 1936, kuru later and most recently fatal familial insomnia (FFI).

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D. Carleton Gajdusek provided the first detailed description of kuru. This stimulated the scrapie researcher William Hadlow to speculate that kuru might be the human equivalent of scrapie. He proposed conducting transmission experiments in primates. This was successfully done by Gajdusek and colleagues, and Gajdusek was awarded the Nobel Prize in Physiology or Medicine in 1976. He was able to transmit kuru and later CJD and GSS syndrome to monkeys, thus demonstrating the infectious nature of these human neurodegenerative diseases.

It soon became apparent that the pathogens of these infectious neurodegenerative diseases exhibit very unusual properties: all virus-inactivating procedures, which destroy nucleic acid, had no effect. It was suggested that these pathogens do not require nucleic acid, and that they transmit their infectivity only through proteins. Later, this "protein-only" hypothesis was experimentally corroborated by Stanley B. Prusiner, for which he was awarded the Nobel Prize in Physiology or Medicine in 1997. The infectious agent was purified from brains of experimentally infected rodents in the 1970s: indeed, it was identified as a protein molecule, the prion protein (PrP), which has been designated PrP^{Sc} in its infectious form. PrP^{Sc} has specific biochemical properties, such as its insolubility, infectivity and resistance to proteases. In 1982, Prusiner introduced the technical term "prion" (derived from *proteinaceous infectious particle*) in order to distinguish these pathogens clearly from viruses and viroids. In 1985, a normal, cellular PrP (PrP^{C}) was found which exhibits fundamentally different biochemical and biophysical properties in comparison with PrP^{Sc}. Some years later, it became clear that the genetic forms of human spongiform encephalopathies are associated with distinct mutations in the PrP gene.

Around 1990, a new epidemic prion disease emerged in animals in England: mad cow disease (bovine spongiform encephalopathy, BSE). It was associated with feeding cattle with slaughterhouse waste (meat and bonemeal) that contained residual material from prion-infected animals. It remained unclear whether the disease originated from scrapie-infected sheep or from cattle spontaneously suffering from BSE. In this context, an important question emerged immediately of whether the pathogen can be transmitted to humans by food. In fact, a new CJD disease emerged in the UK in 1995: variant CJD (vCJD). It has been proven experimentally that the causative agents of BSE and vCJD are identical. Both agents are clearly distinguishable from other prion agents, such as those of sporadic CJD. Thus, the zoonotic transmissibility of the BSE agent from cattle to humans has been proven in the form of vCJD. However, the extent and frequency of transmission cannot be estimated at the moment. In recent years, it has also been shown that vCJD can be transmitted iatrogenically by blood and blood products (secondary vCJD, svCJD).

21.1 Classification and Characteristic Prototypes

Prion diseases are unique because they occur in three manifestations: acquired by infection, genetic and sporadic (Table 21.1). The three manifestations are clearly based on different pathogenic mechanisms. The acquired form is based on the direct contact between exogenous PrP^{Sc} and PrP^{C} of the recipient cell; as a result of this

Table 21.1 Characteristic forms and manifestations of prion diseases

Manifestation form	Disease		Mechanism
	Human	Animal	
Acquired by infection	Kuru Iatrogenic Creutzfeldt–Jakob disease vCJD/svCJD	Scrapie (sheep and goats) Bovine spongiform encephalopathy (cattle) Feline spongiform encephalopathy (cats) Chronic wasting disease (deer, elk)	Conformational change of endogenous PrP^{C} substrate by interaction with PrP^{Sc} template; exogenous
Sporadic	Creutzfeldt–Jakob disease (about 90 % of cases)		Spontaneous conformational conversion of PrP^{C} into PrP^{Sc}; endogenous
Genetic	Creutzfeldt–Jakob disease (roughly 10 % of cases) Gerstmann–Sträussler–Scheinker syndrome Fatal familial insomnia		Spontaneous, conformational change of PrP^{C} and conversion into PrP^{Sc} favoured by mutations; endogenous

vCJD variant Creutzfeldt–Jakob disease, *svCJD* secondary variant Creutzfeldt–Jakob disease, PrP^{Sc} pathological (scrapie) isoform of prion protein, PrP^{C} cellular prion protein/cellular isoform

interaction, PrP^{C} is autogenously converted into PrP^{Sc}. In the genetic form, it is assumed that mutations lead to substitutions of single amino acid residues in PrP^{C}, and these exert a destabilizing effect on the structure of PrP^{C}, thus facilitating the conversion to PrP^{Sc}. The sporadic form might be based on spontaneous conversion of PrP^{C} to PrP^{Sc}.

The various manifestations of human prion diseases are particularly impressive. In humans, the commonest form is sporadic CJD, whereas the genetic forms (familial CJD, GSS syndrome and FFI) are approximately ten times less frequent. The acquired forms, which are caused by infection, include iatrogenic and zoonotic forms such as kuru, iatrogenic CJD, vCJD and svCJD. The prion diseases of animals are mainly acquired forms. In addition to scrapie in sheep and goats and BSE in cattle, further prion diseases are known in animals, including chronic wasting disease (CWD) in deer and elk, transmissible mink encephalopathy (TME) in farmed minks, exotic ungulate encephalopathy in zoo animals and feline spongiform encephalopathy in cats. The latter three diseases are directly related to feeding animals with meat and bonemeal or carcasses that were infected with the BSE agent. It is assumed that sporadic prion diseases also occur in the animal kingdom with very low frequency, whereas purely genetic forms are not known

among animals, with one exception. However, polymorphisms in the PrP gene can significantly modulate the susceptibility of animals to prion infection (e.g. sheep, goats, mice, deer and elk).

After peripheral inoculation and ingestion, for example via nutritional intake, prions initially replicate in the lymphatic system, such as the Peyer patches in the small intestine or the spleen. From there, prions spread into peripheral nerve endings and are retrogradely transported through the spinal cord, the sympathetic and parasympathetic system or directly via cranial nerves into the central nervous system in a very slow neuroinvasion process. The neurodegeneration of the central nervous system is a slow, steadily progressive process, which always ends fatally. Koch's postulates apply to prions with certain restrictions: the causative agent can be isolated from the brain of diseased individuals and cultured in "pure culture" such as in cultured cells. In these cells, it can propagate for several years and be continuously detected as PrP^{Sc}. Finally, animals can be inoculated with it, and they develop the disease with pathognomonic symptoms. The causative agent can be re-isolated from the brain of diseased individuals.

21.2 Structure of the PrP Gene (*PRNP*/*Prnp*)

Prions do not contain a coding nucleic acid in the infectious agent. They use an epigenetic mechanism to pass on their infectivity. For this purpose, they use the cellular isoform of PrP (PrP^{C}) and convert it to the pathological isoform PrP^{Sc}. The PrP coding gene (referred to as *PRNP* and *Prnp* in humans and in animals, respectively) is highly conserved, and is located on chromosome 20 in humans. All known mammalian PrP genes consist of two or three exons, and the coding region with the complete open reading frame is located within the last exon. The promoter that controls the expression of the PrP gene contains GC-rich sequence elements and binding sites for transcription factors, but no classic TATA box. The resulting messenger RNA encompasses 2,100–2,500 nucleotides and directs the translation of a protein comprising approximately 250 amino acids. In mammals, the amino-terminal part contains a non-structured region comprising five octarepeats of largely identical amino acid sequences [PQGGGGWGQ] $[PHGGGWGQ]_4$, which are located between amino acids 51 and 91 (Fig. 21.1). Human PrP^{C} contains polymorphisms at positions 129 and 219. These amino acids influence conversion of PrP^{C} into PrP^{Sc} and the susceptibility to prion infections. If both alleles of the PrP gene encode the genetic information for the amino acid methionine at codon 129, then conversion into PrP^{Sc} seems to be favoured. At this position, 50 % of the white population are heterozygous for the amino acids methionine and valine, whereas 40 % are homozygous for methionine and 10 % for valine. To date, all individuals who contracted vCJD were homozygous for methionine at position 129. This constellation is also found as a genetic predisposition in most patients with sporadic CJD, iatrogenic forms of CJD (transmitted by contaminated growth hormone or dura mater grafts) and in kuru patients. The polymorphism at position 219, which affects less than 10 % of the Asian population, has been described only as a modulating factor in Asian patients so far.

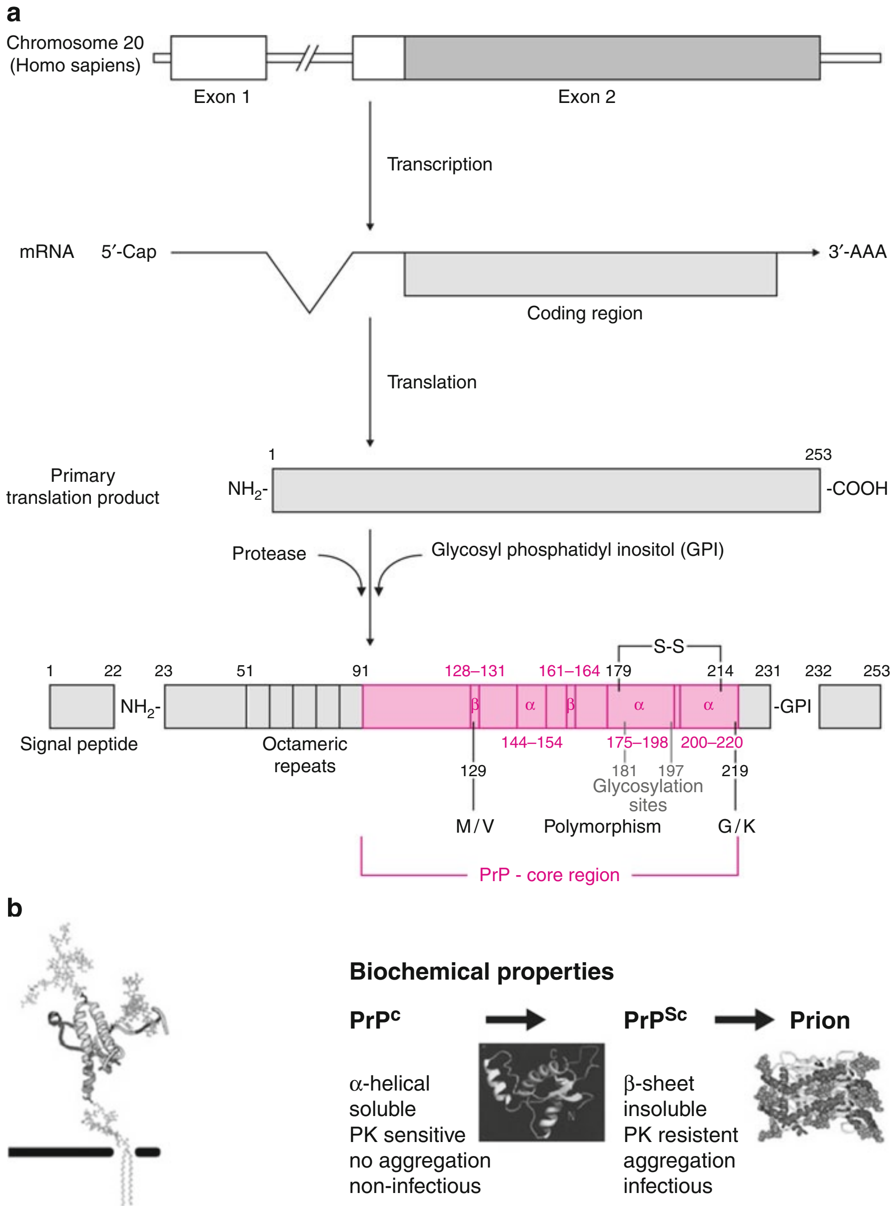


Fig. 21.1 Prion protein (*PrP*) structure and organization. (**a**) Structure and organization of the human PrP gene, its transcription and translation, as well as post-translational protein modifications. The signal peptide is cleaved during translocation into the endoplasmic reticulum (ER) lumen, and the carboxy-terminal signal peptide (amino acids 232–253) is co-translationally removed, in order to attach the glycosylphosphatidylinositol (*GPI*) anchor. The octarepeat region covers amino acid residues 51–90. A disulphide bridge and two N-glycosylation sites are located

21.3 Structure of PrP Isoforms

Prions are aggregates of many PrP^{Sc} molecules, which arise by conformational conversion of PrP^{C}. The highest infectivity has been found in prion preparations that are made up of 14–28 PrP molecules. If PrP^{Sc} is purified from infected brain material that has been subjected to proteinase K digestion, fibrillar forms can be observed in electron-microscopic analyses (scrapie-associated fibrils or prion rods). PrP^{Sc} accumulates in extracellular amyloid plaques in the brain, and these are detectable histologically and immunohistochemically by staining.

The translation product of the human PrP gene (*PRNP*) consists of 253 amino acids, whereas rodent genes (*Prnp*) usually comprise 254 residues. Twenty-two amino acids are co-translationally removed from both termini. Carbohydrate groups are post-translationally added to asparagine residues 181 and 197, a disulphide bond is formed between cysteines at positions 179 and 214, and a glycosylphosphatidylinositol anchor is attached at position 230/231 in the carboxy-terminal domain (final molecular mass 33–35 kDa). Fully mature PrP^{C} is located at the outer leaflet of the plasma membrane, preferably in lipid rafts. It is anchored by the glycosylphosphatidylinositol moiety and can be removed from the cell surface by phospholipase treatment. PrP^{C} is able to bind Cu^{2+} ions, mediated mainly by amino acids of the octarepeats.

NMR analysis was used to decipher the structure of recombinant PrP^{C}. Kurt Wüthrich of ETH Zurich was awarded the Nobel Prize in Chemistry in 2002 for this and his basic contributions of using NMR for deciphering protein structures. These analyses revealed that the protein consists of an unstructured and flexible amino-terminal part, which comprises about 100 amino acids (residues 23–120; Fig. 21.1). This part is followed by a structured domain (residues 121–231) that is composed of three α-helices and two short β-sheet regions. In the pathological isoform PrP^{Sc}, this structured region forms significantly more ß-sheet structures; it is responsible for the proteinase K resistance of the amino-terminally truncated PrP core (amino acids 90–231, 27–30 kDa), which is referred to as PrP27-30 owing to its molecular weight. PrP27-30 is sufficient for the known pathological properties of PrP^{Sc}. Since PrP^{C} and PrP^{Sc} differ apparently neither in the primary structure nor in their post-translational modifications, both PrP isoforms are found in different glycosylated forms in brain material of infected animals and humans, namely non-glycosylated as well as monoglycosylated and diglycosylated forms. Accordingly, three bands appear for

Fig. 21.1 (continued) in the carboxy terminus. The proteinase K (*PK*)-resistant part of PrP^{Sc} is shown in *red*. It has the same primary structure and post-translational modifications as the cellular PrP isoform (*PrP^{C}*), from which it differs significantly in terms of spatial arrangement and biochemical properties (*bottom centre*). The PrP^{C} regions containing secondary structures (two short β-sheet regions and three α-helices) and the polymorphisms at positions 129 and 219 are also indicated. (**b**) The putative PrP^{C} structure on the cell membrane is shown at the *bottom left*, the NMR structure of recombinant PrP (Riek et al. 1996.) is shown in the *middle* and a model for the spatial arrangement of PrP27-30 (Govaerts et al. 2004) is depicted at the *bottom right*. *mRNA* messenger RNA

PrP^{C} and PrP27-30 in Western blot analysis. The relative intensity of the various glycosylated forms seems to be associated with different prion strains. This property is also used for diagnosis of TSEs and is employed in epidemiological studies, such as in investigation of the transmission of BSE from cattle to sheep or humans (vCJD).

Prions are unequivocally the most resistant human and animal pathogenic infectious agents. Neither antimicrobial disinfection methods nor nucleic acid degrading enzymes (RNase, DNase) can reduce their infection potential. They are also resistant to boiling (heating to 100 °C); treatments with microwaves, formalin, acids or autoclaving have only a limited effect. Dry heat is effective at temperatures above 300 °C. Only agents that denature proteins (such as 3–6 M guanidine hydrochloride, 1 M NaOH, 6 M urea) are able to destroy the infectivity of prions. Treatment with alkaline solutions (e.g., 1–2 M NaOH for at least 30 min), autoclaving at 132–136 °C for at least 30 min at elevated pressure (3 bar) and the combination of both methods are very effective inactivation methods. Only these methods can reliably inactivate the infectious potential of prions, even in very high doses.

21.4 Prion Propagation

Prions are composed of the pathological isoform (PrP^{Sc}) of the normal isoform PrP^{C}. The conformational conversion, which is associated with the acquisition of completely different biochemical properties, constitutes the key pathogenetic basic principle of prion disease. PrP^{Sc} is formed by intramolecular conformational changes in the carboxy-terminal domain of PrP^{C}, whereby the biochemical properties of the proteins change dramatically: PrP^{C} is soluble, has mainly an α-helical conformation, is sensitive to proteinase K digestion and is not infectious. In contrast, PrP^{Sc} is characterized by high β-sheet content, is prone to aggregation and is largely resistant to degradation by proteases (Fig. 21.1). There are two basic models that explain the mechanism for conformational conversion of PrP^{C} into PrP^{Sc}: the heterodimer model postulated by Prusiner and colleagues involves a conversion intermediate that is formed from PrP^{C}; the crystallization or seeding model, originally postulated by Gajdusek, and then further developed by Peter Lansbury and Byron Caughey, is based on the notion that PrP^{C} molecules attach to PrP^{Sc} oligomers and adopt the PrP^{Sc} conformation without generating a folding intermediate (Fig. 21.2).

PrP^{C} is expressed in many cell types and cells and is localized within lipid rafts at the plasma membrane. The highest amounts are found in the central nervous system and in neurons. Its exact function is unknown. PrP-knockout mice (PrP$^{0/0}$ mice) exhibit no significant phenotypic alterations in comparison with wild-type mice. There is evidence that PrP^{C} possesses neuroprotective functions, and may also be active in immune cells. PrP^{C} binds Cu^{2+} ions, and might also be involved in signal transduction. PrP^{C} is essential for susceptibility to prion diseases, as PrP$^{0/0}$ mice do not develop prion disease after intracerebral

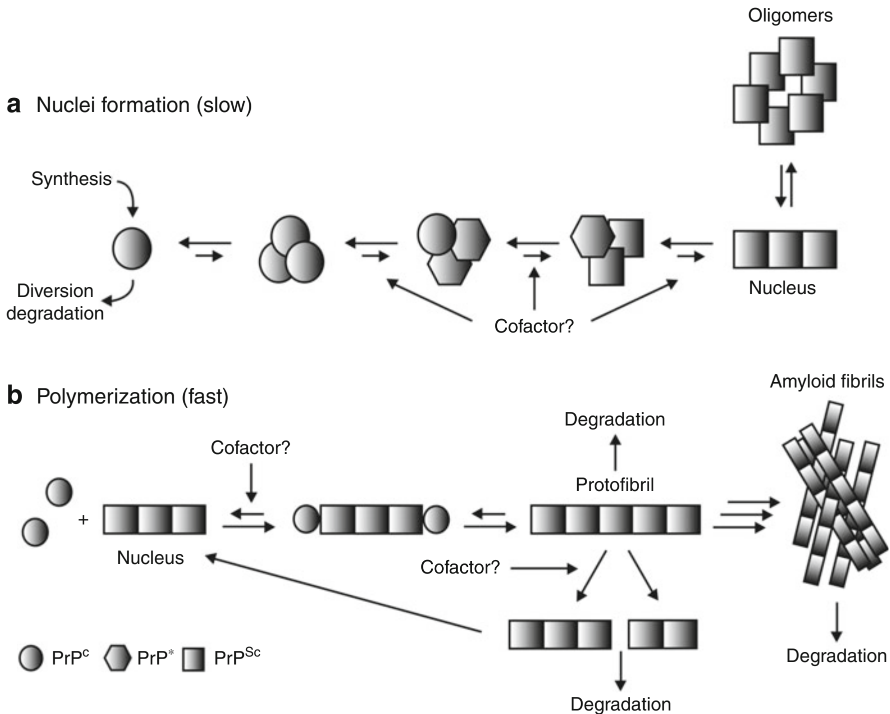


Fig. 21.2 PrP^C to PrP^{Sc} conversion process according to the crystallization or seeding model. The formation of amyloids can be divided into two steps. The initial step (**a**) is important for the formation of nuclei, and requires partially unfolded proteins and leads to aggregates consisting of a few oligomers. This rare event is followed by a much more rapid polymerization phase (**b**), in which the native proteins are embedded in the growing nucleus (seeding). The resulting fibrils must be broken up and degraded in order to create new nuclei. This eventually leads to exponential growth. The spontaneous conversion of PrP^C into PrP^{Sc} is a very rare event, which leads to sporadic disease forms. Mutations in the *PRNP* gene probably render the protein more susceptible to aggregation (genetic prion diseases). In acquired prion diseases, the exogenous uptake of PrP^{Sc} seeds induces the conversion of recipient-encoded PrP^C

inoculation with prions. Approximately 1–5 % of newly synthesized PrP^C is continuously converted to PrP^{Sc} in the cell (Fig. 21.3). This process requires direct physical contact between the two PrP isoforms, which occurs at the cytoplasmic membrane or in endosomal compartments. It is unclear whether and how far cellular cofactors are involved in the PrP conversion process. PrP^{Sc} is degraded only to a limited extent, accumulating in lysosomes. On the other hand, cells do have a clearance capacity for prions, and cellular autophagy seems to play a role here. To maintain the continuous conversion following a "domino principle" without chain termination, PrP^{Sc} should be subject to a subcellular recycling process. The release of prions from cells occurs predominantly through apoptosis. However, at least in cell culture, there is evidence for a continuous, possibly exosome-mediated release of PrP^{Sc} without cell death. Extracellular PrP^{Sc} is taken

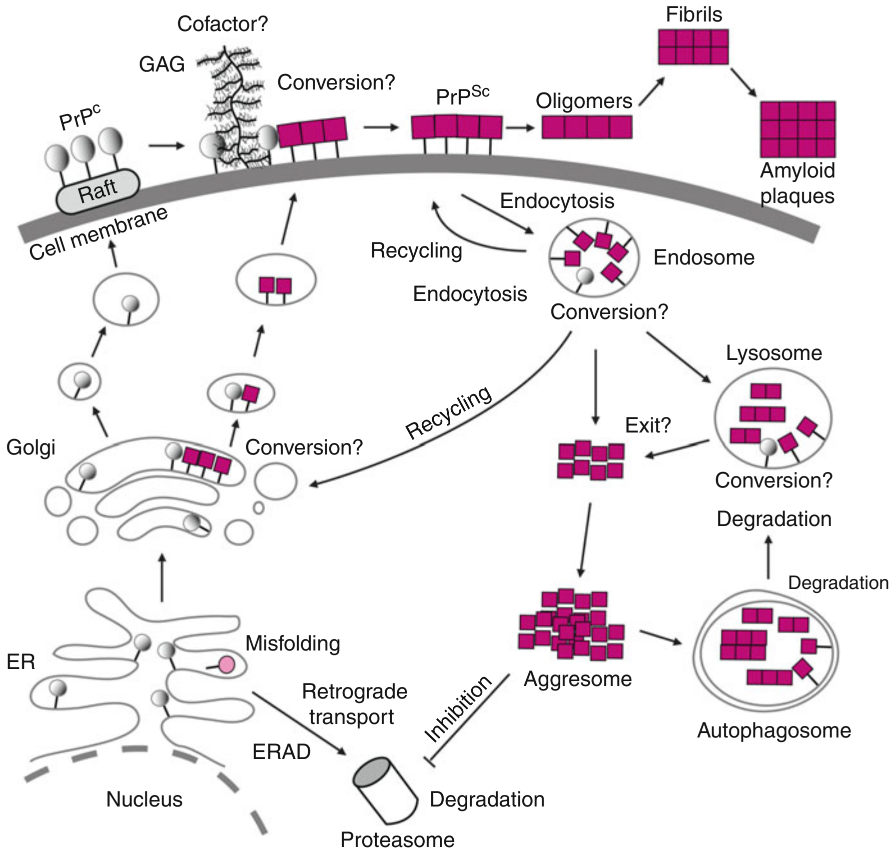


Fig. 21.3 Cell biology of PrP^C and PrP^{Sc}. After its synthesis at the ER membrane, fully mature PrP^C migrates via Golgi vesicles and the *trans*-Golgi network (TGN) to the outer cell membrane, where it accumulates in lipid rafts. From there it is recycled via endocytic pathways and is eventually degraded in lysosomes. The conformational conversion of PrP^C into PrP^{Sc} occurs at the cell membrane or in compartments of the endocytic pathway. Possibly, cofactors such as glycosaminoglycans (*GAG*) are also involved. Approximately 1–5 % of PrP^C is continuously converted into PrP^{Sc} in persistently prion-infected cells in vitro. PrP^{Sc} is only incompletely degraded in the lysosomes, it has a half-life of more than 24 h and ultimately accumulates in the cell. However, the cell possesses the ability to eliminate PrP aggregates and prions, and induction of cellular autophagy was shown to be a contributing factor. PrP^{Sc} should also be subject to a subcellular recycling process in order to maintain the continuous conversion following a “domino principle” without chain termination. The neurotoxic nature of PrP^{Sc} is still unclear as is the exact mechanism of how the infectivity is transmitted from cell to cell. PrP misfolding in the ER and compromising of the degradation process via ER-associated degradation (*ERAD*) and proteasome, possibly by formation of aggresomes, may play a role in neurotoxicity in some genetic forms of prion diseases

up by other cells. Several receptors have been postulated for this process, e.g. the laminin receptor precursor protein. When cells are infected with brain homogenate in vitro, they incorporate the infectivity mainly by phagocytosis.

The zoonotic potential of BSE demonstrated clearly that prion infections are able to overcome species barriers. Although transgressions are the rule rather than the exception in experimental animal models, they nevertheless still require human intervention. TSEs probably occur in most animal species at low frequency, similar to the sporadic occurrence of CJD in humans. However, human intervention may lead to massive prion dissemination, thus resulting in epidemic outbreaks within a species, for instance BSE in the cattle population by “neo-cannibalism”. Under certain circumstances, appropriate exposure and predisposition can give rise to crossing of a species barrier, leading, for example, to zoonotic spread of BSE to humans. In turn, there is a risk of transmission of the disease within the newly infected species, as now occurs with svCJD.

21.5 Human Prion Diseases: Kuru, CJD and Similar Disorders

21.5.1 Epidemiology and Transmission

21.5.1.1 Kuru

An endemic disease was found among members of the Fore tribe in the eastern highlands of Papua New Guinea in the 1950s, and is referred to as kuru (“cold shivering in fear”). Particularly adult women and children contracted kuru; a total of 2,500 cases have been recorded. Kuru was transmitted during mortuary feasts when deceased relatives were consumed by close relatives as a mark of respect and mourning (ritual endocannibalism). Even though these rites were banned by the Australian government in the 1960s, patients still occasionally die of kuru because of the very long incubation periods (since 2000, six patients have died of kuru, the last in March 2005). However, people who were born after the ban on cannibalism have not contracted the disease.

21.5.1.2 CJD, GSS Syndrome and FFI

CJD was first described by Hans-Gerhard Creutzfeldt and Alfons Maria Jakob in 1920 and 1921, respectively. It has a low incidence, with one case per million people per year worldwide. Most cases occur sporadically; about 10 % are passed on genetically to descendants owing to autosomal dominant inheritance (familial CJD). CJD is not contagious in normal social interactions; however, about 140 iatrogenic transmissions from person to person occurred after cornea and dura mater transplants. Individual cases have been attributed to contaminated neurosurgical instruments and intracerebral electrodes. About 165 cases of iatrogenic CJD occurred in patients who were treated with prion-contaminated growth hormones that were extracted from pituitary glands of human corpses. Sporadic CJD commonly becomes symptomatic after the age of 50 or 60 years. From the first symptoms to the fatal outcome, the disease lasts only a few months, rarely a few years (genetic forms).

GSS syndrome was described for the first time in 1936. It is a rare, autosomal dominant inherited neurodegenerative disease. FFI, which was discovered only in 1986, is a rapidly progressive, fatal autosomal dominant inherited disease with a life expectancy of 6–36 months after diagnosis. The occurrence of familial CJD/GSS syndrome/FFI is clustered regionally; overall, it is about ten times less frequent than sporadic CJD. Regions with a higher prevalence can be found, for example, in Libya, Israel, North Africa, Slovakia and Germany.

21.5.1.3 Variant CJD

Variant CJD (vCJD) appeared in the UK for the first time in 1996. More than 220 cases have been recorded as of the time of writing, 176 of them in the UK, 27 in France, five in Spain, four in the Republic of Ireland, and three in the Netherlands (http://www.cjd.ed.ac.uk/vcjdworld.htm). The rest were found in European countries, Japan, Saudi Arabia, Taiwan, Canada and the USA. The latter are probably “imported” cases, as the patients are thought to have been infected in the UK. Some countries with rather high BSE prevalence such as Germany have reported no cases of vCJD as yet. Five cases of secondary vCJD (svCJD) have occurred through blood transfusions since 2004. The first infection of a haemophilia patient with svCJD was described in the UK in 2009. The cause of transmission was presumably a contaminated plasma factor VIII product.

In the case of human prion diseases, there are usually neither vertical nor horizontal transmissions (except iatrogenic transmissions or transmission by cannibalism). With the exception of cerebrospinal fluid, these diseases cannot be transmitted through blood products and secretions or excrements. In the case of vCJD, transmission is possible by altered lymphatic tropism, resulting in a horizontal spread of vCJD through blood products among the human population. The incubation period of human prion diseases ranges from a minimum of 4 years (some kuru and iatrogenic CJD cases) to more than 50 years. In the genetic forms, the incubation period usually lasts four to five decades. Even sporadic kuru cases with such long incubation periods are still appearing today. The incubation period of vCJD is estimated to be at least 10 years, and periods of 20–30 years are also considered probable.

21.5.2 Clinical Features

21.5.2.1 Creutzfeldt–Jakob Disease

Sporadic CJD usually becomes apparent after the age of 50 or 60 years. The period from the first symptoms until the fatal end usually lasts only a few months, rarely a few years. Initial symptoms include mental abnormality with irritability, apathy, depressive mood or even paranoid traits; they are followed by gross cognitive performance deterioration with cerebellar disorders such as ataxia and speech disorders. The terminal phase is characterized by steadily progressive loss of higher intellectual capacity (progressive dementia), loss of movement and disturbance of

vegetative functions. In patients with genetically determined CJD, underlying point mutations (e.g., E200K, V180I, V210I, M232R, D178N-129V) or octarepeat insertions in the *PRNP* gene have been determined.

21.5.2.2 GSS Syndrome

Patients with GSS syndrome develop symptoms of a progressive dysfunction of the cerebellum during midlife (cerebellar dysfunction), which become apparent as vacillation, clumsiness, loss of coordination and increasing gait disorder. Progression of the disease leads to ataxia, dysarthria and nystagmus. Whereas dementia predominates in CJD, it is only slightly pronounced in GSS syndrome, or it is masked by the cerebellar dysfunction. Molecular genetic studies have revealed that families with GSS syndrome regularly exhibit mutations in the *PRNP* gene. The most frequent substitution is proline to leucine at position 102; however, positions 105, 117, 145, 187, 198, 202, 212, and 217 can also be affected.

21.5.2.3 Fatal Familial Insomnia

FFI is characterized by non-influenceable insomnia, an increased sympathetic tone and other autonomic and endocrine disorders. Histopathologically, thalamic nuclei are principally affected. The *PRNP* gene of FFI carries a mutation in codon 178 (D178N-129M), which leads to the replacement of aspartic acid by asparagine, whereby a methionine is located at position 129 of the same allele.

21.5.2.4 Kuru

The disease starts with loss of coordination, unsteady gait and tremor (kuru means “trembling” in Fore, a Papuan language). Finally, after about 1 year, the highly cachectic patients die of exhaustion, aspiration pneumonia or as a consequence of pressure sores that are caused by immobility.

21.5.2.5 Variant CJD

Typically, pronounced psychiatric symptoms are in the foreground at the onset of the disease. It particularly affects young people. The clinical course is considerably prolonged, and ataxia and not dementia is the primary symptom.

21.5.2.6 Pathogenesis

All human prion diseases are inevitably fatal. Since different centres of the central nervous system can be affected with differing magnitudes, several definable histopathological forms can be distinguished. The polymorphism at position 129 of the *PRNP* gene is also histopathologically important and decisive for the formation of specific clinical–pathological types and forms of CJD. However, an explanation at the molecular level is still pending. The cerebral cortex and other centres typically exhibit diffuse spongiform (vacuolizing) alterations that are caused by neuronal cell death, which can be differently pronounced and localized at different sites. Immunohistochemical staining with PrP-specific antibodies shows localized or diffusely distributed extracellular plaques composed of PrP^{Sc} aggregates, which are particularly strongly marked in kuru and vCJD. PrP plaques

cause a proliferation of astrocytes or all glial cells (gliosis) and a progressive atrophy of the central nervous system with loss of neurons. The familial forms of CJD and kuru show also plaques in the cerebellum; spongiosis is generally less than in CJD. In contrast to viral encephalitis, all prion diseases show no infiltrating lymphocytes, and there are no other signs of local or systemic immune response.

21.5.3 Immune Response and Diagnosis

Except for the genetic forms, preclinical diagnosis of CJD is not possible. Prions can be detected neither in blood nor in the cerebrospinal fluid with currently available diagnostic methods. In addition, there are no signs of inflammation or immune mediators in the brain or cerebrospinal fluid, PrP^{Sc}-specific antibodies are not produced. Cellular degradation products (e.g. 14-3-3, NSE and S100) can be detected in the cerebrospinal fluid, and used in supportive tests, however, are not specific for prion diseases. Since, except for vCJD, only the central nervous system is affected, the examination of a brain biopsy or autopsy is essential to confirm the diagnosis. Histological and histopathological examinations are performed with this material (e.g. the presence of PrP plaques). The histopathological identification of PrP^{Sc} and immunoblot analysis the gold standard for detection of PrP^{Sc} (Fig. 21.4). They exploit conditions (e.g., by proteinase K digestion) where only PrP^{Sc} and not PrP^{C} is detectable. PrP^{Sc} is regularly detected also in lymphoid tissues such as tonsils, lymph nodes, appendix and Peyer patches only in vCJD. Protein misfolding cyclic amplification (a new PCR-like method), which uses PrP^{Sc} as a template from the material to be examined and PrP^{C} from non-infected brain as a substrate, is very sensitive and can potentially detect one infectious PrP^{Sc} molecule. This method might also be suitable for blood and cerebrospinal fluid. However, because of its sensitivity, there are still "specificity" problems, which question its diagnostic application. More recently, a similar in vitro amplification method was developed which seems to be more specific (real-time quaking-induced conversion). EEG studies and, more recently, magnetic resonance tomography (particularly for vCJD) have proven to be helpful in diagnosis.

21.5.4 Therapy and Prophylaxis

There is no therapy for human prion diseases. In the clinical stage, the pathological alterations in the central nervous system are far advanced and probably irreversible. There is a variety of therapeutically oriented approaches in several experimental models in vitro and in vivo. Prophylactic vaccines are not available, although an effect has been shown by active and passive vaccination in several experimental animal studies. However, the necessity to overcome the self-tolerance to PrP may lead to considerable side effects.

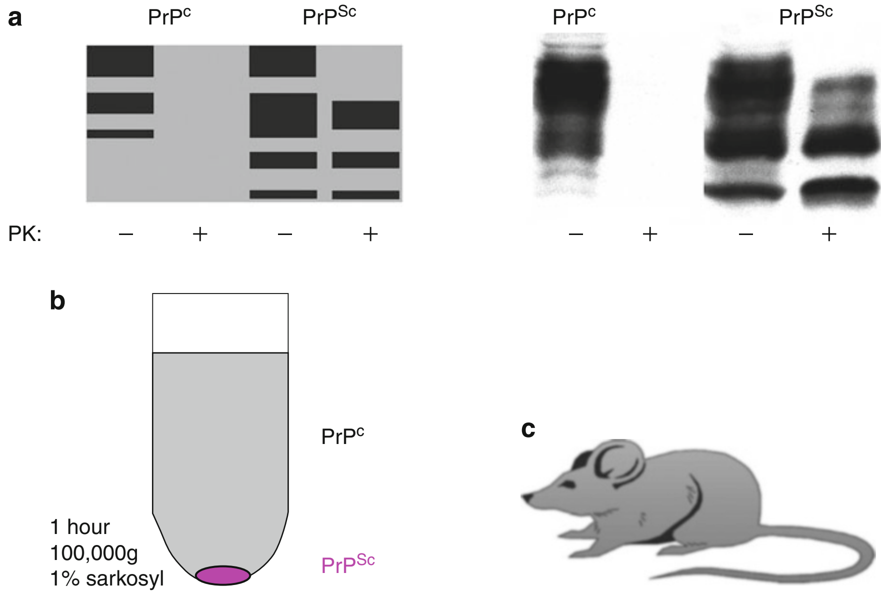
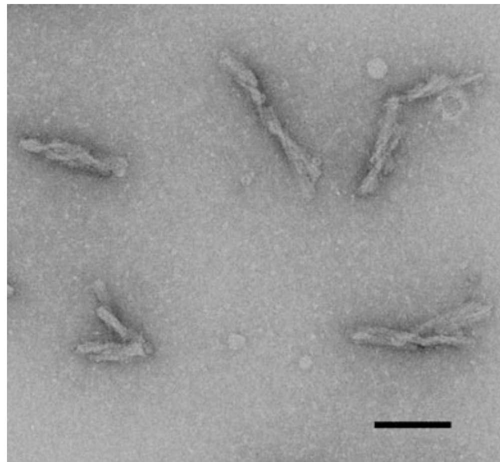


Fig. 21.4 Diagnostic and experimental ways to distinguish between PrP^C and PrP^{Sc}. (**a**) Relative proteinase K (*PK*) resistance of PrP^{Sc}. Lysates of cells or tissues are digested with PK under defined standard conditions and analysed in an immunoblot test. PrP^C is completely sensitive to PK digestion. PrP^{Sc} is only partially sensitive and becomes degraded solely at the amino terminus. (**b**) Another way to distinguish biochemically is based on the insolubility of PrP^{Sc} in non-ionic detergents. (**c**) The specific prion infectivity can also be tested in appropriate animal experiments

21.6 Animal Prion Diseases



Prion diseases exist as a natural disease in some animal species, but they can be transmitted experimentally to a variety of species. In addition to these naturally occurring forms, by some measures (feeding, vaccine contamination, breeding) humans repeatedly contributed to increased spread of the naturally occurring forms of prion diseases (e.g., scrapie) or even to transgression of species boundaries, which may result in new diseases such as TME, or the epidemic form of BSE. Recent developments include the massive spread of prion disease in deer and elk (CWD) in North America and the worldwide occurrence of atypical BSE or scrapie cases. The latter probably represent sporadic forms.

The most important animal prion diseases worldwide are scrapie and BSE, both notifiable diseases in most countries. Scrapie is endemic in Europe, Asia and North America. Starting from the UK, BSE has become a worldwide problem often with catastrophic economic consequences. Recently, Canada experienced this, for example. In European Union countries, all healthy slaughtered cattle older than 30 months have been tested for BSE since 2001 in active surveillance. From 2009, examinations were restricted to slaughtered cattle older than 48 months, and since 2011 they have been restricted to slaughtered cattle older than 72 months. If the present trend with very low BSE cases in such healthy slaughtered cattle continues, the European Union is likely to lift BSE testing completely in such cattle. In a country such as Germany, more than 21 million cattle have been tested for BSE since 2000, resulting in 406 confirmed cases of BSE. In the UK between 2001 and 2012, almost six million cattle were tested, with over 1,800 positive cases. Passive surveillance occurs when animals have clinical signs suggestive of BSE and includes BSE testing and farmer compensation. Countries such as the USA and Canada perform passive surveillance and target higher-risk animals. These are cattle that are non-ambulatory or are displaying signs of neurological diseases, older animals, and fallen cattle or downer cattle. This strategy is thought to adequately protect consumers and has resulted in over 40,000 cattle being tested in the USA and over 30,000 cattle being tested in Canada. The following text summarizes the most important clinical, pathological and epidemiological knowledge of these infectious diseases.

21.6.1 Scrapie

21.6.1.1 Epidemiology and Transmission

Scrapie has been described in small ruminants such as sheep, goats and mouflons (*Ovis musimon*). The disease has been documented since the early eighteenth century; however, linguistic research suggests that it was already known in antiquity. The disease has different names in various countries, and they refer to the characteristic symptoms of the disease: "scrapie" (from "to scrape") refers to the itching symptoms during the illness, *Traberkrankheit* and *rida* describe the pronounced ataxia and hypermetria, and *tremblante* refers to the tremor or the myoclonus. In scrapie, the existence of defined pathogenic prion strains and genetic susceptibilities has long been ascertained. Sheep populations have been selected which are almost resistant against classic scrapie.

Scrapie has a worldwide distribution. The sole exceptions are Australia and New Zealand, which are considered to be free of scrapie. The prevalence of infection in endemic areas is estimated to be up to 10 %, the clinical incidence is lower. This is also reflected by the fact that significantly higher numbers are found in Europe and other countries, since the European Union and the World Organisation for Animal Health (OIE) have prescribed examination of part of the national sheep populations using TSE tests. In these monitoring programmes, sheep and goats over 12 or 18 months old are randomly examined for TSE. In European countries such as Germany, approximately 40,000 sheep and 4,000 goats are involved in the monitoring programmes each year. Although only 12 cases were described in Germany from 1990 to 2000, testing increased the number of cases to over 120 in 5 years (2003–2007). In the UK between 2002 and 2012, about 350,000 animals were tested, which resulted in about 250 cases of classic scrapie and 280 cases of atypical scrapie being confirmed. In the USA in 2010, about 48,000 animals were tested, resulting in 72 cases of classic scrapie and five cases of atypical scrapie being confirmed.

Scrapie can be transmitted vertically and horizontally. Transmission is promoted by direct, close contact between animals. The extremely high stability of the pathogens in the environment explains why the disease can recur in farms in which, after culling, no sheep were kept for 1 year or longer.

Three transmission pathways are mainly discussed or have already been proven experimentally:

1. Oral ingestion of traces of infected material (brain, placenta), e.g. during grazing. Oral transmission by feeding animals with infected material has been demonstrated experimentally and is corroborated by detection of PrP^{Sc} in the small intestine and tonsils. Infection of sheep by feeding them with scrapie-containing placentas has also been demonstrated. Pastures contaminated with placentas have led to infection of susceptible sheep in Iceland.
2. Infection through skin wounds. Transmission of the pathogen through skin wounds (scarification) has been demonstrated at least in the mouse model. A possible cofactor for horizontal transfer of scrapie prions via excretions and faeces may be chronic viral or bacterial infections in the kidney and udder. It is been shown that mastitis caused by maedi–visna virus infections favours scrapie transmissions (▶ Sect. 18.1.6). PrP^{Sc} is mainly detectable in inflamed mammary glands. Whether and to what extent prions are actually excreted in milk is not clear.
3. Intrauterine transmission from ewe to lamb. Large pathogen concentrations have been detected in placenta samples; thus, a diaplacental or congenital transmission is likely.

21.6.1.2 Clinical Features

The incubation period is very variable with a range of 3–24 months and depends decisively on the type (scrapie strain) and amount of inoculum and the genetics of the sheep (sheep breed). The disease lasts a few weeks to 6 months. The clinical symptoms are multifarious, and reflect the central nervous system perturbations of

sensorimotor functions. Besides ataxia, itching, scratching and the resulting damage to the fleece of the animals are common signs of scrapie-infected sheep. In contrast, scratching movements of the hind legs or head are commoner in goats.

21.6.1.3 Pathogenesis

The different susceptibility of several sheep breeds is important for the pathogenesis of classic scrapie. This phenomenon became evident by the very different incubation periods, and led to the attempt to eradicate scrapie by selection of resistant sheep in some countries. Although this was not successful, it has led to the insight that the sequence of the PrP gene is essential for resistance or susceptibility of certain sheep breeds to classic scrapie. Polymorphisms at a minimum of three codon sites have been identified by genetic analysis of the *Prnp* gene of different sheep breeds from flocks with defined breeding programmes: positions 136, 154 and 171 significantly influence the susceptibility to classic scrapie. Variations of the three corresponding amino acids explain the differences in natural infections of sheep and also the various courses in experimental infections of ovine PrP transgenic mice.

Sheep breeds encoding valine at position 136 in PrP show high susceptibility to classic scrapie, whereas sheep coding for alanine at this position become sick only after exposure to high infection doses. However, amino acids polymorphisms at position 154 (arginine vs. histidine) and 171 (arginine to histidine or glutamine) determine only a slight susceptibility. So far, scrapie-infected sheep with the phenotype ARR/ARR (amino acid residues 136A, 154R and 171R in both alleles) have never been found in Europe and the USA. The same seems to apply to the AHQ polymorphism, although sheep with this genotype are extremely rare. Interestingly, these natural susceptibilities correlate with the infectivity of isolated PrP proteins in transgenic mouse models: PrP^{Sc}-VRQ induces the disease much more efficiently in mice than PrP^{Sc}-ARQ. PrP^{Sc}-ARQ transforms PrP^{C}-VRQ and PrP^{C}-AQH equally efficiently into the corresponding PrP^{Sc} isoforms. By contrast, PrP^{C}-ARR has not been converted by any of the different PrP^{Sc} variants.

The Hope for Resistant Breeds Wanes with Atypical Scrapie Isolates

Recently, it has become apparent that the genetic rules regarding resistant animal breeds apply only to the known "classic" forms of scrapie. Beginning with the atypical isolate scrapie Nor98, which was detected in Norway in 1998, a series of other atypical isolates have been identified, even in New Zealand, They are characterized by the fact that the diseased animals have significantly less PrP^{Sc} in the brainstem region (obex) than sheep that are infected with the previously known, typical forms of scrapie. These new PrP^{Sc} versions are also more susceptible to proteinase K degradation. However, the atypical forms of PrP^{Sc} have proven to be at least as infectious as the classic forms in animal experiments. Since the atypical scrapie isolates are present in those sheep breeds with PrP genotypes that

are considered resistant, the selection of these breeds may achieve just the opposite goal: not the disappearance of PrP^{Sc}, but a selection of atypical scrapie isolates. Atypical cases are not a rare event any more. In the UK, more atypical scrapie than classic scrapie cases were detected in the last 10 years.

21.6.1.4 Diagnosis

Diagnosis is performed after death by detecting PrP^{Sc} by immunoblot analysis, immunohistochemistry or rapid TSE tests originally developed for cattle. Normally, brainstem (obex) is used as the examination material. Because of the pronounced lymphotropism of scrapie, intralymphoid tissues can also be examined intravitally, for example by rectal, third eyelid or tonsil biopsy.

21.6.1.5 Therapy and Control

Scrapie is a notifiable disease in most countries worldwide. The European Union and the World Organisation for Animal Health demand active surveillance and examination of part of the national sheep populations using TSE tests. In these monitoring programmes, sheep and goats over 12 or 18 months old are randomly examined for scrapie and countries are requested to fulfil defined test numbers per year. In addition, passive surveillance and testing in animals with suspicious clinical signs is requested. Many European countries which culled infected herds turned to procedures based on the genotyping results for the *Prnp* gene. The aim is to kill sheep with the highly susceptible genotype VRQ, and to use only bucks for breeding which have an ARR/ARR genotype. Sheep with an intermediate genotype can still be used, but not slaughtered. In the USA several federal programmes were implemented which intend to decrease the prevalence of scrapie: the voluntary Scrapie Flock Certification Program, the National Accelerated Scrapie Eradication Program, which involves live animal testing, active slaughter and clean-up strategies including genotyping, and the National Scrapie Surveillance Plan, which includes the Regulatory Scrapie Slaughter Surveillance. Regulatory Scrapie Slaughter Surveillance started in 2003 and is designed to identify infected flocks. Almost 300,000 samples have been collected and 445 confirmed positive cases have been identified. Canada launched a national scrapie surveillance programme in 2005.

21.6.2 Chronic Wasting Disease

21.6.2.1 Epidemiology and Transmission

CWD is the only prion disease which is found in free-ranging and farmed animals. It is currently considered the most infectious and the most difficult to control form of the TSEs. CWD is endemic in various cervid species (black-tailed deer, white-tailed deer, mule deer, Rocky Mountain elk and moose) in the northwest of

the USA. Elisabeth Williams initially diagnosed CWD in mule deer in northern Colorado in 1967 and then in Wyoming in 1979, and realized that this is a TSE in cervids. CWD is now endemic in free ranging and farmed deer populations in Rocky Mountains states (Wyoming, Colorado, Utah, Montana, New Mexico), but also in Wisconsin and Virginia, in 18 states in total 2013. It is also endemic in two provinces (Alberta and Saskatchewan) in Canada. A prevalence of over 90 % is observed in affected farmed herds and up to 50 % in free-ranging populations. Outside North America, CWD has been found only in South Korea: it was imported by animals from Canada. Individual European countries and Japan have examined the prevalence of CWD in native deer populations without having found positive cases. Experimental transmission of CWD to European red deer, roe deer and reindeer is possible by the oral route. Since the CWD prions are detectable in muscle tissue and blood, and deer and elk are hunted and widely consumed, the question concerning the zoonotic potential becomes more important. Currently, it is still not clear whether the causative agent of CWD is also pathogenic for humans. Relevant investigations are currently under way, including, for example, inoculation of monkeys and transgenic mice carrying the human *PRNP* gene with the CWD pathogen.

The ways in which CWD is transmitted probably resemble those of scrapie: horizontal transfers are obvious, and also infections via contaminated pastures. There is a lot of infectivity outside the central nervous system, with prions being shed into the environment, which plays a leading role in transmission. Particularly striking is the massive salivation of sick animals, which is considered to be a key factor for efficient transmission from animal to animal. Because of the pronounced social contacts among young deer, CWD seems to be transmitted very efficiently. The infectivity has been unequivocally detected in the urine and faeces of diseased and preclinical animals. Since migration of North American free-ranging deer populations cannot be restricted nor can they all be culled or tested in vivo, CWD will remain a serious and long-term problem. Of particular concern is whether CWD will be introduced into caribou and reindeer herds, which are highly migratory and are of particular importance for Native American populations.

21.6.2.2 Clinical Features

The clinical picture is substantially similar to that of scrapie in sheep, although there is no itching, but rather general emaciation, depressive behaviour, difficulty in swallowing, excessive urination and strong salivation of animals. Diseased animals are on average 3–5 years old.

21.6.2.3 Pathogenesis

In addition to severe spongiform alterations, especially in the medulla oblongata, plaques are particularly striking, and are similar to the florid plaques in kuru and vCJD. However, the distribution of these plaques is different from that in BSE

in cattle. All tests, including the determination of a histopathological lesion profile in the brain of CWD-infected mouse strains, strongly suggest that the causative agent of CWD is identical neither with the causative agent of BSE nor with one of the known scrapie strains. The origin of CWD is presently unclear.

21.6.2.4 Diagnosis

Diagnosis is performed after death by detecting PrP^{Sc} from the obex region by immunoblot analysis, immunohistochemistry or TSE tests analogous to those used for BSE and scrapie. CWD can be relatively well diagnosed intravitally from biopsy material from lymphoid tissue (rectoanal mucosa-associated lymphoid tissue, retropharyngeal lymph nodes, tonsils).

21.6.2.5 Therapy and Control

Affected farmed herds (captive animals) are culled. However, there is currently no way for efficient control of free-ranging deer and elk populations. Sharpshooting of suspicious and clinical animals was tried, but had no effect on the prevalence of CWD. The type of soil might play a role in the persistence of CWD prions in the environment. Environmental measures such as ploughing up the earth from an affected research facility and repeatedly treating the area with disinfectants for a 6-month period were also ineffective. There are specific guidelines for hunters in various US states, but all are on a voluntary basis. It is recommended to harvest only animals which look and behave normally and to not consume brain, spinal cord, eyes, spleen and lymphoid tissue. Dressing of animals should be done in a way which minimizes the risk of infection. Suspicious animals should be reported to health officials and complimentary testing of elk and deer is available in many states.

21.6.3 Transmissible Mink Encephalopathy

21.6.3.1 Epidemiology and Transmission

TME is a rare disease of farm minks. It has been described sporadically in individual farms in North America and Europe. The last outbreak was recorded in 1985 (in Stetsonville, Wisconsin, USA). The origin of the TME agent is the subject of controversial discussion. One hypothesis postulates that the scrapie agent was transmitted by feeding minks with infected sheep tissues. However, histological analysis in minks revealed a distribution pattern different from that of the corresponding lesions in scrapie-infected sheep or mice. Furthermore, feeding with sheep meat can be virtually excluded in the last well-documented case in 1985. Another hypothesis postulates a bovine source for the agent. This is supported by experimental data. If minks are fed with brain from TME-infected cattle, the minks become ill after a very short incubation period of only 7 months. In line with this, intracerebral inoculation of cattle with materials containing the TME agent resulted in a very short incubation period until the onset of clinical symptoms. Both facts suggest the lack of or a reduced species barrier and imply an identity of the causative agents. This hypothesis also leads to the conclusion that BSE occurs sporadically in North America.

21.6.3.2 Clinical Features

Affected mink show striking behavioural changes, which are primarily manifested in the form of increased aggressiveness and hyperaesthesia. Paralysis and injuries caused by self-mutilation are frequently found in the final phase.

21.6.3.3 Pathogenesis

It has been shown that TME prions can exist as various strains with different PrP^{Sc} conformations. In the hamster model, two prion strains were isolated which were distinguishable physically and biologically, and stemmed from minks that were sick during the last outbreak in 1985: DY (drowsy) and HY (hyper). These isolates cause very different symptoms in hamster, and can be distinguished by PrP^{Sc} immunoblot analysis as well by their physical properties. These facts show that prion strains result from different conformers of PrP^{Sc}, although their primary structure and that of the PrP^{C} substrate are identical. They also show that such conformers can have different biological properties.

21.6.3.4 Diagnosis

The diagnosis is made after death by histology, immunohistology or immunoblot analysis.

21.6.3.5 Therapy and Control

Infected minks are culled and farms are depopulated.

Mad Cow Disease Still Has Extensive Political Consequences

BSE has had far-reaching political consequences, and many compromise solutions purely based on trade-policy considerations have turned out to be dangerous for the consumer. BSE will be historically remembered as an infectious disease that is man-made by negligence (Carleton Gajdusek coined the term "neo-cannibalism"), and whose development has been made possible and even promoted through policy decisions (often contrary to numerous expert suggestions) and finally by inadequate controls at all levels of animal disease control and consumer protection. The trade-policy dimensions are still of concern in some countries, such as Japan and Canada, whose cattle or beef exports have come to a complete standstill, and who thus have experienced immense economic disadvantages. Because the import of beef from the USA became permitted again, the South Korean government was almost overthrown by the people in the summer of 2008. The certainly questionable handling of BSE in South Korea is perhaps because almost 95 % of the Korean population carries the genetic constellation Met/Met in codon 129 of the PrP gene; hence, the population is considered to be particularly susceptible to BSE/vCJD.

Table 21.2 Scenarios for spread of a prion infections illustrated by the example of bovine spongiform encephalopathy (BSE)

Forms of spread/emergence	Example
Sporadic/spontaneous emergence (cofactors?)	Sporadic BSE
Intraspecies spread (with or without human influence)	Epidemic BSE
Trans-species infection (zoonosis)	vCJD
Intraspecies spread (e.g. iatrogenic)	Secondary/iatrogenic vCJD/svCJD

21.6.4 Bovine Spongiform Encephalopathy

21.6.4.1 Epidemiology and Transmission

BSE, which occurred in its epidemic form in the UK for the first time in 1986, has led to enormous financial losses for the local husbandry sector and started a critical discussion on the limitations of an "industrialization of agriculture", first in Europe and then throughout the world.

In 1986, this disease was first diagnosed in a cow that had central nervous system disorders. A histopathological examination of tissues from the central nervous system showed great similarity to scrapie in sheep. Thus, BSE was established as a new prototype of TSEs. In the following years, an epidemic developed in the UK, which reported up to 3,500 new clinical cases per month at its climax in 1992. Today, after an extensive reduction of the incidence to only eight cases in 2011 and one in the first half of 2012, more than 184,000 clinical cases have been recorded since the first appearance of BSE, affecting more than 50 % of UK cattle farms. However, the real significance of BSE is as a zoonosis. To date, over 220 cases of human vCJD have been described in England, France and many other countries (Sect. 21.5.1.3); vCJD can be transmitted iatrogenically from person to person, e.g. through blood donations and blood products, causing svCJD (Table 21.2).

BSE has now been detected almost worldwide, including in countries such as Canada, Israel, Oman, Japan and the USA. Besides the UK, Ireland, Portugal, France, Spain, Switzerland and Germany have exhibited the most detections of BSE (Table 21.3).

The emergence of BSE is ascribed to feeding animals with scrapie- and/or BSE-contaminated meat and bonemeal. Around 1980, the production of meat and bonemeal was considerably changed in the UK. For example, the absolute temperature for inactivation of pathogens was decreased to below 100 °C, and the use of organic solvents was reduced or even discontinued. The result was a completely inadequate heating and inactivation of PrP^{Sc}, which was then fed in the form of meat and bonemeal to cattle. Two further factors contributed to the emergence of BSE. On the one hand, the sheep population was very large and scrapie spread widely in flocks around 1980. On the other hand, prices for soy and fishmeal rose strongly, so more meat and bonemeal was added as a crude protein source in concentrate feed for calves. This was especially true for calves of dairy breeds, which should be fed with protein-rich feed after weaning from the mother cow's milk.

Table 21.3 Number of verified BSE cases in various countries

Country	Number of cases (as of July 2012)
Austria	8
Belgium	133
Canada	18
Czech Republic	30
Denmark	16
Germany	406
Falkland Islands	1
Finland	1
France	1,020
Greece	1
Ireland	1,658
Israel	1
Italy	142
Japan	36
Liechtenstein	2
Luxembourg	3
Netherlands	88
Oman	2
Poland	73
Portugal	1,080
Slovakia	25
Slovenia	8
Spain	779
Sweden	1
Switzerland	467
UK	183,317
USA	4

Source: World Organisation for Animal Health (http://www.oie.int/?id=505). Data can contain imported/exported cases and atypical cases. Some data are estimates and depend on how imported/exported cases were calculated.

In other European countries, there were far more stringent regulations for the production of meat and bonemeal than in the UK. Animal carcasses that were not considered for human consumption were heated to 133 °C for at least 20 min, conditions that largely inactivate prions. However, meat and bonemeal from animals considered suitable for human consumption was not heated above 100 °C. Therefore, every cow that did not exhibit clear symptoms at the time of slaughter could be used for production of meat and bonemeal. Feeding ruminants with meat and bonemeal was banned throughout the European Union in 1994. However, feeding all farm animals (poultry, pigs) with meat and bonemeal was prohibited in the European Union only in December 2000.

The hypothesis that transgression of species boundaries by scrapie prions from sheep to cattle is the cause of BSE is not without controversy because experimental

infection of cattle with scrapie induces a different clinical picture and different histopathological features. Nevertheless, these experiments are not sufficient to refute this hypothesis: the properties of the agents of spongiform encephalopathies may change in a new host in the course of several passages. Since only a limited number of scrapie strains have been used to infect cattle, it cannot be excluded that another, previously not investigated scrapie strain was transmitted to cattle. Altogether, it is currently considered far more likely that endogenous, sporadic, even if rare cattle BSE has been spread by changed feeding conditions ("neo-cannibalism") within cattle populations. This initial spark was epidemically extended by further feeding of infected carcasses.

Two measures by the British government have to be mentioned in this context. In 1988, a statutory feeding ban was imposed on meat and bonemeal for ruminants. This action effectively broke the infection chain. Positive changes appeared with a delay of 3–5 years, the incubation period of BSE in cattle. Horizontal transmission plays no significant role in cattle, as the BSE agent does not have a marked lymphotropism in bovines. Vertical transmission can occur in 5–10 % of newborn calves; however, it depends on the incubation age of the mother cow. As shown by epidemiological studies in the UK, this is not sufficient to maintain the epidemic.

Specified risk materials from cattle were no longer introduced into the human food chain only in 1989. These include all parts of the central nervous system, the spine and some visceral organs. Although at that time it was not yet known that prions can be present in muscle, this measure dramatically reduced the exposure of the human population in the UK. Before that measure, which was later followed by others concerning the processing of cattle products, the risk was presumably 1,000–100,000 times higher.

However, many rules were initially implemented only slowly and inadequately in the UK. This also applies to the import prohibitions of the European countries, which were evidently eluded. After meat and bonemeal could no longer be used in the UK, attempts were made to export it to other member states of the European Union. When this was officially no longer possible, the material was still sold in often dubious ways to non-European countries. Therefore, it can be assumed that BSE has been exported to many countries. However, only countries possessing an active and extensive surveillance system (including BSE tests) will be able to detect these cases. Many European countries were regarded as BSE-free before the introduction of compulsory BSE testing. Countries such as the USA and Canada reacted rather quickly and implemented prevention programmes. In 1992, Canada implemented a national BSE surveillance programme which was adapted several times later, most recently in 2011, which focuses on higher-risk animals and includes active testing. Canada was hit hard by BSE when a cow tested positive in May 2003 in Alberta and the USA immediately stopped importing beef from Canada because of this. So far, Canada has had 18 confirmed cases. The US government imposed import restrictions for live ruminants and ruminant products from countries with known BSE in 1989. These restrictions were expanded to all European countries in 1997. The FDA consequently banned most mammalian proteins as a food source for ruminants. The US policy also focuses on targeted

surveillance within the country. High-risk animals are adult animals with neurological signs, non-ambulatory or downer cows, and cattle dying on farms. Presently, around 40,000 animals are actively tested per year, which may be considered low for a country with such a big cattle population. The USA has had four confirmed BSE cases as of 2012. The first three were officially labelled as non-endogenous, imported cases from Canada and therefore had no negative impact on the American beef industry. Later it was considered more likely that these cases represent atypical BSE cases. The first was detected in December 2003 in a 6.5-year old Holstein cow in Washington state. In one of these cases a *Prnp* gene mutation was detected (E211K) which corresponds to the pathological human E200K mutation which is linked to familial CJD. This is so far the only reported case in animals which might be caused by a mutation in *Prnp*, in analogy to familial prion diseases in humans. The prevalence of the E211K mutation in the US cattle population seems to be extremely low. The fourth case, again considered to be an atypical one, was detected in April 2012 in an over 10-year-old animal that was sampled at a rendering facility in central California.

In recent years, atypical BSE has been found in several countries worldwide, which, like atypical scrapie, has altered histopathological and biophysical properties, such as a reduced proteinase K resistance. Therefore, it can easily be overlooked with the usual tests for BSE. This form of BSE was found to be clearly infectious in animal experiments. Therefore, some researchers hold the opinion that this form of BSE also possesses zoonotic potential. The exact distribution and frequency of these forms of BSE must be elucidated in future studies. It remains to be seen whether atypical BSE corresponds to sporadic cases in cattle.

BSE and Consumer Protection

Besides the UK, there were some European countries which early on had confirmed BSE cases, such as Ireland, Portugal and Switzerland. Other European countries such as Spain and Germany which later had rather high numbers of BSE cases were thought to be free of BSE at that time. There was a lot of political pressure to keep this impression alive and great efforts were made to signal to consumers that their beef is free of BSE. This situation changed dramatically when the European Union introduced BSE testing in 2000. After the report of the first BSE cases in Germany in November 2000, beef consumption declined dramatically. This was similar in other European countries. Consumers demanded controls and non-hazardous, “safe” foods. One consequence was the implementation of mandatory testing of all healthy cattle older than 24 months being sent for slaughter in Germany (elsewhere in the European Union the limit was 30 months). Only in 2011 was this changed to 72 months. In Germany, since that time, almost 22 million BSE tests have been performed on cattle originating from normal slaughter, emergency slaughter and possibly symptomatic animals, of which a total of 406 cases were confirmed to be positive. This corresponds to an incidence of more than

1:50,000 or an incidence of less than two cases per 100,000 animals (equivalent to 0.002 % of all animals tested). Thus, such comprehensive supervision of a rare infection is unprecedented. Nevertheless, the financial expense of BSE testing, which amounts to billions of euros, is justified owing to the importance of consumer protection. Most diagnosed German BSE cases were in cattle born between 1995 and 1996, and only two cows were younger than 30 months. Most of these cases were clinically normal cattle going to slaughter. On the other hand, of the 406 cows that tested positive, about one third were killed because of clinical abnormalities, most likely in conjunction with BSE, and would have been seen in passive surveillance. Since a few of these cows were born after the ban on meat and bonemeal as animal feed in 2000, the question arises how these animals had become infected. Given the fact that vCJD is transmitted within the human population (e.g. by blood donation) and that such iatrogenic, svCJD cases are very difficult to prevent and require immense financial and logistical efforts, the various measures aimed at protecting people (also in medicine and blood banking) are certainly adequate. Overall, prevention of transmission of BSE to humans is much easier than dealing with vCJD in the human population.

21.6.4.2 Clinical Features

In contrast to the pathogen of scrapie, the BSE agent appears to be very uniform; pathogens originating from different outbreaks show a consistent histopathological lesion profile in the brains of infected mice, and a homogeneous and constant behaviour in immunoblot tests, as well as in animal experiments with transgenic mice. The outbreak of the disease occurred almost simultaneously in different regions of the UK after 3–5 years of the mean incubation period. The manifestation of the disease begins with behavioural disorders, such as increased sensitivity to acoustic or tactile stimuli, and progressively develops into ataxic gait, as well as falling and lying. The duration of the disease is relatively short, only a few weeks; however, the first symptoms are often overlooked or misinterpreted, and the cattle are slaughtered before final diagnosis. Histologically, the typical changes associated with spongiform encephalopathies are found predominantly in the midbrain, pons, medulla oblongata and hippocampus. Like all TSEs, BSE is invariably fatal.

21.6.4.3 Pathogenesis

The pathogenesis of oral BSE infection in cattle has been examined in detail in very complex pathogenesis studies in several countries (e.g. in the UK, Germany and Japan). For this purpose, experimentally infected cattle were slaughtered under controlled conditions at specific times, and a variety of biological materials were retained (including faeces, urine, secretions and excretions) and examined with various test systems. In particular, focus was directed on the presymptomatic period and it was found that infectivity precedes clinical symptoms. Depending on the

detection method, prion infectivity can be detected in peripheral nerves long before it can be detected in the central nervous system. Furthermore, PrP^{Sc} and prion infectivity have been detected at low levels in muscle meat.

21.6.4.4 Diagnosis

The diagnosis is performed on dead animals by detecting proteinase K resistant PrP^{Sc} from the obex region (brainstem) in “rapid BSE tests” (Fig. 21.4). It is necessary that the test results are obtained within a very short time so that the processing of meat and meat products can proceed. In addition, intensive research is being conducted in order to develop “live tests”. Owing to the lack of lymphotropism in cattle, lymphatic materials cannot be used intravitally. Furthermore, the PrP genotype plays no role in BSE. In positive cases, an immunohistochemical analysis is performed, and additional detection of scrapie-associated fibrils is done by immunoblotting (usually at national reference laboratories) for confirmation.

21.6.4.5 Therapy and Control

Besides culling, there is neither therapy nor prophylaxis. All animal TSEs are notifiable diseases in almost all countries. Every suspected case of disease must be notified to the relevant veterinary authorities by a veterinarian or another person, such as the owner of the animal. The control of BSE is usually regulated by the national animal health authorities and by the World Organisation for Animal Health. Luckily, the BSE epidemic was a manageable and well-controllable problem. Only a few animals in a herd became sick. Since horizontal transmission seems to be very inefficient, it was possible to eradicate the infection source by eliminating a herd in which a case had occurred. After official ascertainment of the disease, so-called cohort killing was practised in several countries for a long time. That means that all animals of the same herd were killed that were born 1 year before and 1 year after the diseased animal, as well as all animals that received the same feed during the first 12 months of life. The remaining animals in the herd were not subject to special regulations. Milk and meat of these animals can be traded freely. As virtually almost all animals of a “cohort" proved to be negative, cohort killing is no longer practised. Instead, these animals are just recorded. The animals of the cohort can be used without restrictions, but they must not be slaughtered. They are killed after they have been used, and the carcasses are destroyed and eliminated harmlessly.

21.6.4.6 BSE-Related Diseases in Ungulates and Felids

BSE was transmitted to exotic ungulates and great cats in zoos by exposure to the BSE agent by feed and feed supplements such as mineral preparations containing meat and bonemeal. Between 1996 and 2004, 16 cases in zoo bovids (e.g., nyala, oryx, greater kudu, gemsbok and American bison) and 24 cases in zoo felids (e.g., cheetah, puma, ocelot, leopard, tiger and lion) were reported in the UK. Additional cases were found in other countries.

Similarly, BSE was transmitted to domestic cats in the UK, first reported 1990 (feline spongiform encephalopathy). Between 1990 and 2005, 90 cases of feline spongiform encephalopathy were reported in the UK, and four cases were reported

in the rest of Europe. There were no transmissions in dogs. Feline spongiform encephalopathy was obviously a transient event as producers of cat food accordingly changed their food production when the first cases were reported.

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Appendix 1 – Transmission Electron Microscopy in Virology: Principles, Preparation Methods and Rapid Diagnosis

Hans R. Gelderblom

Formerly, well-equipped virology institutes possessed many different cell culture types, various ultracentrifuges and even an electron microscope. *Tempi passati*? Indeed! Meanwhile, molecular biological methods such as polymerase chain reaction, ELISA and chip technologies—all fast, highly sensitive detection systems—qualify the merits of electron microscopy within the spectrum of virological methods. Unlike in the material sciences, a significant decline in the use of electron microscopy occurred in life sciences owing to high acquisition costs and lack of experienced personnel. Another reason is the misconception that the use of electron microscopes is expensive and time consuming. However, this does not apply to most conventional methods. The cost of an electron-microscopic preparation, the cost of reagents, contrast and embedding media and the cost of carrier networks are low. Electron microscopy is fast, and for negative staining needs barely 15 min from the start of sample preparation to analysis. Another advantage is that a virtually unlimited number of different samples can be analysed—from nanoparticles to fetid diarrhoea samples.

A.1 Principles of Electron Microscopy and Morphological Virus Diagnosis

In transmission electron microscopy, accelerated, monochromatic electrons are used to irradiate the object to be imaged. This leads to interactions: the beam electrons are scattered differentially by atomic nuclei and electron shells, losing some of their energy. After magnification through a multistage lens system, a 1,000-fold higher resolution is obtained with a transmission electron microscope than with a light microscope (2 nm vs. 2 μm) owing to the much shorter wavelength of electrons in comparison with visible light. Hence, in contrast to light microscopy, transmission electron microscopy is capable of visualizing even the smallest viruses. Scanning electron microscopy depicts surfaces, but no internal structures. Even though confocal laser scanning light microscopy can localize individual viruses, the fluorescence signal does not image the particle. However, identification

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of viruses and description of virus–cell interactions frequently require structural information in high resolution, as can be supplied only by electron microscopy.

Electron microscopes are also used in the diagnosis of viral diseases: although a modern diagnostic laboratory allows high sample throughput, it requires a clinical diagnosis and pathogen-specific reagents. By contrast, electron microscopy “illuminates,” after rapid and simple contrasting, and provides a high-resolution “open view” of the finest structures in a sample. Thereby, every pathogen is rendered visible also in the case of multiple infections or unknown pathogens, or agents that have never been considered by the clinician, and even genetically engineered organisms. Infectious agents exhibit constant morphological characteristics such as size, shape, subunits, perhaps an envelope, surface projections or protrusions and specific nature of the interaction with the host cell. These criteria help to assign the object in question to a specific pathogen family. Although the morphological diagnosis “viruses of the herpesvirus family” does not indicate the viral species, as a “family diagnosis” it gives the clinician or epidemiologist—along with the previous clinical history—a rapid perspective on the action required, e.g. antiviral therapy, quarantine or vaccination. If the pathogen family is ascertained, if necessary, even a “fine diagnosis” by molecular methods or by immune electron microscopy can be significantly accelerated. Independence from pathogen-specific reagents is a great advantage for diagnosis of infections, epidemics and bioterrorism hazards, but also in the characterization of laboratory products and biological preparations, vaccines, therapeutic antibodies and in the frequently neglected internal quality assurance.

A disadvantage of electron microscopy is that it has a very narrow field of view, owing to the very high magnification; therefore, high particle concentrations are required for detection (more than 10^5 particles per millilitre). Samples with particle concentrations below this threshold need to be effectively and rapidly enriched before use. Furthermore, electron microscopy does not allow high-throughput analyses. The examiner must be concerned only with one preparation for at least 20 min before it can classify it as “negative.”

A.2 Transmission Electron Microscopy: Thin Preparations Are Required

In preparations with a layer thickness of more than 80 nm, the imaging electrons are scattered several times; the consequences are loss of image sharpness and information. The necessary thinner specimens are obtained after embedding the samples in resin (Epon) in ultrathin sections or by negative staining of particle suspensions. Both methods result de facto in a resolution of 2 nm. Viruses are biological macromolecules which consist essentially of light atoms with low mass density. In the transmission electron microscope, they appear highly transparent and with low contrast. Detailed and contrast-rich images can be obtained by absorption and scattering of electrons only at high mass density of the objects, as can be created by negative or positive staining (Fig. A.1).

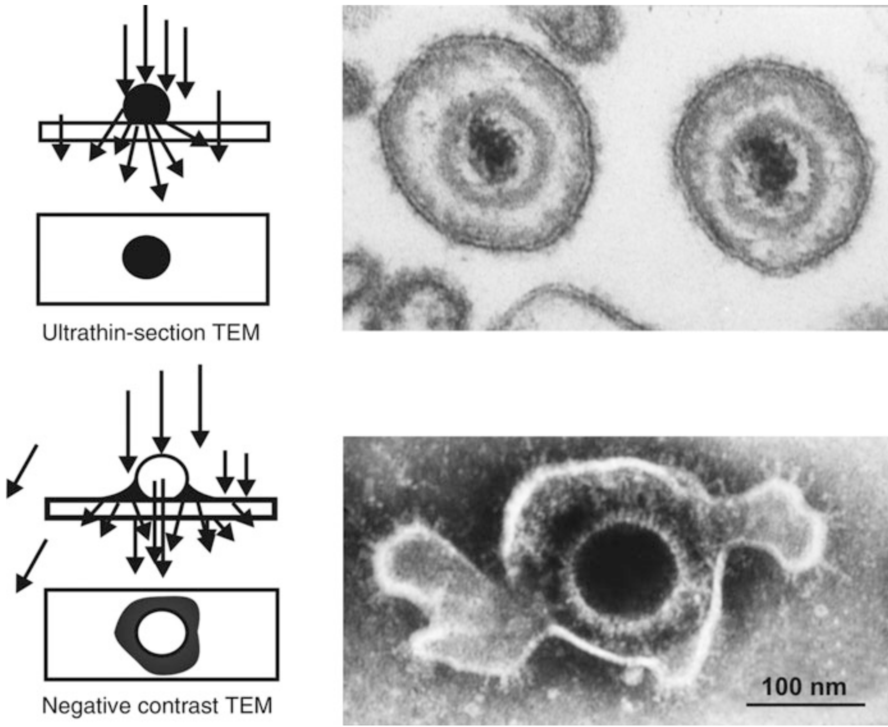

Fig. A.1 Preparation for transmission electron microscopy (TEM). Principle of ultrathin-section and negative-contrast TEM using the example of a herpesvirus. The *upper half* shows the ultrathin section of a specimen embedded in Epon. For this purpose, in vitro infected cells were fixed with glutaraldehyde, incubated with heavy metal salts, dehydrated and finally embedded in resin. The hardened samples were cut in an ultramicrotome into 50-nm-thick specimens, treated again with contrast medium and analysed by TEM. The following viral structures can be recognized from outside to inside: the viral envelope as a lipid bilayer with glycoprotein protrusions, a moderately contrasted tegument, the hexagonal viral capsid and deep inside the viral DNA complex. Heavy metal salts are also used as contrast media in negative staining (*lower half*) (see Fig. A.2). In this case, the short exposure leads only slightly to chemical bonds. The viral components are transparent, they appear bright, negatively contrasted against the dark, electron-dense contrast medium, and we can recognize the fragile envelope of a herpesvirus with its glycoprotein processes and inside the contrast-medium-filled capsid with capsomeres and a typical diameter of 100 nm

Negative staining requires particle suspensions, as they can be directly prepared from swab samples from patients, cell culture supernatants, disrupted cells, pulverized tumours, urine, serum or stool samples, bacterial colonies and bioterrorism-suspected "powder." In principle, any organic or inorganic material is suitable if it can be resuspended. Rough cell debris is removed by low-speed centrifugation.

The sample is then stained (Fig. A.2). Usually, a copper grid (carrier network) is placed on a drop of the suspension. The grid carries a stable and very electron transparent plastic carrier film with regularly dispersed holes. After short

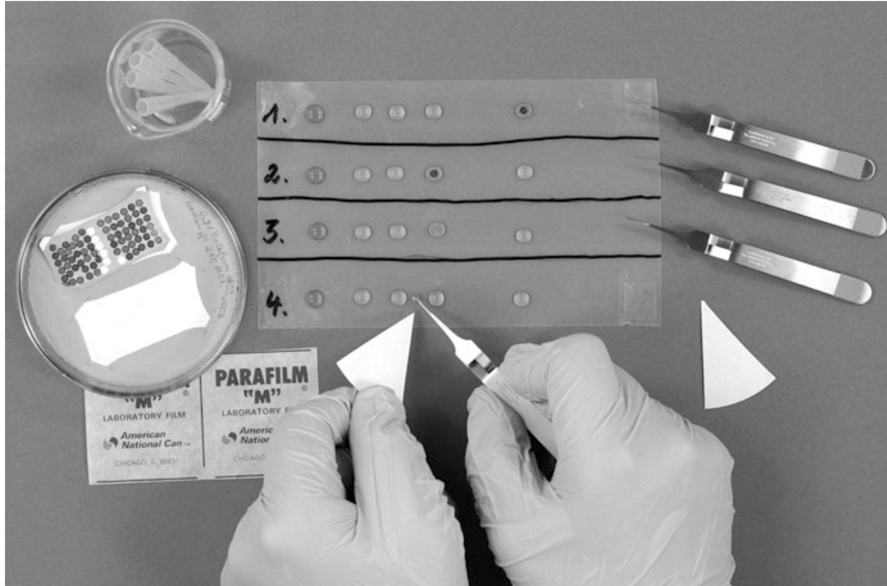

Fig. A.2 Negative staining procedure. Drops of the suspension to be examined, washer fluid and contrast medium are applied from left to right in a horizontal row on an inert surface. Grids (in the Petri dish) are placed on the sample using a pair of tweezers for 10 s or longer, are then washed with drops of double distilled water and are finally stained with contrast medium for 10 s. After excess contrast medium has been removed and subsequent air drying, the grids are well prepared for TEM. (From Gelderblom 2003)

adsorption, the sample excess and interfering ions are removed with filter paper and the grid with the adsorbed material is placed for a few seconds on the contrast medium, which consists of a 0.5–4.0 % heavy metal salt solution with high mass density, such as phosphotungstic acid or uranyl acetate. Excess contrast medium is removed from the "contrasted" grid, briefly air-dried and is then prepared for electron-microscopic examination. The dried, electron-dense contrast medium coats the "transparent" structures on the carrier network and renders visible surface details with very high resolution—comparable to the X-ray contrast technique. However, it can also penetrate into labile samples, facilitating the representation of internal structures. Advantageous for a natural image is that viruses, like other unstable biostructures, are closely enveloped, and thus are stabilized by the contrast medium.

Thin specimens make possible, apart from negative staining, also the more complex ultrathin-section technique. For this purpose, small blocks of solid tissues, cell culture conglomerates or ultracentrifuge sediments (edge length under 1 mm) are chemically fixed by aldehydes, and incubated with heavy metal salt solutions (osmium, uranium, lead). The heavy metal salts bind to different groups in the material and this results in high mass densities and high-contrast images even of

internal structures, which become visible later. After dehydration, e.g. in an ascending ethanol series, the samples are embedded in resin and cut in an ultramicrotome: sections with a thickness of 40–70 nm are restained with lead salts and analysed by electron microscopy. The entire conventional preparation requires 4–5 days. In contrast, modern, rapid methods reduce the preparation time to a few hours, but they still require considerably more effort and time than the negative staining procedure.

A.3 Perspectives for Transmission Electron Microscopy in Virology Research

When promptness, high resolution and the "open view" of electron microscopy are to be used reasonably in laboratory medicine and cell biology research, a "frontline" application is required. Therefore, this method should be used together with complementary techniques to increase its diagnostic value. An electron-microscopic laboratory should also provide more services for other institutions. Between services, routine research and basic research, there are a myriad of worthwhile tasks. Such an open-use approach should inspire the next generation of scientists, and ensure the long-term survival of electron microscopy within the life sciences.

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Appendix 2 – Information on the "Prototypical Electron-Microscopic Portraits" of Individual Virus Families

Hans R. Gelderblom

Poliovirus type 1 (▶ Sect. 14.1) as a prototype of the family *Picornaviridae*, grown in cell culture, purified by gradient centrifugation and represented by negative staining with 2 % phosphotungstic acid (PTA). Picornaviruses are isometric, have no envelope, are 28–30 nm in diameter and do not exhibit fine structures, even at high resolution. Primary magnification × 60,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Human astrovirus type 1 (▶ Sect. 14.2), the prototype of the family *Astroviridae* (Greek *aster*, "star"), grown in cell culture, purified by gradient centrifugation and represented by negative staining with 1 % uranyl acetate. The isometric, non-enveloped astroviruses are 27–30 nm in diameter, and occasionally show a clear, star-shaped five- or sixfold symmetry. Primary magnification × 60,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Rabbit haemorrhagic disease virus (▶ Sect. 14.3) as an example of the family *Caliciviridae* (Greek *kalix*, "chalice"); partially purified particle suspension from the liver of diseased rabbits, represented by negative staining with uranyl acetate. The isometric caliciviruses have a diameter of 28–34 nm and have capsid structures, which can be used diagnostically: striking cup-shaped depressions in rabbit haemorrhagic disease virus and sapoviruses. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Hepatitis E virus (▶ Sect. 14.4), the prototype of the family *Hepeviridae*, prepared from the bile of a diseased patient after negative staining with PTA. The isometric particles are 27–30 nm in diameter, and aggregated in vivo by the action of the antibodies from the patient. Primary magnification × 40,000, *bar* 100 nm. (Kindly provided by Bärbel Hauröder, Central Institute of the Bundeswehr Medical Service, Koblenz, Germany)

West Nile virus (▶ Sect. 14.5) as a prototype of the family *Flaviviridae*, grown in cell culture, represented in ultrathin section. The spherical virus particles are approximately 50 nm in diameter, have short glycoprotein processes and are tightly enclosed by a lipid envelope. The dark-stained core of flavoviruses is determined by the viral nucleic acid. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

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Sindbis virus (▶ Sect. 14.6) as a prototype of the family *Togaviridae*, grown in cell culture, partially purified by gradient centrifugation and negatively contrasted with uranyl acetate. The virus particles have a diameter of 60–70 nm. In the image, some viruses have been penetrated by the contrast medium; viral glycoprotein protrusions are also partially visible on the lipid envelope. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Equine arteritis virus (▶ Sect. 14.7) grown in cell culture, represented in ultrathin section as a representative of the family *Arteriviridae*. The virions are rounded ovoid and have a diameter of 40–60 nm, are surrounded by a lipid envelope and have a narrow electron-dense ribonucleoprotein core. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

SARS-associated coronavirus (▶ Sect. 14.8) as a prototype of the family *Coronaviridae*, grown in cell culture, without pre-treatment, represented after negative staining with 2 % PTA. The virion is surrounded by a lipid envelope, is 90–140 nm in diameter and has striking, club-like surface processes (20 nm, peplomers), which can be relatively easily released from the surface of the virus particle by shedding (see the virion at the *top right*). Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Vesicular stomatitis virus (▶ Sect. 15.1) as an example of the family *Rhabdoviridae* (Greek *rhabdos*, “rod”, “stick”, or “staff”), grown in cell culture, enriched and represented after negative staining with uranyl acetate. Animal rhabdoviruses, unlike plant rhabdoviruses, are “bullet”-shaped particles that are flattened at one end. They are 75 nm × 190 nm and have an envelope which is studded with viral glycoprotein protrusions. The helically wrapped ribonucleoprotein core resides inside—“like a beehive.” It has a diameter of 50 nm. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Equine Borna disease virus (▶ Sect. 15.2) as a prototype of the family *Bornaviridae*, propagated in cell culture, represented in ultrathin section. The rotund virions are 120 nm in diameter and mature by budding at the cell surface. They are enclosed by an envelope containing glycoprotein protrusions of 7 nm in length. Occasionally, thin strings of viral ribonucleoprotein complexes are recognizable inside the virus. In addition to the 120-nm-diamter particles, the virus preparations frequently also contain smaller, subviral particles of 75 nm in diameter. Primary magnification × 40,000, *bar* 100 nm. (Kindly provided by Kouichi Sano, Department of Microbiology and Infection Control, Osaka Medical College, Japan)

Human parainfluenza virus 1 (▶ Sect. 15.3) as a prototype of the family *Paramyxoviridae*, depicted in ultrathin section. Paramyxoviruses are released by budding at the cell surface; they are round-polymorphic and very heterogeneous in size (90–400 nm). Their envelope is densely occupied with viral glycoproteins (HN and F proteins). Cross sections of viral ribonucleoproteins are recognizable inside the virus, beneath the envelope (diameter 18–22 nm). Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Marburg virus (▶ Sect. 14.8) representing the family *Filoviridae*, grown in cell culture, without pre-treatment and represented after negative staining with 2 % PTA. The virus particles are clumped with cell debris. Their envelope contains

glycoprotein projections; the helical ribonucleoprotein complex is located inside. The diameter of filoviruses is 80 nm; their length can differ considerably: Marburg virus is 700–800 nm long, whereas Ebola virus can reach up to 15 μm. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Lymphocytic choriomeningitis virus (▶ Sect. 16.1) representing the family *Arenaviridae* (Latin *arenosus*, "sandy"), grown in cell culture. The release of virus particles occurs by budding at the cell surface. The envelope of arenaviruses carries glycoprotein protrusions and is flexible, so the released virus particles appear pleomorphic. Arenaviruses incorporate cellular ribosomes during particle assembly, and thus exhibit typical, high-contrast inclusions. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Puumala virus (▶ Sect. 16.2), a member of the genus *Hantavirus*, representing the family *Bunyaviridae*, grown in cell culture. The image shows the ultrathin section of a virus-producing cell with accumulation of released particles. The viruses are surrounded by an envelope, and have a diameter of 80–120 nm. The ribonucleoprotein strands of their segmented genome are recognizable inside the virus particles. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin).

Influenza A virus (▶ Sect. 16.3) as a prototype of the family *Orthomyxoviridae*. The image shows the ultrathin section of a virus-producing cell. The plump, ovoid particles are 80–120 nm in diameter and carry HA and NA glycoprotein processes. The matrix protein is located directly under the envelope as a moderately dark layer. Some of the segmented ribonucleoprotein complexes can be observed inside. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Avian infectious bursal disease virus (▶ Sect. 17.1), representing the family *Birnaviridae*, grown in cell culture, purified by gradient centrifugation and represented by negative staining with uranyl acetate. The isometric particles are 60 nm in diameter, possess an envelope and reveal the typical capsomere structure of their capsids. Primary magnification: ×60,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Human rotaviruses (▶ Sect. 17.2) partially purified from a stool specimen and shown by negative staining with uranyl acetate. Rotaviruses are a subfamily of the family *Reoviridae*; the actual reoviruses are 80 nm in diameter and thus markedly larger. Rotaviruses are 70 nm in diameter and have two isometric protein capsids that lie one above the other. They occasionally show an external, spoke-shaped zone around a large inner hub (a wheel-like structure, hence their name from the Latin *rota*, meaning "wheel"). For most virus particles only in the surface is revealed by the contrast medium; however, the isometric core can occasionally be seen because here uranyl acetate is infiltrated into the virion. Primary magnification × 60,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Human immunodeficiency virus 1, a lentivirus as a prototype of the family *Retroviridae* (▶ Sect. 18.1). The image shows the ultrathin section of a virus-producing T cell with different viral maturity stages. The viruses are about

120 nm in diameter, and have a very fragile envelope. At the *bottom right* is a late budding phase with only scarce interconnection of the virion to the host cell: the viral envelope is densely covered with gp120 protein processes of 9–10 nm in length. Beneath the envelope, the uncleaved Gag–Pol precursor protein and the viral RNA form a 20-nm-thick, dark layer. Their cleavage by the viral protease generates the Gag proteins of the mature virion and causes a massive morphological transformation, and the virion become infectious. Mature lentiviruses (*top left*) have a conical core, which always contains two molecules of the viral ribonucleoprotein. The glycoprotein projections of human immunodeficiency virus and other retroviruses are lost in a time- and temperature-dependent manner: this "shedding" leads to loss of infectivity. Lentiviruses of humans and monkeys have lateral bodies consisting of the structural proteins that are not required for remodelling. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Hepatitis B virus (▶ Sect. 19.1) represented by negative staining with PTA in immune aggregates. The image shows three spherical virions with a diameter of 42–45 nm (Dane particles), which are coaggregated with a variety of non-infectious HBsAg particles (20-nm particles, Australia antigen). The virions have a tight-fitting envelope that surrounds the viral capsid (diameter 28–30 nm). The serum of a hepatitis B infected patient was used for aggregation of hepatitis B virus and HBs particles; cross-linking antibodies hide finer details. Primary magnification × 60,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

BK polyomavirus (▶ Sect. 19.2), a human polyomavirus representing the family *Polyomaviridae*. BK polyomavirus was imaged directly from the urine of an immunosuppressive-treated patient after negative staining with uranyl acetate. The virus particles are 45 nm in diameter and have no envelope. Their isometric capsids consist of 72 pentameric capsomeres. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin)

Papillomavirus (▶ Sect. 19.3) from a chicken tumour (chaffinch), representing the family *Papillomaviridae*. The specimen originated from a biopsy, was isolated by "rubbing" with sea sand and was directly depicted by negative staining unpurified. These enveloped virus particles are isometric, have a diameter of 55 nm and consist of 72 pentameric capsomers. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Fowl adenovirus 2 (▶ Sect. 19.4) as an example of the family *Adenoviridae*, highly purified by gradient centrifugation and represented by negative staining with uranyl acetate. Adenoviruses are 75–80 nm in diameter; their non-enveloped capsids clearly show their perfect icosahedral symmetry and their structure made up of capsomers, which, depending on the position in the capsid, have different properties (a total of 240 hexon and 12 penton capsomers per virion). The pentons are located at the 12 vertices of the icosahedron, and each penton base is associated with a rigid-fibre protein with a terminal knob. These fibres are anchored in poultry adenoviruses and several human adenoviruses in double form on the penton base. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Equine herpesvirus 1 (▶ Sect. 19.5) from the family *Herpesviridae*, represented in ultrathin section. The image shows six virus particles in cross section. They are approximately 180 nm in diameter, but are in different planes, sometimes only peripherally sliced, and thus do not show all the known structural components of herpesviruses. The envelope carries a dense crown of glycoprotein processes. Below the envelope is the moderately electron-dense viral tegument layer, and further inside is the viral capsid (diameter 100 nm), which contains the highly electron-dense viral DNA genome. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Murine ectromelia virus (▶ Sect. 19.6), an orthopoxvirus as an example of the family *Poxviridae*, grown in cell culture and represented by negative staining. Like variola and vaccinia viruses, ectromelia viruses are cuboid. Their dimensions are 250 nm × 350 nm and they have short crests on their surfaces. On the basis of these morphological criteria, orthopoxviruses can easily be distinguished from the smaller, ovoid parapoxviruses by rapid electron-microscopic diagnosis. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

African swine fever virus (▶ Sect. 19.7) is the sole member of the family *Asfarviridae*, represented in ultrathin cryosection. The four virus particles show different stages of maturity: the left virion is still in the process of being released from the cell by budding. The virus particles have a diameter of 200 nm and are isometric. Around the dense DNA-containing core, they exhibit a multilayer structure consisting of a protein capsid located between two lipid envelope membranes. Primary magnification × 40,000, *bar* 100 nm. (Kindly provided by Germán Andrés, Centro de Biologia Molecular Severo Ochoa, Madrid, Spain)

Porcine parvovirus (▶ Sect. 20.1) as prototype of the family *Parvoviridae*, grown in cell culture, partially purified by gradient centrifugation and depicted after negative staining with uranyl acetate. The naked, isometric viruses are 25 nm in diameter and were occasionally penetrated by the contrast medium. Primary magnification × 40,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Porcine circovirus 1 (▶ Sect. 20.2) representing the family *Circoviridae*, grown in cell culture, purified by gradient centrifugation and represented by negative staining using 1 % uranyl acetate. Porcine circoviruses are 17–18 nm in diameter, constituting the smallest autonomously replicating mammalian viruses. They are considerably smaller than avian circoviruses: chicken anaemia virus has a diameter of 22–25 nm and, in contrast to porcine circoviruses, has a clear capsid structure. Primary magnification × 60,000, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Scrapie-associated fibrils (Chap. 21) purified and enriched roughly 10,000-fold from brains of scrapie-infected hamsters, represented by negative staining with 1 % uranyl acetate. The slightly "twisted" glycoprotein fibrils are proteinase K resistant and also characteristic of other transmissible spongiform encephalopathies in humans and animals. Primary magnification × 31,500, *bar* 100 nm. (H.R. Gelderblom, Robert Koch Institute, Berlin, Germany)

Glossary

Aetiology The theory of the causes; in medicine and epidemiology, aetiology is the totality of factors that have led to a specific disease.

Agglutination Clumping (adhesion) of antigen-carrying particles, such as red blood cells (hemagglutination), bacteria, viruses and viruses and infectious agents complexed with antibodies.

Allergy (Greek *αλλερία*, "foreign reaction") The exuberant immune response to certain normally harmless environmental substances (allergens). An allergy manifests itself as a typical symptom caused by inflammatory processes.

Anamnesis (Greek *ανάμνησις*, "memory") The history of a patient with regard to his/her current afflictions.

Anergy Lack of immune response by switching off the immune response against an antigen. Anergy is a permanent mechanism with which the immune system prevents T lymphocytes from attacking the body's own tissues. T cells mature in the thymus during embryonic development. Autoreactive T lymphocytes, which recognize the body's own antigens, and thus would attack the body's own tissue, are generally eliminated. However, a few T cells reach the periphery of the embryonic organism. If these cells bind to an antigen through their T-cell receptor there, they become activated only in the presence of co-stimulatory molecules. If these co-stimulatory signals are lacking, the T cell remains permanently inactivated and inoperative.

Antigen A substance that is recognized as foreign by the immune system, such as a protein, a carbohydrate structure or another chemical compound.

Antigenicity Recognizability of a protein or other substance by the immune system. Even minor changes and variations in the amino acid sequence of a protein can alter antigenicity, thus changing serological detection by antibodies.

Apical The term means "located at the top" and is used to indicate the side of a polarized cell which is located on the surface or in a lumen. The other side of a polarized cell is referred to as the basolateral side.

Apoptosis Programmed cell death.

Apparent/inapparent (Latin *apparere*, "become visible"). Becoming visible or invisible. The term is often used in connection with infections with or without symptoms.

Arthralgia Joint pain.

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Arthritis Acute or chronic, specific or nonspecific joint inflammation, articular rheumatism.

Atrophy Tissue shrinkage.

Arthropods Invertebrate, articulated animals having an exoskeleton, including insects, millipedes, crabs, spiders, scorpions and mites.

Assembly See "Self-assembly" and "Viral assembly".

Attenuated viruses Virus mutants with a weakened virulence which arise spontaneously or by continuous cultivation in cell culture. Infections with such viruses are usually either without or with significantly weakened symptoms. Attenuated virus strains are often used as vaccine viruses.

Autocrine Direct effect of a molecule (cytokine, hormone, growth factor, etc.) released by a cell on the same cell, e.g. by binding to receptors on the cell surface.

Basolateral The base of a polarized cell that is remote from the lumen in a cell layer and its lateral portions; see also "Apical."

Bilirubin Yellowish degradation product of haemoglobin which is coupled to glucuronic acid in the liver, and is excreted through the bile into the intestine.

Bronchial lavage Lavation of the bronchial tree with isotonic solution for therapeutic or diagnostic purposes.

Bronchiolitis Inflammation of the bronchioles, i.e. the cartilage-free branches of segmental bronchi.

Bronchitis Acute or chronic inflammation of the mucous membrane in the region of large and medium-sized bronchi, i.e. the continuation of the trachea for passage of air into the lungs.

Bronchopneumonia A focal pneumonia without reference to anatomical lung limits. A common feature of inflammation foci, which can have various sizes and development stages, is exudate-filled alveoli in the infiltrated lung regions.

Budding The process of releasing virus particles from cellular membranes by extrusion or evagination.

Cap group (5′-cap structure). 7-Methylguanosine modification of eukaryotic messenger RNA (mRNA) molecules attached at the 5′ end, which is linked to the 5′-OH group of the next nucleotide by a 5′–5′ bond. This and the adjacent nucleotide are modified by a methyl group at the 5′-OH residue of the ribose.

Capsid Icosahedral or helical virus particle structure which is composed of proteins.

Capsomers Protein components that make up the capsids, composing one or more viral structural proteins.

Carcinoma Malignant neoplasm of epithelial origin.

Chaperone Catalysts of protein folding. Chaperones have the function of binding specifically to other proteins and of preventing non-specific aggregation or misfolding.

Cholestasis Extrahepatic bile congestion in the large intrahepatic or extrahepatic bile ducts as a result of outflow obstruction, or with intrahepatic obstruction in the bile canaliculi as a result of a metabolic disorder of liver cells that alters their directional permeability (e.g. in viral hepatitis). It leads to an increase of the

levels of bile acid and certain liver-specific enzymes in the blood (e.g. glutamyl-transpeptidase and alkaline phosphatase).

Chorioretinitis Inflammation of the choroid (choroiditis) with secondary inflammation of the retina (retinitis).

Cirrhosis Progressive chronic disease which leads to induration by fibrosis and to a scarring contraction of an organ (usually the liver), resulting in death of functionally active tissue (parenchyma) by connective tissue proliferation due to chronic inflammation.

Co-infection The concurrent infection of a human or animal with another pathogen (virus, bacterium, parasite, fungus).

Confluence Coalescence or merging of exanthemas and skin efflorescence.

Conjuntivitis Inflammation of the conjunctiva.

Contagious Tending to spread, infectious.

Contamination Infection/contagion of skin (hands), surfaces and objects by contact with materials that (possibly) contain infectious agents.

Convulsions (Latin *convolvere*, “to curl,” “to roll together,” “to convolve”). Repeated, serial and involuntary muscular contractions.

Croup A cough illness which particularly affects children aged between 6 months and 3 years because their larynx is very narrow owing to the still immature laryngeal development. During or as a result of infectious diseases, the mucous membrane of the larynx and the vocal chord region becomes inflamed and swells. In this way, the children develop respiratory difficulties and sticky mucus additionally constricts the airways. The term “croup” originally referred only to the cough that is associated with diphtheria.

Cull Government-ordered killing of individual animals or the entire animal population of livestock without bleeding (see “Slaughter”) and with destruction of the undamaged carcasses. This measure prevents spread of pathogens through blood contact or aerosols. It is the usual process in the case of an outbreak of foot-and-mouth disease (picornavirus infection) or classical swine fever (flavivirus infection).

Cytopenia (granulocytopenia, leucocytopenia, erythrocytopenia, lymphocytopenia, monocytopenia, neutrocytopenia, thrombocytopenia) Reduction of the number of the respective individual cells in the peripheral blood.

Degermination (sterilization). See “Disinfestation.”

Dermatitis Acute inflammation of the skin. It can be manifested by skin redness (erythema), swelling (oedema), lymph secretion (exudation) and the formation of blisters, crusts and scales (efflorescence).

Diagnosis (Greek *διάγνωση*, “examination” in the sense of distinction, decision, knowledge). Diagnosis is, in the ambit of health care (medicine, nursing, physiotherapy, psychology), the exact assignment of findings, diagnostic signs or symptoms to a specific illness or to a set of symptoms in the sense of a syndrome. The syndrome determined, together with the suspected cause and pathogenesis of the disease, results in the diagnosis. Diagnosis is the assignment of phenomena to a category and their interpretation.

Disinfection A procedure in which the number of infectious agents is reduced to such an extent that an infection or its transmission can be excluded.

Disinfestation (very rarely or hardly used in English in the German sense of *Entwesung*). Extermination of harmful rodents (particularly mice and rats). It plays an important role in control of epidemics, and is mandatory after culling of a herd to prevent dissemination of pathogens by rodents.

Dyspnoea Breathlessness, respiratory distress, shortness of breath.

Efflorescence Noticeable change in the skin as a result of disease ("skin blossom").

Embryopathy Damage to the embryo before birth, e.g. by infectious diseases of the mother.

Encephalitis Acute or chronic inflammation of brain tissue.

Encephalomyelitis Inflammatory alteration of the nerve tissue in the brain and spinal cord.

Encephalopathy A non-inflammatory change of nerve tissue in the central nervous system. Characteristic of transmissible spongiform encephalopathies such as bovine spongiform encephalopathy.

Endemic Constantly increased incidence of infections in a confined region or population. The prevalence of infection in this region/population (the incidence of all cases of a particular infectious disease in a population at the time of examination) remains largely the same, but is increased in relation to other regions/populations. The relevant geographical area is referred to as the endemic region.

Enteritis Acute or chronic inflammation of the small intestine.

Enterocytes By far the commonest cells of the epithelium of the small intestine, responsible for the absorption of different substances from food. Enterocytes have a characteristic apical brush-shaped membrane with microvilli. These increase the surface area tremendously, and are the basis for absorption.

Envelope (viral envelope) External lipoprotein bilayer derived from cellular membranes (plasma membrane, nuclear membrane, membrane of the endoplasmic reticulum or the Golgi apparatus) in which the viral partially glycosylated envelope proteins are incorporated. The viral envelope surrounds the capsid or nucleocapsid as a membrane.

Enzootic Endemic occurrence of an infectious disease in animals.

Epidemic (Greek *επιδημία*, "stay," "arrival") The temporal and spatial accumulation of an infectious disease within a region and a human population. An epidemic occurs when the prevalence, i.e. the number of infections and the associated morbidity, increases in a given period. An endemic is the ongoing frequent occurrence of a disease in a confined area.

Epidemic wave See "Panzootic."

Epitope Structure accessible to the immune system (antigenic determinant). Epitopes that are recognized by the variable domain of antibodies (immunoglobulins) are mostly located on the surface of particles and macromolecules such as proteins. They can be constituted by a region of a protein of four to six amino acids (sequential epitopes) or by structural, folding-dependent parameters (structural or discontinuous epitopes). Even protein modifications (such as sugars or phosphates) are recognized by antibodies as epitopes. However, epitopes recognized by T lymphocytes are peptide segments of proteins that form complexes with

MHC proteins and are derived from protein structures which are not exposed on the surface. These complexes are recognized by T-cell receptors.

Erythema Skin redness with a more or less distinct boundary. It arises as a result of expansion and increased filling of blood vessels. It usually disappears under pressure.

Exanthem Skin rash.

Exudation Release of certain portions of the blood through altered vessel walls into adjacent tissue or into inner or outer surfaces of the body during inflammatory processes.

Food and Agriculture Organization A special agency of the United Nations, with headquarters in Rome (Italy), founded in Canada in 1945. It comprises 192 member countries today. It has the task of improving worldwide the production and distribution of agricultural products in general and food in particular to ensure human nutrition and to improve living standards. It establishes, inter alia, the international standards for food safety.

Fulminant (Latin *fulminans*, "like a flash," "glossy") Commonly used in connection with infections associated with very severe symptoms.

Ganglion (ganglion nervosum) Nerve cells and fibres surrounded by a capsule and with enclosing glial sheath cells, which are present as bulges along cranial nerves and spinal nerves or as cholinergic switches within the autonomic nervous system.

Gastroenteritis Acute or chronic inflammation of the small intestine.

Genotype The exact genetic configuration, i.e. the nucleotide sequence and the individual set of genes that are present in the genetic information (genome) of a virus. Distinguishable viral genotypes are characterized by a defined percentage of different nucleotides, which is usually determined specifically for each virus type.

Genus Taxonomic category ranking below family and above species, and which contains one or more species. Here, it is used to refer to virus classes (genera).

Glomerulonephritis Inflammatory alterations in the kidneys which affect the renal glomeruli and tubules. This can lead to complete renal dysfunction. It is frequently found in immune complex diseases, since the deposition of complexes in glomeruli impairs the filtration processes.

Haematogenous Dissemination of pathogens into organs through the blood.

Haemorrhage/haemorrhagic Bleeding, associated with bleeding.

Heat/rut Rutting period in sows (heat, hogging) or cows (rutting, bulling). During that period, animals exhibit striking behavioural changes, which indicate increased sexual receptiveness or willingness to mate. Rut occurs at the beginning of a sexual cycle, and is interrupted during pregnancy. A premature termination of pregnancy leads to a re-entry into oestrus, which is specifically characterized by the German husbandry terms *umrauschen* (premature re-entry or return into heat for a sow) and *umrindern* (premature re-entry or return into the rutting period for a cow).

Helminths (Greek *ελμινσ*, "worm") General term for multicellular, endoparasitic organisms (worms).

Hepatitis Liver inflammation.

Herd immunity The existing protection against infectious disease in a population.

Hexon A protein surrounded by six neighbouring proteins in the faces of icosahedral virus particles.

Humoral/humoral defence system Responses of the immune system related to body fluids (especially serum) and mediated by their contents. It refers to the non-cellular components of the immune system, i.e. particularly immunologically active molecules in the blood serum, e.g. immunoglobulins (antibodies).

Hydrops Pathological accumulation of fluid in body cavities or in the interstitial space.

Hyperaemia Increased blood volume in part of the circulatory system or in the organ circulation; e.g. the additional circulation in an organ.

Hyperplasia Abnormal enlargement of a tissue or organ caused by increased cell division, and the associated extraordinary increase in the number of cells.

Hypertrophy Increase in the size of an organ or tissue by cell enlargement or by cell swelling (e.g. by increased water influx) when the original number of cells is maintained.

Iatrogenic (Greek *iatros*, "physician") Infections and diseases transmitted by physicians or through instruments used by them (surgical instruments, needles, etc.).

Icosahedron A regular polyhedron (particle) with 20 equilateral triangles as faces and 12 vertices.

Icterus (jaundice) A symptom that occurs in several different diseases (e.g. hepatitis). It describes the yellowing of the skin, mucous membranes and the conjunctiva of the eye caused by an increased concentration of bilirubin.

Immunoglobulins (antibodies) Proteins that are produced in vertebrates in response to specific antigens (pathogen structures).

Immunosuppression (Latin *supprimere*, "suppress," "repress," "withhold") A reduction or repression of the body's own immune defence mechanisms. Immunosuppression can be caused by viral infections (e.g. by human immunodeficiency virus) or by the use of medication (e.g. by administration of corticosteroids, cyclosporine in organ or bone marrow transplantations and in the treatment of autoimmune diseases). Furthermore, there are also hereditary forms of immunodeficiency.

Incidence The number of new disease cases in a time unit.

Incubation period The time between infection (contact) with a pathogen and the onset of disease symptoms.

Infarction Local tissue destruction (necrosis) caused by impaired circulation, commonly as a result of acute vessel occlusion (thrombus).

Infection Attachment, penetration and proliferation of pathogens (viruses, bacteria, fungi, parasites) in an organism (human, animal, plant). If the organism develops disease symptoms during this process, then it is an infectious disease.

Inflammation Specific or non-specific immune responses to various noxious agents (disease-causing agents, pathogens). Inflammations can be caused by chemical, mechanical, electrical, radiological or biological agents. The latter include infections with viruses, bacteria or parasites and their products. An inflammation

is characterized by sequentially structured phases: vascular reaction, increased vascular permeability, exudation, leucocyte migration (chemotaxis or phagocytosis) and proliferation of connective tissue. Classic signs of inflammation are redness, warmth, swelling, pain and restricted function.

Infiltration Pathologically increased, frequently localized penetration or migration of normal, abnormal or foreign cells into certain regions of the body and/or organs. It is generally associated with immunologically active cells that migrate into the infected organs as a result of viral replication.

Inoculation Introduction or transmission of pathogens or cellular material (inoculum) into an organism or a culture medium.

Interstitium/interstice Space between the body organs or tissues. It is usually filled by (interstitial) connective tissue.

Keratitis Inflammation of the cornea.

Keratoconjunctivitis Inflammation of the cornea and the conjunctiva.

Latent infection Form of infection where the virus remains in the body after primary infection without producing infectious viruses or causing disease symptoms. The virus can be stimulated to replicate by certain internal or external stimuli, leading to recurrences of symptoms of primary infection. Latent infections are caused, for instance, by herpesviruses.

Leader Leader sequence at the beginning of proteins (leader peptide) or transcripts (leader RNA).

Lesion Wound, injury or disruption of tissue structures in a living organism.

Lethality Number of deaths in proportion to the number of new disease cases in a particular illness.

Limitation (of an infection) Delimitation of an infection or viral replication at the entry site of the pathogen or at a specific organ, usually by the functions of the immune system.

Lymphohaematogenic Dissemination of infectious agents into various organs via the blood and/or the lymph fluid.

Mammillitis Inflammation of the nipple, or the teat in cattle.

Maternal immune protection Immune system of newborns based on antibodies (immunoglobulins) that are transferred from the maternal circulatory system into that of the foetus during pregnancy, and which remain detectable up to the age of 4–6 months in humans. IgA antibodies are also transferred to nursing infants by colostrum and breast milk (enteral maternal immune protection).

Meningitis Inflammation of the membranes (meninges) of the brain and/or spinal cord.

Meningoencephalitis Inflammation of the brain and/or spinal cord membranes (meninges) concomitantly with inflammation of adjacent brain tissue.

Metastasis/tumour metastasis A secondary focus of disease (secondary tumour) which is caused by spreading of single cells from a primary, usually persisting focus of disease or tumour cells into other body regions.

MicroRNA See “RNA interference.”

Morbidity Number of diseases (such as a result of an infectious disease) in relation to the total population in a given time span.

Mortality Number of deaths (such as a result of an infectious disease) in relation to the total population in a given period.

Mummification After death of a foetus during pregnancy, it may be "fossilized," remaining primarily in this form in the uterus, and may be delivered on schedule. This is a typical symptom of parvovirus infection in pigs.

Myocarditis Inflammation of the heart muscle (myocardium).

Necrosis Local death of cells of a tissue in a living organism.

Needlestick injury Bleeding and non-bleeding puncture, cut or scratch injury caused by medical personnel (physicians, nurses, laboratory personnel) with needles, scalpels or similar objects which were contaminated with patient blood or body fluids. Needlestick injuries are the commonest occupational injuries of health-care workers. These injuries can lead to transmission of infectious agents (e.g. human immunodeficiency virus, hepatitis B virus and hepatitis C virus).

Neoplasm/neoplasia Formation of new tissue by abnormal growth of body tissues owing to unregulated, autonomous and unrestrained cell proliferation.

Nosocomial (referring to hospital). Nosocomial infections are infections that can be acquired in health-care service units, hospitals or similar establishments.

Notification In animal disease control, notification is mandatory for important, especially contagious diseases such as foot-and-mouth disease. In such a case, the suspicion of an outbreak must be reported to the veterinary authority. Even animal owners are subject to this requirement. This process should make possible the immediate detection of an outbreak, and minimize the risk of dissemination of the disease.

Nucleocapsid Complex of capsid proteins and the viral genome (DNA or RNA).

Obligation to notify According to legislature in most countries, certain infections are notifiable. In the case of viral infections, this means that detection of viruses, suspicion of infection, illness or death caused by diseases which are mentioned in the law should be reported to the respective authorities either personally or anonymously. The treating physician as well as hospitals and laboratories are obliged to report cases of such diseases.

Oedema (Greek *οίδημα*, "swelling") Discharge of fluid from the vascular system and accumulation in the interstitial space. Oedema manifests itself as swelling of the tissue with subcutaneous fluid accumulation or in specific organs (e.g. pulmonary oedema, cerebral oedema).

Opportunistic pathogens/infection Viruses, bacteria, fungi and parasites which infect immunologically weakened organisms in order to spread very efficiently in them. The state of immunosuppression may be genetically determined, may be caused by infection with other pathogens (such as human immunodeficiency virus) or may be caused by therapeutic measures (e.g. after organ transplants).

Outbreak-control vaccination (see "Ring vaccination") A vaccination measure which is undertaken after outbreak of a disease in a population and can be regionally limited. Outbreak-control vaccination is required if the herd immunity in a population has fallen below a certain percentage. Outbreak-control vaccination is aimed at preventing further dissemination of the pathogen by

inducing a rapid protective immunity among people who could come into contact with it (neutralizing antibodies, etc.).

Pandemic Country- and continent-wide spread of an infectious disease. A pandemic is not geographically confined to a particular region; this feature distinguishes a pandemic from an epidemic.

Panzootic Pandemic spread of an infectious disease in animals.

Paracrine The immediate effect of a molecule (cytokine, hormone, growth factor) released by a cell on neighbouring cells, e.g. by binding to receptors on their cell surface.

Parenterally A general term for the way through which pathogens or substances (drugs, injections, infusions) penetrate into the human or animal body, circumventing the gastrointestinal tract, e.g. by intravenous (through a vein), intra-arterial (by an artery), intramuscular (through a muscle) or intraperitoneal (through the abdominal cavity) injection.

Parotid glands The largest of the salivary glands located anterior and inferior to the ears.

Parotitis Inflammation of the parotid glands.

Pathogenicity The genetically determined ability of viruses (also bacteria or parasites) to induce a disease in humans or animals.

Penton Viral protein associated with five neighbouring proteins at the vertices of icosahedral particles.

Perinatal Refers to the period immediately before and after birth.

Peritonitis Localized or diffuse inflammation of the peritoneum (membrane lining the abdominal cavity) which is usually associated with secretion and may lead to ascites (fluid accumulation in the abdominal cavity). It is a typical symptom of a feline infectious peritonitis.

Persistent (Latin *persistere*, "to remain," "to persevere") Commonly associated with infections during which the pathogen cannot be eliminated by the immune system of the organism, remaining in the body for long periods and continuously multiplying, even though frequently only with a low replication rate.

Petechiae Tiny, punctated skin or mucous membrane bleeding (capillary bleeding); single efflorescence of the purpura.

Pharyngitis Inflammation of the pharynx.

Pharynx The "throat" or "gullet" as a common section of the trachea and oesophagus. The pharynx is a muscle–mucosal tube which begins at the skull base and leads into the oesophagus. It is an open connection to the nasal and oral cavities and the larynx, and is accordingly divided into nasopharynx, oropharynx and laryngopharynx.

Phylogenesis/phylogeny The evolutionary development and the genetic relationship of all living organisms at all levels of biological taxonomy. Within the frame of this book, it is mainly used for the relationships of viruses within a family and the evolution of viral traits.

Planum nasolabiale The fusion of upper lip and nose in the muzzle of cattle.

Planum rostrale The fusion of the upper lip and nose to the front plate in the muzzle of swine.

Pleuritis/pleurisy Inflammation of the pleura.

Pneumonia Diffuse or focal inflammation of the lungs. Interstitial pneumonia is a form of pneumonia in which the inflammatory exudate occurs especially in the pulmonary interstitium, i.e. the connective tissue in the lungs.

Polykaryocyte See “Syncytium.”

Polyserositis Inflammation of serous membranes, such as the peritoneum or the pleura. A typical symptom of feline infectious peritonitis, a coronavirus infection.

Prenatal Before birth, related to the child.

Prevalence Frequency of all cases of a particular disease in a population at the time of examination.

Prodromal stage Preliminary phase to the actual disease, usually accompanied by non-specific symptoms such as malaise, headache and fever.

Purpura Spontaneous, speckled petechial capillary bleeding in the skin, mucous membrane and subcutaneous tissue (subcutaneous tissue). In contrast to erythema, it does not disappear under pressure.

Quarantine Statutory and limited period for which people or animals (including people and animals they come in contact with) with certain infectious diseases or suspected of being infected are isolated. The duration of the preventive quarantine depends on the incubation period of the suspected disease.

Quasispecies A population of very similar but not identical viruses, especially RNA viruses. It arises by high mutation rates based on the low fidelity of RNA-dependent RNA polymerases of RNA viruses, which incorporate incorrectly pairing nucleotides with an error rate of about 10^{-4}.

Reading frame (1) Coding region of a gene that is translated into a protein. The start codon is in almost all cases an ATG triplet, which codes for methionine. An open reading frame is a coding sequence which does not contain a stop codon on the mRNA over a long distance after the start codon.

Reading frame (2) One of the three possible alternatives to read the nucleotide sequence of a gene as a series of non-overlapping triplets (codons) which direct its translation into the corresponding amino acid sequence of a protein.

Recurrence (Latin *recurrere*, “to come back,” “to run back,” “to return”) Relapsing (recurrent) symptoms of an infection. It is known particularly in herpesvirus infections, in which the pathogens remain latent in the organism. External stimuli can induce reactivation of the virus from latency, resulting in lytic viral replication, which is associated with clinical symptoms similar to those of the initial infection.

Reinfection (secondary infection) A second infection with the same pathogen (virus, bacterium, etc.).

Relapse Recurrence of a disease after complete healing or recovery. Relapses of infectious diseases can be caused by reinfection with the original pathogen (e.g. by herpesviruses that remain latently present in the organism after a primary infection) or by pathogens from a new disease focus.

Rheumatoid factors Antibodies of different subclasses (IgM, IgG, IgA, IgE) which are directed as autoantibodies against specific epitopes of the body’s own IgG immunoglobulins.

Ring vaccination (German definition) A localized vaccination measure after outbreak of an infectious disease in an animal population. Animals residing within a radius of 3–10 km (depending on the infection) of the infected zone are vaccinated. This measure prevents spread of the disease, and additionally allows the use of vaccinated animals. However, trade restrictions must usually be accepted.

Ring vaccination (English definition) The vaccination of all susceptible individuals in a prescribed area around an outbreak of an infectious disease. Ring vaccination controls an outbreak by vaccinating and monitoring a ring of people around each infected individual. The idea is to form a buffer of immune individuals to prevent the spread of the disease (see "Outbreak-control vaccination").

RNA editing Post-transcriptional modification of the mRNA sequence by targeted insertion or excision of single nucleotides.

RNA interference (RNA silencing) RNA interference is one of the mechanisms through which cells can regulate gene expression at the post-transcriptional level by controlling the sequence-specific degradation and thus the amount of mRNAs. RNA interference is based on the formation of microRNAs (miRNAs) or small interfering RNAs. These are single-stranded RNA molecules of 21–23 nucleotides in length which result from cleavage of longer pre-miRNA molecules. Pre-miRNAs are encoded in the genome of humans, mammals and plants in specific genes that are transcribed by RNA polymerase II and are post-transcriptionally modified with 5′-cap groups and polyadenylated. They contain sequence regions that form intramolecular double-stranded hairpin structures, which are cleaved by the nuclear protein complex Drosha/Pasha, which possesses RNase III activity. The resulting pre-miRNAs are exported to the cytoplasm, where they are further processed into double-stranded miRNA molecules with a 3′ overhang by Dicer, a cytoplasmic RNase III. A helicase unwinds miRNA duplexes to single-stranded miRNAs, each of which interacts with the RNA-induced silencing complex (RICS). The miRNA/RISC complex binds by the miRNA moiety to mRNAs that contain miRNA complementary sequences, directing the degradation of such transcripts. Besides pre-miRNAs, precursor miRNA sequences are also found within the introns of protein-coding sequences of mRNA molecules, which frequently induce the degradation of their own precursor molecules.

Sarcoma Malignant, locally destructive, metastasizing tumour originating from mesenchymal tissues (soft tissue, skeletal and neurogenic tissue and the interstitial connective tissue of organs).

Self-assembly Orderly autonomous assembly of various components (proteins and nucleic acid) into functionally intact units without requiring additional enzymatic activities (e.g. the formation of ribosomal subunits, and infectious viruses; see also "Viral assembly").

Seroconversion The occurrence of antibodies in the serum of a patient who had been free of the corresponding immunoglobulins. Seroconversion occurs as a result of infection or vaccination.

Seroprevalence The frequency of occurrence of antibodies which indicate a past or an existing infection in the blood in a group of people or a population.

Serotype Serologically distinguishable variation within a virus subspecies owing to the ability to interact with specific antibodies. The serotypes of a viral species differ in their antigenic properties (surface structures that are recognized by the immune system). The classification into serotypes allows a subdivision of viral species, which is of particular interest from an epidemiological perspective. Because of the increasingly automated methods of nucleic acid sequencing, the different viral species are distinguished by determining their genotypes.

Slaughter Killing of animals for human consumption, including stunning-bleeding slaughter. Killed animals are released for human consumption within the framework of the detection of specific animal diseases. An example is enzootic bovine leucosis, a retrovirus infection.

Small interfering RNA See "RNA interference."

Squalene An organic, unsaturated compound of the group of triterpenes which is produced by all higher organisms. It is an adjuvant in vaccines, i.e. an additional immunological agent that increases the effect of vaccines.

Stroma The interstitial connective tissue of an organ.

Subarachnoid space The space containing cerebrospinal fluid between the arachnoid (i.e. the poorly vascularised, connective tissue, bilaterally covered with endothelial cells which sheathes the brain and spinal cord) and the pia mater (the connective tissue which lies directly on the brain and spinal cord surfaces and is tightly connected with the underlying membrane).

Superinfection The concurrent or chronologically only slightly delayed secondary infection of an organism (human, animal, plant) with a different strain of the same pathogen or another pathogen (virus, bacterium, parasite, fungus). Because the first infectious agent causes damage to the host organism, other infectious agents can invade and spread very efficiently.

Symptom Sign of disease.

Symptomatic infection Infection associated with symptoms.

Syncytium A multinucleated cell (polykaryocyte), also known as a giant cell. A syncytium can arise by fusion of several individual cells. This process is induced by some viruses (e.g. paramyxoviruses, herpesviruses) which promote the fusion of infected cells with uninfected cells by using the fusogenic activity of their surface proteins. Alternatively, syncytia can also arise by nuclear division without subsequent division of the cytoplasm.

Syndrome (Greek *σύνδρομος*, "coming together," "path," "run"). In medicine, syndrome means the simultaneous presence of various characteristics (symptoms) which usually have a common cause.

Synovial fluid The clear, viscous and ropy fluid produced by the synovium (joint-lining membrane) that reduces friction between the articular cartilages during movement.

Tachypnoea Increased respiratory rate by stimulating the respiratory centre in the case of increased oxygen demand, e.g. during physical exertion, fever or reduced supply of oxygen (hypoxaemia)

Tegument Proteinaceous layer between the envelope and capsid in herpesviruses.

Tonsils (Latin *tonsilla*, “almond”) Almond-shaped organs that are parts of the lymphoepithelial tissue of the lymphatic pharyngeal ring. Four different tonsils can be distinguished: pharyngeal tonsils (tonsilla pharyngealis or adenoidea), tonsils of the tongue (tonsilla lingualis), palatal tonsils (tonsilla palatina) and tubal tonsils (tonsilla tubaria).

Tracheitis Inflammation of the trachea.

Trailer Non-coding RNA sequence region at the 5′ end of the genome of negative-sense RNA viruses (analogous to the 5′ untranslated region).

Transaminases (aminotransferases) Enzymes that catalyse the transfer of an α-amino group from a donor molecule, usually an amino acid, to an acceptor molecule, commonly an α-keto acid, e.g. pyruvate and oxaloacetate (transamination reaction), using pyridoxal phosphate as a coenzyme, including an exchange of the respective redox states. These enzymes include, inter alia, aspartate aminotransferase and alanine aminotransferase. The detection of elevated transaminase activity in serum is used as diagnostic evidence of liver inflammation or other damage to this organ.

Tropism (Greek *τροπός*, “change,” “turn”) Refers to the ability of a virus to infect a particular cell type (cell tropism), the cells of a specific tissue (tissue tropism) or an organ (organ tropism) and to multiply there. Host tropism means the preferred or exclusive infection of a given species as the host organism.

Tumour (Latin *tumor*, “swelling,” “lump”) General term for any distinct swelling of body tissues (neoplasm).

Ulcer (from Latin *ulcus*). Lesion of the skin or mucous membranes with disintegration and necrosis and loss of epithelial tissue, which usually heals by rejection of the necrotic tissue, leaving cicatrices.

Ultrafilterable Designation for substances or particles which cannot be filtered by bacterial filtration systems, and thus are smaller than 0.2 μm.

Undulating fever Wave-like disappearing and recurring fever. It is typical of infectious diseases in which a change of surface antigens occurs, leading to an immune response against the altered “new” pathogen. An example is equine infectious anaemia, a retrovirus infection.

Vaccination (protective vaccination) A preventive measure against various infectious diseases by application of a vaccine. Vaccination stimulates the immune system to induce specific immunological defence mechanisms in order to establish a specific immunity against the corresponding infectious disease.

Vaccine A biological preparation composed of an attenuated (weakened) or inactivated (killed) pathogen that generates an immunity to a particular disease by stimulating antibody production or cellular immunity against the respective pathogen.

Vascular Concerning blood vessels.

Viraemia The occurrence and presence of virus particles in the blood.

Virion Infectious virus particle.

Virulence Sum of all the properties of a pathogen (virus) that contribute to disease development in a human or an animal. Virulence is quantified as the LD_{50} value,

i.e. the number of viruses (or other pathogens) which are sufficient to kill 50 % of the animals or cells in a tissue culture.

Viral assembly The orderly autonomous assembly of the viral structural proteins and viral genomes into infectious virus particles at the end of the viral replication cycle.

World Health Organization A specialized agency of the United Nations that acts as a coordinating authority concerning international public health. It has its headquarters in Geneva (Switzerland). It was founded on 7 April 1948 and has 194 member states.

World Organisation for Animal Health (**OIE**) The authority responsible for international animal health, with headquarters in Paris (France). It was founded in 1924 as the Office International des Epizooties on the occasion of a rinderpest outbreak (1920 in Belgium), and includes 178 member countries today.

Zoonosis Infectious disease in which pathogens can be transmitted from animals to humans, causing a disease in them.

Additional Information

In the various chapters, data concerning the notificability and epidemiology of individual virus infection refer mainly to the situation in countries of Central Europe. For readers with interest in respective data in other countries or regions worldwide, we attach a list of web sites that will supply actual data in English. For individual countries we would like to ask the readers to contact the web sites of global agencies or national health authorities and/or departments of health.

Human infectious diseases
Global:
WHO
Infectious diseases: Global alert and response, databases and information system
http://www.who.int/csr/resources/databases/en/index.html
WHO – Europe:
Centralised information system for infectious diseases CISID
http://data.euo.who.int/cisid/
European Center for Disease Prevention and Cotrol (ECDC), Stockholm, Sweden
Information on surveillance of infectious (communicable) diseases in Europe
http://www.ecdc.europa.eu/en/activities/surveillance/disease_specific_surveillance/Pages/disease_specific_surveillance.aspx

National:
Australia: Department of Health and Aging, Australian Government, Information and surveillance of communicable diseases
http://www.health.gov.au/internet/main/publishing.nsf/Content/ohp-communic-1
Austria: Bundesministerium für Gesundheit, Notifiable infectious diseases and statistics
http://www.bmg.gv.at/home/Schwerpunkte/Krankheiten/Uebertragbare_Krankheiten/
Canada: Public Health Agency of Canada: www.publichealth.gc.ca

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Infectious diseases, infection control guidelines and surveillance
http://www.phac-aspc.gc.ca/id-mi/index-eng.php
Germany: Robert-Koch-Institut, Berlin: List and numbers of notifiable infectious diseases in Germany
http://www.rki.de/DE/Content/Infekt/SurvStat/survstat_node.html
Great Britian: Health Protection Agency, London, Infectious diseases, infection control guidelines and surveillance
http://www.hpa.org.uk/Topics/InfectiousDiseases/
Ireland: Health Protetion Surveillance Centre, Dublin, Information, surveillance and data of notifiable and communicable diseases http://www.hpsc.ie/hpsc/NotifiableDiseases/
New Zealand: New Zealand Public Health Observatory, Auckland, Information on notifiable communicable diseases http://www.nzpho.org.nz/NotifiableDisease.aspx and surveillance: http://www.surv.esr.cri.nz/
Sweden: Smittskyddsinstitutet (smi), Solna, Infectious diseases, infection control guidelines and surveillance
http://www.smittskyddsinstitutet.se/in-english/statistics/
Switzerland: Eidgenössisches Departement des Inneren, Bundesamt für Gesundheit, Surveillance and numbers of notifiable infectious diseases
http://www.bag.admin.ch/k_m_meldesystem/index.html?lang=de
USA: Centers of disease control and infection, Atlanta, Informations about infectious diseases, notification and case numbers
http://www.cdc.gov/
National Center for Emerging and Zoonotic Diseases:
http://www.cdc.gov/ncezid/
National Center for Immunization and Respiratory Diseases: http://www.cdc.gov/ncird/
National Center for HIV/AIDS, Viral hepatitis, STD and TB prevention:
http://www.cdc.gov/nchhstp/

Animal infectious diseases
Global:
OIE (World Organisation for Animal Health):
Collection and analysis the latest scientific information on animal disease control and OIE-listed diseases.
http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2013/

National:
Australia: Australian Animal Health Laboratory (CSIRO), East Geelong.
http://www.csiro.au/en/Organisation-Structure/Divisions/Animal-Food-and-Health-Sciences.aspx
Belgium: Veterinary and Agrochemical Research centre (VAR-CODA-CERVA,) Brussels.
http://www.coda-cerva.be

Canada: Canadian Food Inspection Agency (CFIA), Reportable and notifiable diseases:
http://www.inspection.gc.ca/animals/eng/1299155513713/1299155693492
North American Plan for Animal and Pandemic Influenza:
http://www.publicsafety.gc.ca/prg/em/pandemic/2012-napapi-eng.aspx
Terrestrial Animal Disease Surveillance:
http://www.inspection.gc.ca/animals/terrestrial-animals/diseases/surveillance/eng/1313720601375/1313720675875
Denmark: Technical University of Denmark, National Veterinary Institute (DTU Vet), Lindholm.
http://www.vet.dtu.dk/
France: L'Agence nationale chargée de la sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES), Paris.
http://www.anses.fr/en
Centre de coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Paris, Montpellier.
http://www.cirad.fr
Germany: Friedrich-Loeffler-Institut (FLI), Insel Riems.
http://www.fli.bund.de/
Great Britain: Veterinary Laboratories Agency (VLA), now part of Animal Health and Veterinary Laboratories Agency (AHVLA):
http://vla.defra.gov.uk/
Research and Surveillance Virology:
http://vla.defra.gov.uk/science/sci_virology.htm
Wildlife Diseases:
http://vla.defra.gov.uk/science/sci_wildlife.htm
Pirbright Institute (PI), Pirbright.
http://www.pirbright.ac.uk/
Italy :Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emillia Romagna (IZSLER), Brescia.
http://www.izsler.it/
Istituto Zooprofilattico Sperimentale delle Venezie (IZS-Ve), Legnaro.
http://www.izsvenezie.it/
The Netherlands: Central Veterinary Institute (CVI), Lelystad,
http://www.wageningenur.nl/nl/Expertises-Dienstverlening/Onderzoeksinstituten/central-veterinary-institute.htm
Poland: National Veterinary Research Institute (PIWet; NVRI), Pulawy.
http://www.piwet.pulawy.pl/piwet7/index_a_eng.php
Spain: Centro De Investigación en Sanidad Animal (CISA),
http://www.nadir-project.eu/nadir_project/call_for_access/open_facilities_available_for_access/centro_de_investigacion_en_sanidad_animal_cisa
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Valdeolmos, Madrid.
http://www.inia.es/IniaPortal/verPresentacion.action

Sweden: Statens Veterinarmedicinska Anstalt, Uppsala.
http://www.sva.se/
Switzerland: Institute of Virology and Immunoprophylaxis/Federal Veterinary Office, Mittelhäusern, Bern.
http://www.bvet.admin.ch/index.html?lang=en
USA: Animal and Plant Health inspection service (APHIS), United States Department of Agriculture (USDA) and Centres for Epidemiology and Animal Health (CEAH), Information on notifiable communicable animal diseases.
http://www.aphis.usda.gov/animal_health/index.shtml

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