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Introduction

Cancer plays a major role in human morbidity and mortality. Unfortunately a considerable proportion of cancer is not amenable to surgery and needs to be treated by chemotherapy and/or irradiation. These approaches are characterized by an extremely narrow therapeutic index and major efforts in medical oncology are dedicated to treating their adverse effects. Viruses provide an alternate biological approach to cancer therapy. Initial attempts at the clinical application of viruses during the middle part of the 20th century were fraught with significant side effects and large variability in antitumor activity, likely due to the use of wild-type virus, passage-attenuated virus or infected cell lysates.

With the increase in our understanding of the molecular underpinnings of malignant cells and viruses it has been possible to exploit viruses for cancer therapy. The malignant behavior of a tumor cell is based on genetic alterations that create an imbalance between growth and growth control. The transformed phenotype provides a permissive environment for some viruses or functions to complement viral mutations. Oncolytic viruses are able to selectively replicate in tumor cells and kill them. A major advantage of such replication-competent viruses is this in situ amplification and subsequent spread within the tumor. However, cytotoxicity must be limited or controlled so that normal tissue is not harmed and pathology is minimized.

This book reviews many of the replication-competent viruses currently being pursued for cancer therapy, including those in clinical trial, and highlights features of viral biology that can be harnessed for therapy. These viruses cover the spectrum of animal viruses from RNA to DNA, single-stranded to double-stranded and enveloped to non-enveloped (table 1). Targeting of herpes simplex

Table 1. Replication-competent viruses for cancer therapy

Virus	Genome	Genome size, kb	Virion size, nm	Envelope
Autonomous parvovirus	Single-stranded DNA	5	20–25	No
Newcastle disease virus	Negative-strand RNA nonsegmented	15	150–300	Yes
Reovirus	Double-stranded RNA 10 segments	24	60–80	No
Adenovirus	Double-stranded DNA	36	70–90	No
Herpes simplex virus	Double-stranded DNA	153	150–200	Yes
Vaccinia virus	Double-stranded DNA	192	300–400	Yes

virus (HSV) and vaccinia virus is mainly accomplished by mutating genes required for DNA replication in nondividing cells, such as ribonucleotide reductase and thymidine kinase, or virulence. Mutations in adenovirus E1a and E1b genes create viruses that can replicate in cells lacking Rb and p53 activity, respectively, which are common alterations in cancer cells but not normal cells. Therefore, the transformed phenotype is permissive for these mutants, as it is for reovirus which utilizes an activated Ras pathway, autonomous parvoviruses and Newcastle disease virus. Viruses can also be engineered to selectively replicate in tumor cells by transcriptional regulation of essential genes with tumor-specific promoters/enhancers, such as prostate-specific adenovirus and hepatoma-specific HSV. These examples illustrate the variety and complexity of viral strategies for cancer therapy, and how virus–host interactions can be exploited. The field of oncolytic viruses is in its infancy and this monograph provides an overview of those viruses being employed and how this approach is being translated to the clinic.

We would like to thank the authors who have been instrumental in moving the field forward, those patients who have participated in clinical trials in the hope of a better treatment for cancer, and the ‘Frankfurter Stiftung für Krebskranke Kinder’ that has promoted this new approach to cancer therapy by generously sponsoring this book.

P. Hernáiz Driever
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Replication-Competent Herpes Simplex Virus Vectors for Cancer Therapy

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Introduction

During the last 40 years viruses have been used to treat cancer as an alternative approach to conventional methods such as surgery, chemotherapy and irradiation. Viral therapy of cancer, as a strategy, is based on direct cell killing due to the lytic cycle of the virus and induction of an immune response to the tumor, concomitant to the inflammatory response generated against viral infection of the tumor. Initial viral therapy approaches used wild-type virus, passage-attenuated virus, spontaneous mutants, or infected cell lysates, which were highly variable and accompanied by considerable side effects that impeded broad clinical application [10, 45, 243, 315]. Recent advances in genetic engineering have provided the means to manipulate viruses so their oncolytic, immunomodulatory, and gene transfer activities can be specifically targeted to tumor cells while sparing surrounding normal tissue.

One of the viruses being actively pursued for cancer therapy is herpes simplex virus (HSV). Replication-deficient HSV vectors, for the delivery of cytokines [75, 166, 339], 'suicide' genes [212, 238, 244], and other antitumor agents [76, 183], are being explored for cancer therapy. However, in this review we will confine our discussion to replication-competent or conditionally replicating HSV vectors. In general for these vectors, HSV is mutated so that it has reduced virulence and neuropathogenicity, yet it is still able to replicate in tumor cells. Targeting of viral replication to tumor cells can be achieved at the level of: (i) virus entry, through adsorption to tumor-specific cell surface molecules;

(ii) viral transcription, through tumor-specific transcriptional regulation, or (iii) viral replication, through dependence on proliferating cell nucleotide metabolism. The latter is the predominant strategy used so far, where viral genes that facilitate HSV DNA replication in nondividing cells are inactivated so the virus is only able to replicate in proliferating tumor cells [26, 219, 235]. Viral replication is a major advantage of this strategy, because HSV replication is not only inherently cytotoxic, but results in a large amplification of infectious virus that is then able to spread and infect new tumor cells. This cycle should repeat itself as long as tumor cells are accessible to viral infection.

HSV has many features that make it attractive for cancer therapy: (i) it naturally undergoes a lytic infection that is cytotoxic; (ii) it infects most cell types in a broad range of species, including those used as experimental tumor models; (iii) it can exist in a latent state within neurons without causing detectable damage to the infected cell [358]; (iv) its genome has been sequenced and most of the genes have been identified and characterized [222, 363]; (v) its genome is very large, with many nonessential genes that can be replaced with therapeutic transgenes [6, 49, 178, 256]; (vi) numerous nonessential genes have been identified which affect pathogenicity [230, 249, 336], and (vii) antiviral drugs are available to treat adverse events [14].

HSV Biology

In order to maximize the therapeutic effectiveness and safety of HSV vectors it is important to understand the biology of the virus and in particular the effects on the infected cell and organism. HSV is a human neurotropic virus of the α-herpesvirus subfamily and consists of two serotypes, type 1 (HSV-1) and type 2 (HSV-2). The viral particle, ~200 nm in diameter, is composed of four components: (i) an electron-dense core containing the viral genome, a linear double-stranded DNA molecule of about 153 kb with a G + C content of 68% (HSV-1) [20, 171], which is packaged into (ii) an icosahedral nucleocapsid containing 162 capsomeres, surrounded by (iii) the tegument, an amorphous proteinaceous layer, and (iv) a lipid envelope containing at least 10 glycoproteins.

The HSV genome consists of two segments, a unique L (long) and S (short) region bracketed by inverted repeats (fig. 1). The two segments invert relative to each other so that HSV DNA exists in four equimolar isomers differing in the relative orientation of the L and S segments [128]. Genes encoded within the inverted repeats (ICP0, ICP4, γ34.5) are diploid, and present as two copies. Each end of the genome contains a direct repeat, the 'a' sequence, which is also present in an inverted orientation at the internal L–S junction [309, 357, 359],

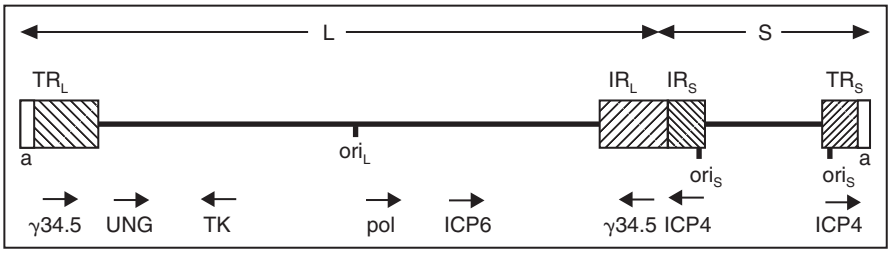

Fig. 1. HSV DNA structure. Schematic arrangement of genes mutated in different replication-competent vectors. The boxes represent the inverted repeat sequences (TR and IR) flanking the long (L) and short (S) unique regions. Arrows indicate orientation and general position of indicated transcripts.

and serves as a DNA cleavage/packaging signal (pac) [80]. The HSV genome contains over 80 genes, of which only 4 contain introns and are spliced (ICP0, ICP22, ICP47, UL15) [284, 363]. The relative lack of overlapping and intron-containing genes simplifies genetic manipulation of the genome.

HSV Infection

Viral infection involves attachment of the virion to the cell surface, fusion of the viral envelope with the plasma membrane, entry of the virion, transport of the capsid to the nuclear pores and release of viral DNA into the nucleus. The initial binding to the cell surface is through heparan sulfate proteoglycans and viral glycoproteins gC and/or gB [319]. The ubiquitous nature of heparan sulfate proteoglycans may explain the large variety of cell types HSV can infect. Entry of the viral capsid follows pH-independent fusion of the viral envelope and plasma membrane [372] and requires glycoproteins gB, gD, and heterodimer gH-gL [36, 105, 201, 293]. A number of cell surface receptors have been identified that interact with gD to mediate viral entry, and they are termed herpesvirus entry mediator (Hve) A, B, and C. HveA (HVEM) is a member of the tumor necrosis factor receptor superfamily that participates in both HSV-1 entry and induced cell fusion [241, 334]. It is expressed in many human tissues such as liver, lung and kidney, but is most highly expressed on human lymphoid cells [184, 241]. The cellular ligands for HveA are the secreted lymphotoxin-α and LIGHT, a transmembrane protein produced by activated T cells [221]. HveB and HveC are poliovirus receptor-related members of the immunoglobulin superfamily [57, 114, 364]. HveB mediates HSV-2, but not HSV-1 entry, whereas HveC mediates both [114, 364]. HveC is expressed in a variety of cell lines, including neuroblastoma, fibroblasts and keratinocytes, with high levels of expression in the brain [58, 114].

Upon entry, viral capsids are rapidly transported to nuclear pores. In neurons, capsids are retrogradely transported in axons towards the cell body via microtubules [179, 208, 349]. Cytosolic capsids bind to dynein, a retrograde microtubule-associated motor [316]. In both neurons and Vero cells, the plus ends of microtubules are localized at the cell periphery or synapse and the minus ends at the perinuclear microtubule-organizing center. At the nuclear pore, the viral genome is released into the nucleus and the empty caspids remain at the pore [17]. At this stage, HSV can follow one of two life styles: a lytic infection where the virus replicates and destroys the infected cells, or a latent infection in sensory neurons where the viral DNA persists in a quiescent state in the absence of viral protein synthesis for the lifetime of the host [290].

Two proteins contained in the tegument and released upon viral entry play a role in initiating the lytic cycle; the virion host shut-off protein VHS (UL41) [100, 279] and the transactivator protein VP16 (Vmw65, αTIF, UL48). VHS causes a rapid shut-off of host protein synthesis due to the degradation of mRNA, both cellular and viral [99, 186, 279]. Mutations in *vhs* have only a limited effect on HSV growth in vitro [279, 329]. VP16 is transported to the nucleus where it induces transcription of the immediate-early (IE) or α genes [39, 269], through a cis-acting sequence, TAATGARAT, that is present in all IE promoters [111, 209]. VP16 does not bind directly to this sequence but forms a complex with cellular factors Oct-1 (OTF-1), a sequence-specific DNA-binding protein, and HCF (C1, VCAF-1) [115, 181, 226, 325, 371].

HSV Replication

Synthesis of the IE gene products (ICP0, ICP4, ICP22, ICP27, ICP47), peaking between 2 and 4 h after infection, initiates the temporally regulated expression of the IE, early (E) or β, and late (L) or γ genes [146]. Progression through this growth cycle is dependent on the two essential IE proteins, ICP4 and ICP27 [82, 270, 298]. ICP4 (Vmw175, IE175, IE-3), the major HSV transcriptional regulatory protein, binds to a degenerate consensus sequence resulting in either the repression of IE genes or activation of E and L genes [82, 117, 180, 285]. ICP27 (IE63) functions posttranscriptionally to regulate viral mRNA processing [223, 227, 314] and transport [265, 300], and contributes to the shut-off of host protein synthesis by disrupting splicing [126]. ICP22 is required in some cells, rodent cell lines and confluent human embryonic lung cells, for efficient late gene expression [305] and is necessary for phosphorylation of RNA polymerase II [282]. ICP0 (Vmw110) is a promiscuous transcriptional activator that transactivates IE, E, and L viral promoters [35, 95] and induces a number of cellular genes including p53-responsive genes [139]. It also disrupts nuclear structures termed ND10 (PML nuclear bodies, PODs) [96, 97], induces proteosome-dependent protein degradation [257] and causes cell cycle arrest

[139, 203]. ICP47 (IE12) does not play a role in the regulation of gene expression, but rather prevents MHC class 1 antigen presentation and thereby participates in HSV evasion of the host immune system [379]. ICP47 binds to the transporter associated with antigen processing (TAP), blocking peptide transport into the endoplasmic reticulum (ER) and loading of MHC class 1 molecules [109, 135]. This binding by both HSV-1 and HSV-2 ICP47 is species-specific, inhibiting TAP in pig, dog, cow and primate cells but not in rodent or rabbit cells [163], with the affinity to murine TAP 100-fold less than to human [5].

The synthesis of E or β genes, peaking between 5 and 7 h after infection, is dependent upon and coincides with a decline in IE gene expression. For the most part the E genes encode proteins involved in viral DNA replication. This includes the 7 proteins required for HSV DNA synthesis: the origin binding protein (UL9) [94, 254]; HSV DNA polymerase (pol, UL30) [271, 367], where many drug-resistant mutations map [59, 145], and which forms a complex with the 65-kD DNA-binding protein/polymerase accessory factor (UL42) [113, 123, 258]; the helicase/primase complex (UL5, UL8, UL52) [72], and the ssDNA-binding protein (ICP8, UL29) [202, 368]. The other set of β proteins is involved in nucleic acid metabolism and is important for growth in nondividing cells. These include: thymidine kinase (TK, UL23) which phosphorylates deoxythymidine, deoxyuridine, deoxycytidine, thymidylate, purine pentosides and nucleoside analogs, such as acyclovir, ganciclovir, and bromovinyldeoxyuridine, that are not phosphorylated by cellular kinases [50, 110, 156]; ribonucleotide reductase (RR), consisting of two subunits (the large subunit ICP6 (UL39) and the small subunit UL40) [64, 106, 148], which reduce ribonucleotides to deoxyribonucleotides; dUTPase (UL50) hydrolyzes dUTP to dUMP which is converted to dTMP by cellular thymidylate synthetase [40, 373]; uracil DNA glycosylase (UNG, UL2) repairs deaminated cytosine residues [40], and alkaline exonuclease (DNase, UL12) [169] which is required for processing and correct packaging of replication intermediates [217].

With the synthesis of E proteins, DNA replication is initiated in the nucleus in a limited number of replication compartments [275], at or adjacent to ND10 [220]. The newly synthesized DNA is found in large concatemeric structures, likely arising via a rolling circle mechanism [19, 154] and/or through recombination [308]. The L or γ genes encode mostly structural proteins (virion polypeptides, VP) [132] or those required for packaging viral DNA (UL6, UL15, UL25, UL28, UL32, UL33) [144]. They can be divided into two groups, those that are expressed relatively early, γ1 (i.e., gB, gD), and those whose expression is dependent upon DNA replication, γ2 [141]. The viral capsids assemble in the nucleus as B-capsids, consisting of VP5 (UL19), VP19C (UL38), VP21 (UL26C), VP22a (UL26.5), VP23 (UL18), VP24 (UL26N), and VP26 (UL35) [116], which are converted to C-capsids during the packaging of viral DNA.

The DNA-containing capsids and tegument are enveloped as they pass through the inner nuclear membrane, which contains immature viral glycoproteins [268, 350]. The mature virions are released from the cell by exocytosis after being transported via the ER, Golgi apparatus, and cytoplasmic vesicles to the plasma membrane. It is not clear whether the nuclear envelope is retained as the virion is transported to the surface [350] or if it is lost and a new envelope acquired from the Golgi apparatus [32, 208, 369] or whether both pathways play a role in egress. In neurons, unenveloped capsids and glycoproteins are transported separately by anterograde axonal transport to the axonal termini where they are enveloped and released [260]. A number of viral proteins are required for capsid envelopment and virion egress including: UL11 [12], UL20, an integral membrane protein [13], UL34 [291], γ34.5, especially in stationary-phase fibroblasts [30], and glycoproteins gK (UL53) [151], gD and gH [32, 369]. The viral glycoproteins are glycosylated and modified during transit through the Golgi, in a similar fashion to cellular glycoproteins [161]. In vivo, cell-to-cell spread across cell junctions, which is resistant to neutralizing antibody, is likely the most common route for HSV. Mutants in gE or gI, which form a complex, are compromised in cell-to-cell spread but not in entry as free virus [86].

Effect on Infected Cell

During the viral replicative cycle there is extensive damage to the infected cells resulting in the cytopathic effect, where cells round up and clump together. One of the earliest events is the displacement and disaggregation of nucleoli, and margination of the chromosomes with subsequent fragmentation [290]. Early in infection, microtubules are fragmented at the periphery and later redistributed as the cells assume a rounded shape [11, 131, 251]. There is fragmentation and dispersal of the Golgi apparatus late in infection, during egress of virions, that is dependent upon viral DNA synthesis [38] and may require microtubule redistribution [11]. Interestingly, neither nocodazole, which causes fragmentation of Golgi, nor taxol, which stabilizes microtubules and prevents Golgi fragmentation, affect the exocytosis of infectious virus [11]. During cell rounding or cell fusion, fibronectin is lost from the surface of infected cells [85]. Some viral mutations cause infected cells to fuse into polykaryocytes rather than rounding up and were initially isolated as macroplaque (MP) variants as opposed to the normal microplaque (mP) variant [140]. They are isolatable after viral passage and are referred to as syncytial (*syn*) mutants [31, 280, 297]. *Syn* mutants map to gB [33] which seems to require a wild-type UL45 gene (i.e., UL45 mutants are nonsyncytial) [125], gK (the mutation in MP) [149, 267], gL [292], UL24 [348], and UL20 [150] loci.

As a protective measure against viral spread, infected cells activate double-stranded RNA-dependent protein kinase R (PKR) upon the synthesis of viral

complementary mRNA, and this results in phosphorylation of eIF-2α (which shuts off protein synthesis). In HSV-infected cells, the γ34.5 protein blocks this pathway and precludes shut-off of protein synthesis [53] by directing the dephosphorylation of eIF-2α by protein phosphatase 1α [130]. This function maps to the carboxy terminus of γ34.5 [54], a region homologous to the cellular damage- and growth arrest-inducible gene GADD 34 or MyD116 [142, 381]. Viral mutants in γ34.5 that lack this domain prematurely terminate protein synthesis in human SK-N-SH neuroblastoma cells and foreskin fibroblasts which leads to impaired growth [53, 240]. Extragenic suppressor mutants of these γ34.5 mutations are due to ectopic early expression of the late gene US11, which binds to PKR and prevents phosphylation of eIF-2α [44, 247].

Latency

HSV latency in sensory ganglia comprises 3 phases: establishment, maintenance, and reactivation [358]. After retrograde transport of viral capsids within sensory neurons, latency is established when there is a failure to undergo a productive replicative cycle. This process does not seem to require viral gene expression or replication [213, 307, 323]. Viral genomes are then maintained in the nucleus in a quiescent state, as nonintegrated, circular or concatameric DNA organized into chromatin-like structures [84, 93, 234, 287] for the life of the organism. The viral genome is transcriptionally silent, except for a region in the inverted long repeat encoding a family of poly-A^- latency-associated transcripts (LATs) antisense to the ICP0 and γ34.5 genes [71, 78, 288, 322, 326]. The most abundant LATs are stable introns [98] and no LAT-encoded proteins have been detected. There are about 20 viral genomes/LAT-positive cell in latently infected ganglia [136], with the copy number within individual neurons ranging from <10 to $>1{,}000$ copies/latently infected cell [301]. There is no detectable immune response generated against the latently infected ganglia [77, 261].

The LATs or the LAT region appears to play a minor or no role in the establishment and maintainence of latency [158, 306, 324, 335], but does affect reactivation; the rate of in vitro reactivation, and the probability of spontaneous and induced reactivation in vivo [137, 195, 205, 352, 380]. The effects of genetic alterations on reactivation are somewhat complicated because reactivation has been correlated with the number of latently infected neurons [211] and viral genome copy number [198, 302].

Whether true latency, with reactivation, occurs in the central nervous system (CNS) is not known. It is clear that establishment and maintenance of latency do occur [34, 79, 88, 287, 330]. HSV DNA has been detected in human brain tissue from patients dying without encephalitis [15, 200, 299]. The possibility of latency and potential long-term expression has been a motivation

for the development of replication-deficient HSV vectors for gene therapy in the CNS [104].

Neuropathogenicity

Viral pathogenicity involves an interaction between the host and virus, and is dependent upon the animal type and strain [167, 204], the route of inoculation, and the strain and genotype of the virus [24, 87, 283]. Alterations in neuropathogenicity can be due to viral mutations that affect neurovirulence, spread of the virus within the nervous system and induction of CNS disease, which is usually measured after CNS inoculation, and/or neuroinvasiveness, the ability to spread from the peripheral nervous system to the brain, which is measured after peripheral inoculation. A large number of HSV genes, at least 14, affect neuropathogenicity.

The γ34.5 (RL1) gene, located in the long terminal repeat overlapping the LAT region, is a major determinant of neuropathogenicity [52, 210, 336]. Neurovirulence of γ34.5 deletion mutants is decreased to a 50% lethal dose (LD_{50}) of $>10^6$ plaque-forming units (pfu) in HSV-1 backgrounds; strain F [52], 17+ [210], or McKrae [263]. Mutants in γ34.5 replicate poorly, if at all, in sensory ganglia and the CNS, and establish and reactivate poorly from latency in rodents [286, 321, 370], but reactivate normally in rabbits after high-dose ocular infection [262]. In mice lacking IFN-induced RNA-dependent PKR, or IFN receptors (IFN-$\alpha\beta\gamma R^{-/-}$), γ34.5 mutants have wild-type neurovirulence [196].

HSV genes involved in nucleotide metabolism (TK, dUTPase, UNG, RR) also play a large role in neurovirulence. Mutations in TK have little effect on the ability of HSV to replicate in tissue culture, except when cells are nondividing or serum-starved [102, 156, 159, 165]; however, they greatly limit viral replication in the peripheral nervous system, reactivation from latency, and pathogencity in adult mice [62, 92, 102, 122]. This effect of TK mutations on attenuating neurovirulence does not occur in newborn mice [22, 127, 332]. dUTPase and uracil DNA glycosylase (UNG) mutants also replicate well in culture, and are only about 10-fold attenuated for neurovirulence, but are 1,000- to 100,000-fold attenuated for neuroinvasiveness [272, 273]. RR is a key determinant for pathogenicity in the mouse and guinea pig, after intracerebral, corneal or intraperitoneal inoculation [28, 37, 152, 374]. This attenuation is in part due to decreased replication in the eye, ganglia, and brain, and the inability to reactivate from latency [155, 374].

HSV with mutations in vhs have greatly diminished neuropathogenicity, with decreased establishment of latency and growth in the brain [21, 328, 329]. This decreased virulence could be due to decreased replication at the primary site of infection [329], overexpression of viral IE genes [186], lack of

vhs-dependent downregulation of MHC class 1 expression [337], or suppression of cytokine production which would decrease nonspecific immune responses [331]. A mutant in VP16, that abolished its transactivation activity but not its structural function, has greatly reduced virulence [2] and replication in the nervous system, but is still able to establish latency and reactivate [323]. ICP47 mutants should have decreased neurovirulence in primates, because of their inability to block MHC class 1 presentation and CD8+ T cell responses. This turns out to be the case in mice after corneal infection [118] which is somewhat unexpected due to the species-specificity of ICP47. However, the reduced affinity of murine TAP may still be sufficient in the 'immune-privileged' brain where MHC class 1 expression is very low.

As would be expected, viral glycoproteins play an important role in neuropathogenicity. In order to protect HSV-infected cells from antibody-dependent cytotoxicity [91] and virions from antibody-mediated neutralization [248], HSV expresses two IgG Fc receptors (FcR) [18]; the gE/gI complex binds monomeric IgG and aggregates [160], while gE alone only binds IgG aggregates [90]. In addition, glycoprotein gC binds to complement C3b and blocks complement activation [107], which protects against complement-mediated neutralization [108, 229]. gC mutants that do not bind complement are about 100-fold less virulent than wild-type virus [207]. *Syn*3 mutations in gB confer an increased neurovirulence phenotype on HSV compared to the non-syncytial strains [121, 366]. Similarly, *syn*1 mutations in UL53 have increased neurovirulence [245]. While mutations in gD can convert a nonneuroinvasive strain into a neuroinvasive strain [153].

Replication-Competent HSV Vectors for Cancer Therapy

Oncolytic viruses have been studied for antitumor activity since before the 1950s [243, 296]. In a comparison of a number of viruses, Skinner et al. [70, 311] demonstrated that in vitro and in vivo infection of tumors with HSV-1 HFEM, an attenuated laboratory strain, and MDK, a TK^- isolate (table 1), reduced the tumorigenicity of malignant hamster and mouse cells (table 2). The recent advances in genetic engineering have provided opportunities to specifically manipulate the viral genome, creating defined mutations/deletions or inserting transgenes [289]. This has ushered in a rapid expansion in the use of replication-competent or replication-conditional HSV vectors for cancer therapy (tables 1, 2).

A number of different tumor models have been used to test the in vivo efficacy of these vectors (table 2). Experiments with human tumor cells require xenografts in immune-deficient animals, athymic or nude mice lacking T cells

Table 1. Replication-competent HSV mutants used for tumor therapy

Virus name	Parental strain	Genotype/structure	Reference no.
HFEM	HFEM	UL56$^-$	294
MDK		TK$^-$	89
*dl*sptk	KOS	TKΔ [360 bp Δ of SphI-PstI]	62
*dl*8.36tk	KOS	TKΔ (*dl*sptk)/lacZ$^+$	165
KOS-SB	KOS	TKΔ	313
RH105	F	TKΔ [502 bp Δ]/lacZ$^+$ [α4-lacZ]	138
hrR3	KOS	ICP6$^-$ [lacZ insertion]	120
rRp450	KOS/hrR3	ICP6Δ [CYP2B1 insertion]	49
AraAr13	KOS	pol [AraA resistant]	60
RE6	HG52 (HSV-2) × 17 + (HSV-1)	γ34.5$^-$	336
R7020	F	UL24Δ/1 copy of γ34.5/HSV-2 gG, gJ, gD, gI	231
R3616	F	γ34.5Δ [1 kb Δ of BstEII-StuI]	52
R4009	F	γ34.5$^-$ [translation stop codon]	52
R3659	F	γ34.5Δ (TK insertion)	187
R8309	F	γ34.5ΔMyD116	129
R8306	F/R3659	γ34.5Δ/IL-4	6
R8308	F/R3659	γ34.5Δ/IL-10	6
M002	F/R3659	γ34.5Δ/IL-12	256
1716	17$^+$	γ34.5Δ [759 bp Δ in BamHI k]	210
G207	F/R3616	γ34.5Δ/ICP6$^-$ [lacZ insertion]	236
MGH1	F/R3616	γ34.5Δ/ICP6$^-$ [lacZ insertion]	177
G47Δ	F/G207	γ34.5Δ/ICP6$^-$ [lacZ insertion]/ICP47Δ	344
3616UB	F/R3616	γ34.5Δ/UNG$^-$ [lacZ insertion]	274
Myb34.5	F/MGH1	γ34.5Δ (R3616)/ICP6$^-$ [B-*myb*-34.5 insertion]	55
G92A	KOS/d120	ICP4Δ/US3$^-$/UL24$^-$/TK$^-$ [alb-ICP4, lacZ insertion]	237

TK = Thymidine kinase, UL23; ICP6 = large subunit of ribonucleotide reductase, UL39; UNG = uracil DNA glycosylase, UL2; Pol = DNA polymerase, UL30.

but with normal B cells, natural killer cells and macrophages, or SCID mice lacking both T and B cells. Immune-competent mice, rats or hamsters are used when syngeneic or allogeneic tumor lines are available or can be induced in situ. Many of the syngeneic tumor cell lines were induced in a somewhat artificial fashion, with high-dose carcinogens or viral transformation [264], and many still retain an inherent immunogenicity that can be unmasked in vaccination studies [333]. Tumor fragments or cells can be implanted at a variety of tissue sites: subcutaneous, subrenal capsule, intracerebral, intraperitoneal, and intrasplenic for liver metastases, etc. Subcutaneous implantation is the most

Table 2. In vivo tumor therapy using replication-competent HSV mutants

Virus	Tumor	Model	Cell line	Rodent strain	Reference no.
HFEM	Hamster kidney cells	s.c.	BHK-21	Hamster	70, 311
MDK	Mouse fibroblast	s.c.	NCTC2472	C_3H mice	70, 311
*dl*sptk	Human glioma	s.c., i.c., subrenal capsule	U87MG	NCr/sed (*nu/nu*)	219
*dl*sptk	Human medulloblastoma	s.c.	DAOY	BALB/c (*nu/nu*)	214
*dl*sptk	Human malignant meningioma	Subrenal capsule	Tumor specimen M3	BALB/c (*nu/nu*)	214
KOS-SB	Rat gliosarcoma	i.c.	9L	Long-Evans rat	159
RH105	Rat gliosarcoma	i.c.	9L	Fischer 344 CD rat	26
*dl*8.36tk	Rat carcinoma	i.c.	W256	Sprague–Dawley rat	165
$AraA^r13$	Human glioma	s.c.	U87	NCr/sed (*nu/nu*)	215
hrR3	Human glioma	s.c.	U87MG	BALB/c (*nu/nu*)	235
hrR3	Human retinoblastoma	s.c.	Y79	BALB/c (*nu/nu*)	175
hrR3	Human colon carcinoma	s.c.	HT29	BALB/c (*nu/nu*)	376
hrR3	Human hepatocellular carcinoma	s.c.	Hep3B	BALB/c (*nu/nu*)	218
hrR3	Human prostate adenocarcinoma	s.c.	PC3	BALB/c (*nu/nu*)	218
hrR3	Mouse colon carcinoma	Liver met., s.c.	MC26	BALB/c, BALB/c (*nu/nu*)	378
hrR3	Rat gliosarcoma	s.c.	9L	Athymic mice	55
hrR3	Rat gliosarcoma	i.c.	9L	Fischer 344 CD rat	26
hrR3	Rat glioma	i.c.	D74	CD Fischer rat	134
rRp450	Human glioma	s.c.	U87MG	NCr/sed (*nu/nu*)	49
rRp450	Rat gliosarcoma	s.c.	9L	NCr/sed (*nu/nu*)	49
rRp450	Rat hepatocellular carcinoma	i.v.	McA RH7777	Buffalo rat	259
RE6	Human glioma	s.c., i.c.	U87	NCr/sed (*nu/nu*)	215
RE6	Mouse retinoblastoma	Spontaneous		LHβ-TAg transgenic mice	29

Table 2. (Continued)

Virus	Tumor	Model	Cell line	Rodent strain	Reference no.
R7020	Human epidermoid carcinoma, prostate adenocarcinoma	s.c.	SQ20b PC-3	Athymic mice	3
R3616	Human glioma	i.c.	U87MG	Athymic mice	27
R3616	Human glioma	s.c., i.c.	U87	NCr/sed (*nu/nu*)	215
R3616	Mouse glioma	i.c.	MT539MG	CB17 SCID	48
R3616	Mouse glioma	i.c.	GL261	C57BL/6	6
R8309	Human glioma	i.c.	D54MG	CB17 SCID mice	8
R4009	Human glioma	i.c.	D54MG, U-251MG	CB17 SCID mice	7
R4009	Mouse glioma	i.c.	GL261	C57Bl/6	6
R8306	Mouse glioma	i.c.	GL261	C57Bl/6	6
R8308	Mouse glioma	i.c.	GL261	C57Bl/6	6
R3659	Mouse neuroblastoma	i.c.	Neuro2a	A/J mice	256
M002	Mouse neuroblastoma	i.c.	Neuro2a	A/J mice	256
1716	Human melanoma	s.c.	1205, WM-451-Lu	SCID mouse	277
1716	Human mesothelioma	i.p.	REN	SCID mouse	182
1716	Human medulloblastoma	i.c.	D283	Nude mice	193
1716	Human embryonal carcinoma	i.c.	NT2	Nude mice	170
1716	Human NSCLC	s.c.	NCH-1460	SCID mice	351
1716	Human ovarian carcinoma	i.p.	SKOV3, A2780	CB17 SCID mice	67
1716	Human non-small cell lung cancer	s.c.	NCI-H460	CB17 SCID mice	351
1716	Mouse melanoma	i.c.	Harding-Passey	C57Bl/6	278
1716	Mouse transformed fibroblast	i.p.	EJ-6-2-Bam-6a	BALB/c	190
1716	Mouse lung carcinoma	s.c.	Lewis	SCID, C57Bl/6	189
G207	Human glioma	s.c., i.c.	U87MG	Nude mouse	236
G207	Human malignant meningioma	s.c., i.c.	F5	BALB/c (*nu/nu*)	375

G207	Human breast adenocarcinoma	s.c., i.c.	MDA-MB-435	BALB/c (*nu/nu*)	341
G207	Human head and neck SCC	s.c.	MSK QLL2, SCC15, SCC1483	Athymic rat	42
G207	Human colorectal	s.c.	HCT8, C86, C85	Athymic rat	176
G207	Human head and neck SCC	s.c.	UMSCC-22A, SQ20B, UMSCC-38	Athymic mice	47
G207	Human prostate adenocarcinoma	s.c.	LNCaP, DU-145	BALB/c (*nu/nu*)	360
G207	Human gastric carcinomatosis	i.p.	OCUM-2MD3	Athymic mice	23
G207	Human epithelial ovarian cancer	i.p.	SKOV3	CB17 SCID mice	69
G207	Hamster oral cavity SSC	Cheek pouch	DMBA-induced	Syrian golden hamster	42
G207	Rat hepatoma	Liver met.	Morris hepatoma McA-RH7777	Buffalo rat	176
G207	Mouse colorectal carcinoma	s.c.	CT26	BALB/c mice	340
G207	Mouse melanoma	s.c.	Cloudman S91-M3	DBA/2 mice	340
G207	Mouse neuroblastoma	s.c., i.c.	N18	A/J mice	347
G207	Mouse neuroblastoma	s.c.	Neuro2a	A/J mice	343
G47Δ	Human glioma	s.c.	U87MG	Athymic mice	344
G47Δ	Mouse neuroblastoma	s.c.	Neuro2a	A/J mice	344
3616UB	Human medulloblastoma hemangiosarcoma	s.c.	DAOY, SK-M	CB17 SCID mice	274
G92A	Human hepatoma	s.c.	Hep3B	Nude mouse	239
Myb34.5	Rat gliosarcoma	s.c.	9L	Athymic mice	55
Myb34.5	Human glioma	s.c.	U87ΔEGFR	Athymic mice	55

SCC = Squamous cell carcinoma; NSCLC = nonsmall cell lung cancer; s.c. = subcutaneous; i.c. = intracerebral; i.p. = intraperotineal.

common because of the ease of measurement of tumor growth and of intraneoplastic inoculation. It is important to note that the growth characteristics and pathology of abnormally situated tumors, as well as, the host or induced immune response to them are likely to be altered [355].

HSV Genes Mutated in Replication-Competent HSV Vectors

Thymidine Kinase

The initial studies focused on brain tumors as a target and HSV mutations that decreased neurovirulence. TK is important for DNA replication in nondividing cells, especially in the brain, but not mitotically active cells that have sufficient nucleotide pools for DNA replication. HSV TK^- mutants replicate poorly, if at all, in nondividing cells [102, 156, 165], including neurons [159], and are attenuated in neurovirulence compared to wild-type [62, 92, 122, 332]. Deletion mutants in TK, such as *dl*sptk, replicated well in human glioma cells in culture, including primary glioma cultures, even when cells were infected at very low multiplicities of infection (MOIs) [219]. This demonstrated a key feature of replication-competent vectors, a large amplification in virus and cell killing, and the ability of the virus to spread between tumor cells. Similar in vitro results were obtained with cells from a number of human nervous system tumors: malignant meningioma, neurofibrosarcoma, and medulloblastoma [214]. A single or double intraneoplastic inoculation of *dl*sptk into established intracerebral, subrenal capsule, or subcutaneous human glioma xenografts significantly inhibited their growth [219]. Other TK deletion mutants (KOS-SB, RH105, *dl*8.36tk) have been shown to be efficacious against intracerebral rat tumors in immunocompetent rats [26, 159, 165] (table 2). However, TK mutants still exhibit sufficient neurovirulence to limit treatment doses [215] and the lack of TK makes them resistant to many of the commonly used nucleoside analog antiviral drugs (i.e., acyclovir) [63, 101, 304], although they are still sensitive to other HSV DNA polymerase inhibitors such as, foscarnet and vidarabine (adenine arabinoside) [159, 219].

Ribonucleotide Reductase

RR mutant vectors have a number of attractive features. Like TK, RR is also involved in nucleotide metabolism and the generation of sufficient dNTP pools for viral DNA replication, and is therefore necessary for replication in nondividing cells [49, 119, 356]. The lack of cellular RR expression, which, for example, is low in normal liver but high in colon carcinoma liver metastases, is likely a contributing factor in this and provides a rationale for targeting

RR mutants to tumor cells [43]. RR deletion mutants are also somewhat temperature-sensitive [119] and grow more poorly in mouse cells [155]. The contribution of this species specificity in pathogenesis must be considered in preclinical trials of RR mutants. In contrast to TK mutants, RR mutants retain sensitivity to nucleoside analog drugs such as acyclovir and ganciclovir (GCV). Loss of HSV RR activity actually causes hypersensitivity not only to acyclovir and GCV, but also to aphidicolin and phosphonoacetic acid [61, 235, 320]. This sensitivity is not only a safety feature for RR mutant vectors, but could be applied in a 'suicide' gene strategy (TK + GCV; see below). All studies examining the use of RR mutants for tumor therapy have been with hrR3 [26, 175, 235, 376, 378], where the ICP6 gene, encoding the large subunit of RR, is inactivated by an inframe insertion of the *Escherichia coli* LacZ gene. This results in the N-terminal 434 amino acids of ICP6 being fused to β-galactosidase, and no RR activity [120]. The presence of lacZ in hrR3 provides a sensitive means to track viral infection within the tumor and any potential spread outside the tumor [26, 43, 175, 235].

γ34.5

Most of the serious pathological consequences of HSV infection in humans involve the CNS. Therefore, HSV genes participating in neurovirulence are prime targets for mutation when generating HSV vectors that could be used in humans. The γ34.5 gene is a major determinant of HSV pathogenicity [52]. HSV vectors containing deletions of γ34.5 in two HSV-1 laboratory strains (R3616 in F [52] and 1716 in 17 + [210]) have been prominent in studies of HSV-mediated tumor therapy. The initial use of γ34.5 mutants came in response to the development of encephalitis in mice bearing U87MG glioma i.c. tumors that had been treated with the TK mutant *dl*sptk [215, 219]. Both RE6, an intertypic recombinant, and R3616 were efficacious in prolonging survival of animals bearing human U87MG i.c. tumors, with no premature virally induced deaths at 10^7 pfu, although there was some histopathologic evidence for minimal focal encephalitis in the RE6 treated animals [215]. The efficacy of γ34.5 mutants is not limited to brain tumors. Intratumoral inoculation of 1716 significantly inhibited the growth of human melanoma s.c. tumors, with 30–40% complete cures [277], human malignant mesothelioma i.p. tumors [182], human epithelial ovarian carcinoma i.p. tumors [67], and murine transformed NIH 3T3 i.p. tumors in syngeneic mice, with ~40% cures [190] (table 2). In a mixing experiment, where different ratios of 1716-infected and uninfected Lewis lung carcinoma cells were implanted subcutaneously, it was found that 1 in 100 infected tumor cells was sufficient to significantly inhibit tumor growth in both immune-deficient SCID, and immune-competent C57BL/6 mice [190].

One drawback of γ34.5 mutants is that they grow less efficiently than wild-type virus in many tumor cell types, possibly due to the premature shutoff of host protein synthesis [53]. In a survey of human glioma cell lines (SB18, T98G, U251, U87MG), 1716 yielded ~10-fold less virus than wild-type strain 17+ in single-step growth experiments [225]. R3616 similarly yielded over 10-fold less virus than wild-type strain F in human neuroblastoma SK-N-SH, glioma U373MG, head and neck carcinoma SQ20B [344], human glioma D54MG and U251MG, and mouse glioma MT539MG [7, 48]. The growth of R3616, deleted for both copies of γ34.5, in human glioma cell lines was much less than other γ34.5 mutants such as R4009 with a stop codon and R908 with a 14-amino acid in-frame deletion at amino acid 24 [7]. R4009 was also more effective than R3616 in inhibiting mouse GL261 and MT539MG glioma i.c. tumors [6, 48]. This suggests that the level of viral replication in vitro correlates with antitumor activity in vivo.

Multimutated HSV Vectors

In considering the first clinical application of replication-competent HSV vectors for brain tumor therapy, we were concerned that vectors with only a single mutation might revert to wild-type or pathogenic isolates after amplification in vivo; for example, by excision of LacZ from ICP6 in hrR3, generation of extragenic second-site suppressors of the γ34.5 deletion [164, 240], or recombination with resident HSV. We therefore decided to develop second-generation multimutated HSV vectors, in particular G207, containing the γ34.5 deletions of R3616 and the inactivating LacZ insertion in ICP6 of hrR3 [236]. Pyles et al. [274] created a similar vector, 3616UB, except that in place of the RR mutation they inactivated UNG by inserting LacZ. 3616UB was as efficacious as its parent R3616 in inhibiting the growth of human DAOY medulloblastoma and SK-M hemangiosarcoma s.c. tumors in SCID mice, and had a better safety profile [274]. It caused no deaths after i.c. injection of 10^8 pfu in Swiss Webster mice, whereas R3616 caused 1 death, and like RR mutants, 3616UB was hypersensitive to GCV [274].

G207

G207, our prototypical second-generation vector, has many features that make it attractive for clinical use, both from a safety and efficacy perspective. The genome is very stable, even after numerous passages, and the multiple, widely spaced mutations make it highly unlikely that a neurovirulent isolate could arise, even through recombination with a wild-type HSV. G207 retains hypersensitivity to nucleoside analog antiviral drugs (acyclovir, GCV) due to

the RR mutation [236]. The presence of the LacZ reporter gene makes it easy to detect replicating virus [69, 176, 236, 347, 375] and uniquely identify G207 in patients undergoing treatment [216]. As a result of its propensity to replicate in dividing cells, the cytopathic effects of G207 are preferentially limited to tumor cells. Of 39 human tumor cells we have tested in vitro, only 5 were not susceptible to G207 cytotoxicity and growth at low MOIs (=0.1), and similarly only 1 of 10 human gastric and colorectal cancer cell lines tested in the Fong laboratory [23, 176] was not susceptible. This in vitro susceptibility correlates with the in vivo efficacy of G207 with human xenografts in immune-deficient mice [340, 341]. G207 was efficacious at inhibiting tumor growth in immune-deficient mice harboring subcutaneous, intracerebral, or intraperitoneal human tumors (glioma, malignant meningioma, breast adenocarcinoma, prostate adenocarcinoma, head and neck squamous cell carcinoma, colorectal carcinoma, gastric carcinoma, or epithelial ovarian cancer) [23, 42, 47, 69, 176, 236, 341, 360, 375] (table 2).

In general, mouse tumor cell lines are much less sensitive than human tumor cell lines to G207 replication and cytotoxicity. However, in examining G207 activity in mouse syngeneic tumor models we found that intraneoplastic inoculation of G207, in addition to its oncolytic activity, elicits a powerful and specific immune response against the tumor, that does not occur in athymic mice or after intradermal inoculation [340, 347]. This systemic antitumor immune response is able to inhibit the growth of established noninoculated tumors in the absence of any detectable spread of the virus from the inoculated to the noninoculated tumors. G207 inoculation of subcutaneous tumors caused regression of established tumors even in the brain [347], and in 3 different tumor cell types (CT26 colon carcinoma, M3 melanoma, and N18 neuroblastoma) in 3 different inbred mouse strains (BALB/c, DBA/2, and A/J, respectively) [340, 347]. The induced immune response provides persistent protection against rechallenge with a lethal dose of the treated tumor cell type (N18), but not a different A/J syngeneic tumor cell (Sal/N) [347]. Antitumor immunity was associated with the induction of a tumor-specific T cell response. Tumor cell-specific in vitro cytotoxic T lymphocyte (CTL) activity was generated that persisted for at least 13 months [347], and in the case of CT26 tumors recognized a dominant ‘tumor-specific’ MHC class I-restricted antigenic peptide [340]. This suggests that G207 could be used as an ‘in situ cancer vaccine’, without prior identification or isolation of tumor antigens. Another study, examining 1716 infection of Lewis lung carcinoma in C57BL/6 mice, found no inhibition of distant noninoculated tumor growth [190]. Further studies will be required to determine whether this difference is due to the tumor cell type, mouse strain or virus.

G207 Safety

The safety of G207 was ascertained in 2 HSV-susceptible animal models, young mice and the New World owl monkey *Aotus nancymae* [147, 330, 342]. In BALB/c mice, the highest dose of G207 (10^7 pfu) caused no symptoms or disease when inoculated intracerebrally, intracerebroventricularly, intravenously, or intrahepatically [330]. Furthermore, G207 failed to reactivate 'latent' KOS virus in the brain of mice that survived intracerebral inoculation with a sublethal dose of KOS [330]. *A. nancymae* are exquisitely sensitive to HSV-1 infection [147, 168], similar to human neonates and immunocompromised patients [232], and develop clinical symptoms comparable to humans [147, 233]. Single intracerebral inoculations of G207 at 10^7 or 10^9 pfu caused neither virus-related symptoms nor detectable changes in the brain as assessed by magnetic resonance imaging and pathological study [147]. Intracerebral inoculation does not lead to any viral distribution beyond the brain, nor virus shedding as determined by PCR [342]. Two animals that received 10^7 pfu were reinoculated intracerebrally 1 year after the first G207 inoculation and similarly showed no clinical symptoms of disease. Intracerebral inoculation of G207 generated an anti-HSV antibody response, beginning about 3 weeks after inoculation [342], that was significantly boosted after the second inoculation [147]. These 2 animals were subsequently reinoculated a third time with G207 in the prostate and showed no evidence of pathological changes in the brain, although G207 DNA was detected by PCR [383]. G207 inoculation in the prostate of male animals similarly caused no detectable disease or virus shedding [383]. These studies clearly demonstrate that G207 is safe for clinical evaluation in humans, and this has been supported by the preliminary clinical results [216].

HSV Vectors for Transgene Expression

There are 2 general classes of HSV vectors that can be used for transgene delivery and expression [reviewed in 197]: (i) recombinant vectors where the transgene is inserted into the viral genome, as was done for the reporter gene LacZ in hrR3 and G207 – HSV can accommodate large DNA inserts because of the large size of the genome and the number of nonessential genes, and (ii) plasmid-based defective vectors where the transgene is inserted into an amplicon plasmid that is amplified and packaged into virions in place of the viral genome [317, 327]. The generation of defective vectors (dv) requires a helper HSV genome to provide the viral functions necessary for the production of virus. In the examples described here, that is a replication-competent HSV. A full viral genome length (~150 kb) of amplicon plasmid DNA is packaged so that each defective particle contains approximately 15–30 tandemly repeated copies of the transgene, depending upon the size of the amplicon plasmid [185].

Because the defective genome is not integrated and contains no HSV-coding sequences, transgene expression is regulated by the enhancer/promoter sequences of the construct. Both recombinant and defective HSV vectors transduce dividing and nondividing cells at high efficiency. The expression of transgenes in cells infected with recombinant replication-competent vectors is limited because of rapid cell death and the viral regulatory cascade. In contrast, transgenes should be expressed for longer periods of time from defective vectors which do not kill the infected cell.

'Suicide' Genes

The HSV TK gene has been used in a number of different vector systems for 'suicide' gene or prodrug activating enzyme therapy [73, 242]. Expression of TK in tumor cells converts the nontoxic substrate GCV (9-(1,3-dihydroxy-2-propoxymethyl)guanine, DHPG) to the toxic metabolite GCV-monophosphate [51, 312]. In one tumor cell type it has been reported that GCV treatment after hrR3 (RR^-) inoculation of i.c. 9L rat gliosarcomas increased survival [25]. However, GCV treatment of HSV-infected tumor cells will block viral replication and therefore, GCV treatment will likely not be beneficial in situations where viral replication is important in antitumor efficacy. This seemed to be the case in other studies, where GCV treatment had no effect on tumor growth of hrR3-infected human HT29 colon carcinoma s.c. tumors [376], R3616-infected GL261 glioma s.c. tumors in syngeneic C57BL/6 mice [238], or G207-infected N18 neuroblastoma s.c. or i.c. tumors in syngeneic A/J mice [346].

In addition to TK, a number of enzyme/prodrug combinations have been used for cancer therapy [65]. CYP2B1, a member of the cytochrome p450 family that converts cyclophosphamide into phosphoramide mustard which crosslinks DNA [133], has been inserted into the ICP6 locus of HSV to create rRp450 [49]. In contrast to TK/GCV, the toxic metabolites generated by CYP2B1 do not inhibit HSV DNA replication [49] and are diffusible [365]. rRp450 is efficacious in inhibiting the growth of subcutaneous 9L or U87 glial tumors or diffuse liver tumors [49, 259]. The addition of cyclophosphamide treatment greatly enhanced the antitumor activity, resulting in complete U87 tumor regression in 4 of 5 versus 1 in 5 animals with virus alone [49]. A concern is that cyclophosphamide can be metabolized in the liver where CYP2B1 is expressed [56].

In an effort to increase levels of transgene expression and improve antitumor activity, we have used a combination of replication-competent HSV vectors for oncolytic activity, and defective HSV vectors for transgene expression [238, 338]. This is illustrated in studies with a defective vector expressing TK driven by the CMVIE promoter. There was a 4- to 5-fold increase in TK activity in infected cells compared to those infected with the replication-competent

helper virus alone and this resulted in a significant inhibition of GL261 glioma s.c. tumor growth that was dependent upon GCV treatment [238].

Immune Modulatory Genes

In light of the antitumor immune response generated by intratumoral inoculation of replication-competent HSV vectors, efforts have been directed towards boosting this antitumor immune response. A variety of genes encoding immune-modulatory molecules has been used for cancer gene therapy, usually in the context of vaccination with killed tumor cells [255]. For the most part, these have been delivered ex vivo or in situ using replication-deficient vectors [354], including defective HSV vectors [41, 157, 339]. A number of mouse cytokine genes (IL-4, IL-10, IL-12) have been inserted into the γ34.5 region of R3659 and their efficacy examined in syngeneic mouse brain tumor models. IL-10 expression had no effect on survival, IL-4 significantly increased survival with no cures, and IL-12 significantly increased survival with about 20% cures [6, 256]. There was an increase in CD8+ T cells within the tumor after IL-4 and IL-12 expression, but a decrease after IL-10 [6, 256].

We have used a defective vector/replication-competent HSV combination approach to deliver immune modulatory genes as an adjuvant to in situ tumor vaccination with G207 [338, 343]. A defective vector, encoding the 2 subunits of murine IL-12 (p35 and p40) was generated with G207 as helper virus (dvIL12/G207). Infected tumor cells secreted high levels of heterodimeric IL-12 in vitro (~300–1,500 pg/10^5 cells/24 h) [338]. In an established, bilateral subcutaneous CT26 mouse colon carcinoma tumor model, unilateral intratumoral injection of dvIL12/G207 significantly inhibited the growth of both inoculated and noninoculated tumors and increased survival, compared to a control vector, dvLacZ/G207 [338]. The effect of the control vector dvLacZ/G207, which expresses the reporter gene LacZ also present on G207, was no different from that seen with G207 alone in the same system. Intratumoral IL-12 expression elicited a strong specific CTL response in vitro, that recognized CT26 tumor cells and not A20, another H-2^d tumor cell line, with splenocytes from dvIL12/G207-infected animals secreting about 10 times more IFN-γ than control splenocytes [338].

Most human tumors are poorly immunogenic and are not good antigen-presenting cells (APC) [9]. Induction of CTL requires at least 2 signals: MHC class I or II presentation of tumor antigens and sufficient costimulatory signals [246]. We have examined approaches that target both these signals. The first approach was to delete the ICP47 gene from a replication-competent vector. As ICP47 binds to TAP, thereby blocking MHC class I expression on the surface of infected human cells [109, 135], its expression should reduce the visibility

of infected tumor cells to T cells. A deletion in ICP47 was made in G207 and R3616, creating G47Δ and R47Δ, respectively [344]. The absence of ICP47 in infected human cells led to normal MHC class I expression in fibroblasts and decreased inhibition in melanoma cells. G47Δ-infected melanoma cells were better at stimulating their cognate tumor-infiltrating lymphocytes than G207-infected cells [344]. In melanoma vaccine trials, increased T cell stimulation correlated with prolonged relapse-free survival [362].

In the second, we expressed a soluble B7-1 fusion protein using a defective vector/G207 combination. The B7 family of membrane proteins is amongst the most active costimulatory molecules [112]. Because tumor cells are such poor APCs, we hypothesized that the expression of soluble dimeric B7 within the tumor would enhance T cell activation by professional APCs. At doses of G207 that were ineffective at inhibiting subcutaneous or intracerebral tumor growth (2×10^5 pfu twice), dvB7Ig/G207 was very effective [343]. Depleting animals of CD8+, but not CD4+ T cells abrogated this inhibition of tumor growth. There are numerous ways to enhance an immune response and it is likely that successful therapies will involve some mix of a number of these.

Combinations with Established Cancer Therapies

Much of current cancer therapy depends upon multimodal treatment strategies for maximal efficacy and to overcome inherent or acquired resistance of tumors to therapy. The conventional approaches for cancer therapy consist of chemotherapy, radiotherapy, and surgery. For radiotherapy and most chemotherapeutic agents the therapeutic index is extremely narrow, with significant dose-related toxicities. This is in contrast to second-generation replication-competent HSV vectors which have so far not demonstrated toxicity [147, 216, 330]. Because replication-competent HSV vectors seem to function as anti-tumor agents through pathways different from those targeted by conventional approaches, it is likely that combinations with conventional agents will improve efficacy as long as those agents do not inhibit the activity of HSV vectors. From a clinical standpoint, there are advantages to combining a new therapeutic approach with a conventional treatment.

One of several ways involved in inherent or acquired resistance of tumor cells to chemotherapeutic agents and radiotherapy is through mutation or loss of the p53 gene [1, 194, 206], with a high proportion of human tumors having lost p53 function [143]. Importantly, HSV replication and cytotoxicity do not seem to be effected by the p53 status of tumor cells [68, 237, 377], or whether they are radiation- or chemotherapy-resistant [3, 47]. ICP6$^-$ hrR3 was equally cytotoxic in vitro in human osteogenic sarcoma SAOS-2-LM2 cells with wild-type p53, no p53 or mutant p53 [377]. Similarly, chemotherapy-sensitive human ovarian

cancer cells A2780 and PA-1 and their chemotherapy-resistant clones lacking p53 function were equally sensitive to γ34.5-deficient R3616 [68].

Chemotherapy

The combination of chemotherapeutic drugs with replication-competent HSV vectors augmented treatment efficacy [47, 351]. Human head and neck squamous cell carcinoma (SCC) cell lines (UMSCC-22A, UMSCC-38, SQ20B) with over a 10-fold range in cisplatin sensitivity were similarly sensitive to G207 cytotoxicity in vitro [47]. Cisplatin did not affect G207 replication and cytotoxicity at the highest clinically achievable dose (7.5 μmol/l) even in tumor cells that were resistant to cisplatin (SCC-25/CP). In vivo, cisplatin enhanced the antitumor activity of G207 in subcutaneous human SCC cisplatin-sensitive tumors (UMSCC-38; 100% cures with G207 + cisplatin vs. 42% cures with G207 alone), but not in cisplatin-resistant tumors (SQ20B) [47]. The in vitro cytotoxic activity of 1716 in combination with mitomycin C was synergistic in 2 of 5 human nonsmall cell lung cancer cell lines and additive in the other 3 [351]. The combination treatment in a mitomycin C-sensitive human lung cancer subcutaneous tumor (NCI-H460) was additive [351]. These studies suggest that the combination of chemotherapeutic agents with replication-competent HSV vectors is a promising approach for the clinic, and that chemotherapy is unlikely to antagonize viral therapy.

Radiotherapy

Ionizing radiation is the standard therapy for malignant glioma [192] and other tumors. Weichselbaum et al. [3, 4, 27] have reported that inhibition of tumor growth by R3616 or R7020 is enhanced by ionizing radiation. In a human glioma subcutaneous model (U-87MG), single or triple injections of R3616 followed by 45 Gy (20 Gy day 1 + 25 Gy day 2) of radiation, resulted in 56 and 90% tumor regressions, respectively, whereas radiation or R3616 alone resulted in ~10% regression [4]. In a follow-up study, using a more clinically relevant radiation dose (5 Gy fractions on days 1, 2, 4, 5, 8, 9, 11, and 12 for a 40-Gy total dose), no tumor regressions were seen, only an increase in growth delay [27]. An increase in survival was also seen in animals with intracranial U-87MG tumors after R3616 intratumoral injection + whole brain irradiation (5 Gy every other day for a total dose of 30 Gy), although there were no long-term survivors [27]. Increased levels of virus were present in tumors that had been irradiated with 45 Gy, along with a greater distribution of virally infected cells [4, 27].

In subcutaneous prostate tumor models (human LNCaP and mouse TRAMP), we found no improvement in antitumor activity using a combination

of G207 + radiation (2 Gy over 5 days for LNCaP; 4 Gy over 5 days for TRAMP) compared to single treatments alone [162]. Irradiation of tumor cells (PANC-1 pancreatic carcinoma, U-87MG, CaSki cervical carcinoma) in vitro did not augment viral replication or cytotoxicity (hrR3 or wild-type KOS), regardless of MOI or radiation dose [318]. Further study will be required to determine how ionizing radiation affects HSV-mediated tumor therapy and whether this combination will be of benefit to patients.

Effect of the Host Immune Status

An important concern in the use of human viruses for therapy is the effect of the host's prior exposure and immunity to that virus on antitumor activity. This is particularly true for HSV-1, where approximately 60% of the population in the US are seropositive by adulthood [191, 253, 295, 310]. In humans, even though a robust humoral and cellular immune response is generated by HSV infection, it is not sufficient to block recurrent infections [66, 174, 353, 382]. The level of the response can, however, affect the frequency and duration of recurrences [281] or reinfection [303]. It is difficult to predict whether the impact of HSV immunity, either pre-existing or therapeutically induced, on HSV-mediated tumor therapy will be detrimental or beneficial. The humoral and/or cellular immune response might neutralize virus, limit viral spread, eliminate infected cells, induce an inflammatory response, or be misdirected towards tumor antigens and thereby enhance efficacy.

To address the effects of prior exposure to HSV, a number of studies have examined the effects of HSV immunization on tumor therapy [46, 134, 190, 378]. The number of hrR3-infected tumor cells, as measured by lacZ and TK expression, was greatly reduced in rats that were pre-immunized with hrR3 [134]. However, the therapeutic efficacy of hrR3 in an intracranial rat syngeneic D74 glioma model was the same in immunized and nonimmunized animals. Unfortunately, D74 cells are fairly resistant to hrR3 infection and there was only a small increase in survival of hrR3-treated animals compared to controls [134]. Pre-immunization of mice with HSV had no effect on tumor growth inhibition by: G207 in subcutaneous N18 neuroblastoma in A/J mice or CT26 colon carcinoma in BALB/c mice [46]; hrR3 in MC26 colon carcinoma metastatic to the liver in BALB/c mice [378], or 1716 in intraperitoneal EJ-6-2-Bam-6a Ras-transformed fibroblasts in BALB/c mice [190]. In these studies, G207 was injected intratumorally, hrR3 intravascularly, and 1716 intraperitoneally. Furthermore in nonimmunized mice, multiple inoculations of G207 (biweekly over 3 weeks) were substantially better than a single inoculation, even with a lower total dose, with the cure rate of CT26-bearing animals increased from 10 to 75% [46]. Similarly, 3 intraperitoneal injections of 1716 (every 3rd day) was

substantially better than a single inoculation, with a 50% cure rate compared to no cures with a single inoculation [190].

Most patients with malignant brain tumors are often treated with corticosteroids to reduce vasogenic brain edema surrounding the tumor [173]. Corticosteroids are also immunosuppressive [74, 124]. In light of the immune-mediated component of G207 antitumor activity [340, 347], it would be predicted that immunosuppression would reduce efficacy. Dexamethasone treatment (5 mg/kg) for 7 days from the start of G207 treatment of N18 neuroblastoma in A/J mice had no effect on the inhibition of tumor growth, however there was a delayed tumor regrowth when dexamethasone was given for 16 days [345]. Dexamethasone treatment did suppress the induction of neutralizing antibody and abolished CTL activity, but did not affect the intratumoral replication of G207 [345]. Therefore, corticosteroid immunosuppression did not affect the short-term oncolytic activity of G207 but did affect the long-term efficacy likely through suppression of CTL activity.

Transcriptionally Targeted Vectors

One way to overcome the reduced growth properties of γ34.5-deficient mutants, yet retain safety, would be to limit γ34.5 gene expression to tumor cells using a tumor-specific promoter. Myb34.5 was constructed by inserting a B-myb promoter-γ34.5 transgene into the ICP6 locus of the γ34.5Δ mutant MGH1 [55]. The B-myb promoter is cell cycle regulated, being repressed in G0 cells [188]. Myb34.5 was more cytotoxic than MGH1 in human glioma cells (U87, U343, T98G) and rat 9L cells in vitro (MOI = 0.1), and correspondingly, inhibited subcutaneous 9L and U87ΔEFGR tumor growth to a greater extent than MGH1 [55]. Myb34.5 was quite safe after intracerebral inoculation in BALB/c mice, although 1 of 6 animals died after injection of 10^7 pfu while none of the MGH1-injected animals died at this dose [55].

A separate strategy for targeting HSV cytotoxicity to tumor cells is via transcriptional targeting of viral replication. In this approach, the essential immediate-early ICP4 gene is regulated by a tumor-specific promoter/enhancer sequence. Because ICP4 is required for the synthesis of E and L gene products [81, 270], ICP4 mutant HSVs do not replicate. If the tumor-specific promoter/enhancer is properly regulated, it should drive not only ICP4 expression but also the complete replicative cycle of HSV, in essence amplifying the output. As a proof-of-principle, the albumin enhancer/promoter, which drives expression specifically in liver cells and many hepatocellular carcinomas [83, 252, 266], was used to regulate ICP4 expression [237]. G92A was constructed by inserting an albumin enhancer/promoter-ICP4 transgene into the TK locus of d120, which is deleted for both copies of ICP4 [81].

The cell specificity of G92A was demonstrated in vitro where the plaquing efficiency was over 1,000 times higher on albumin-expressing human hepatoma cells than on nonalbumin-expressing cells, with a virus burst of >50 pfu/cell on Hep3B hepatoma cells and <0.1 on MCF7 breast cancer cells [237]. The expression of ICP4 protein was greatly delayed in G92A-infected hepatoma cells compared to wild-type KOS, as was the kinetics of viral growth. This is indicative of altered regulation by the albumin enhancer/promoter compared to the endogenous ICP4 promoter [237]. In vivo, G92A inhibited the growth of subcutaneous human Hep3B hepatoma tumors to a similar extent as hrR3, but had no effect on human PC3 prostate tumors [239]. Despite the high level of albumin expression in normal hepatocytes, intrahepatic injection of G92A (7×10^6 pfu) caused no symptoms of disease, whereas 10^5 pfu of wild-type KOS caused 50% mortality [239]. This lack of toxicity in the liver is likely due to the mutations in TK and US3, a protein kinase that blocks virally induced apoptosis [199]. A role for US3 in pathogenicity was demonstrated with a HSV-2 US3$^-$ mutant that had greatly reduced virulence after intraperitoneal injection, and was associated with restricted replication in the liver [250]. Transcriptionally targeted HSV vectors should prove useful for metastatic tumors requiring systemic delivery, or benign or slowly growing tumors that may not be sensitive to viral vectors targeting replicating cells.

Clinical Trials

In light of the encouraging preclinical data concerning antitumor efficacy and safety of mutated oncolytic HSV-1, two clinical trials in the US and UK were initiated in recurrent glioma patients [172]. High-grade astrocytic tumors still remain a therapeutic challenge in neurooncology and prognosis continues to be grim with a median survival of approximately 12–18 months for primary tumors and 6–9 months for recurrent tumors [16, 103, 361]. The Glasgow group evaluated 1716, containing a γ34.5Δ in strain 17+ background [276], while the US group (Georgetown University and University of Alabama at Birmingham) evaluated G207 [216]. Both studies were phase-I dose-escalation safety studies with virus injected intratumorally into neoplastic lesions in patients suffering from recurrent or progressing astrocytic tumors, WHO III/IV, who had failed conventional chemotherapy and irradiation.

Age, sex, clinical evaluation of patients as well as inclusion and exclusion criteria before entering the studies did not differ significantly between both studies, except for the inclusion of patients with a Karnofsky score of 60 in the Glasgow trial. Nine patients were treated with 1716 at doses ranging from 10^3 to 10^5 pfu [276]. The G207 doses ranged from 10^6 pfu at a single site to 3×10^9 pfu at 5 sites in 21 patients [216]. γ34.5 mutations in the strain 17+ background (i.e., 1716) are more neurovirulent than those in strain F (i.e., G207) [210, 224].

Intracerebral injection of 10^6 pfu of a 1716 derivative (1716lacZ) caused clinical disease and a severe inflammatory response in rats and mice [228]. While adverse events were noted in both trials, none could be unequivocally ascribed to the virus and there was no induction of encephalitis, confirming the safety profile obtained in animals [147, 330]. Postmortem tissue analysis from both studies confirmed the absence of encephalitis. Biopsy specimens from 2 of 6 patients treated with G207, obtained 97 and 157 days after inoculation contained G207 DNA [216], while 1716 DNA was not detected in specimens from 5 patients [276]. One of 5 seronegative G207 patients seroconverted at the 3×10^9 pfu dose, while the 1 seronegative 1716 patient did not. Neither study was designed to test efficacy, but anecdotal cases of tumor shrinkage or stable disease were reported. These first clinical studies with replication-competent HSV vectors are encouraging and indicate that this therapy is feasible, safe and the clinical responses suggest at least some antitumor activity.

Conclusions

It has been just over a decade since the first experimental studies using replication-competent HSV for tumor therapy were reported. Since that time a number of genetically engineered viruses have been constructed with mutations in viral genes that confer selective replication in tumor cells and attenuate virulence. With the various mutations, there is a fine balance between safety and viral replication. It is likely that the set point for this balance will vary with the target organ and tumor, and the mode of vector delivery. The most promising of these to date contain deletions in the γ34.5 gene, and these have progressed from preclinical efficacy and safety studies to clinical trials in patients with recurrent glioma. While the initial focus of these vectors has been towards brain tumor therapy, it is clear that they hold significant promise for most solid tumors, including prostate, breast, head and neck, and liver metastases. Many questions remain regarding the use of these vectors including: how applicable are the animal models to the human condition; what are the underlying biological features of tumors that make them particularly susceptible to attenuated mutants of HSV; how does viral infection induce a tumor cell-specific immune response, and does this have any relevance to autoimmune or neurodegenerative disease?

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References

1 Aas T, Borresen AL, Geisler S, Smith-Sorensen B, Johnsen H, Varhaug JE, Akslen LA, Lonning PE: Specific P53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. Nat Med 1996;2:811–814.
2 Ace CI, McKee TA, Ryan JM, Cameron JM, Preston CM: Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. J Virol 1989;63:2260–2269.
3 Advani SJ, Chung SM, Yan SY, Gillespie GY, Markert JM, Whitley RJ, Roizman B, Weichselbaum RR: Replication-competent, nonneuroinvasive genetically engineered herpes virus is highly effective in the treatment of therapy-resistant experimental human tumors. Cancer Res 1999;59: 2055–2058.
4 Advani SJ, Sibley GS, Song PY, Hallahan DE, Kataoka Y, Roizman B, Weichselbaum RR: Enhancement of replication of genetically engineered herpes simplex viruses by ionizing radiation: A new paradigm for destruction of therapeutically intractable tumors. Gene Ther 1998; 5:160–165.
5 Ahn K, Meyer TH, Uebel S, Sempe P, Djaballah H, Yang Y, Peterson PA, Fruh K, Tampe R: Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47. EMBO J 1996;15:3247–3255.
6 Andreansky S, He B, van Cott J, McGhee J, Markert JM, Gillespie GY, Roizman B, Whitley RJ: Treatment of intracranial gliomas in immunocompetent mice using herpes simplex viruses that express murine interleukins. Gene Ther 1998;5:121–130.
7 Andreansky S, Soroceanu L, Flotte R, Chou J, Markert M, Gillespie GY, Roizman B, Whitley RJ: Evaluation of genetically engineered herpes simplex viruses as oncolytic agents for human malignant brain tumors. Cancer Res 1997;57:1502–1509.
8 Andreansky SS, He B, Gillespie GY, Soroceanu L, Markert J, Chou J, Roizman B, Whitley RJ: The application of genetically engineered herpes simplex viruses to the treatment of experimental brain tumors. Proc Natl Acad Sci USA 1996;93:11313–11318.
9 Antonia SJ, Extermann M, Flavell RA: Immunologic nonresponsiveness to tumors. Crit Rev Oncog 1998;9:35–41.
10 Asada T: Treatment of human cancer with mumps virus. Cancer 1974;34:1907–1928.
11 Avitabile E, Di Gaeta S, Torrisi MR, Ward PL, Roizman B, Campadelli-Fiume G: Redistribution of microtubules and Golgi apparatus in herpes simplex virus-infected cells and their role in viral exocytosis. J Virol 1995;69:7472–7482.
12 Baines JD, Roizman B: The UL11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells. J Virol 1992;66:5168–5174.
13 Baines JD, Ward PL, Campadelli-Fiume G, Roizman B: The UL20 gene of herpes simplex virus 1 encodes a function necessary for viral egress. J Virol 1991;65:6414–6424.
14 Balfour HH Jr. Antiviral drugs. N Engl J Med 1991;340: 1255–1268.
15 Baringer JR, Pisani P: Herpes simplex virus genomes in human nervous system tissue analyzed by polymerase chain reaction. Ann Neurol 1994;36:823–829.
16 Barker FG 2nd, Chang SM, Gutin PH, Malec MK, McDermott MW, Prados MD, Wilson CB: Survival and functional status after resection of recurrent glioblastoma multiforme. Neurosurgery 1998;42:709–720.

17 Batterson W, Furlong D, Roizman B: Molecular genetics of herpes simplex virus. VIII. Further characterization of a temperature-sensitive mutant defective in release of viral DNA and in other stages of the viral reproductive cycle. J Virol 1983;45:397–407.
18 Baucke RB, Spear PG: Membrane proteins specified by herpes simplex viruses. V. Identification of an Fc-binding glycoprotein. J Virol 1979;32:779–789.
19 Becker Y, Asher Y, Weinberg-Zahlering E, Rabkin SD, Friedmann A, Kessler E: Defective herpes simplex virus DNA: Circular and circular-linear molecules resembling rolling circles. J Gen Virol 1978;40:319–335.
20 Becker Y, Dym H, Sarov I: Herpes simplex virus DNA. Virology 1968;36:184–192.
21 Becker Y, Tavor E, Asher Y, Berkowitz C, Moyal M: Effect of herpes simplex virus type-1 UL41 gene on the stability of mRNA from the cellular genes: Beta-actin, fibronectin, glucose transporter-1, and docking protein, and on virus intraperitoneal pathogenicity to newborn mice. Virus Genes 1993;7:133–143.
22 Ben-Hur T, Hadar J, Shtram Y, Gilden DH, Becker Y: Neurovirulence of herpes simplex virus type 1 depends on age in mice and thymidine kinase expression. Arch Virol 1983;78:303–308.
23 Bennett JJ, Kooby DA, Delman K, McAuliffe P, Halterman MW, Federoff H, Fong Y: Antitumor efficacy of regional oncolytic viral therapy for peritoneally disseminated cancer. J Mol Med 2000;78:166–174.
24 Bergstrom T, Alestig K, Svennerholm B, Horal P, Skoldenberg B, Vahlne A: Neurovirulence of herpes simplex virus types 1 and 2 isolates in diseases of the central nervous system. Eur J Clin Microbiol Infect Dis 1990;9:751–757.
25 Boviatsis EJ, Park JS, Sena-Esteves M, Kramm CM, Chase M, Efird JT, Wei MX, Breakfield XO, Chiocca EA: Long-term survival of rats harboring brain neoplasms treated with ganciclovir and a herpes simplex virus vector that retains an intact thymidine kinase gene. Cancer Res 1994; 54:5745–5751.
26 Boviatsis EJ, Scharf JM, Chase M, Harrington K, Kowall NW, Breakefield XO, Chiocca EA: Antitumor activity and reporter gene transfer into rat brain neoplasms inoculated with herpes simplex virus vectors defective in thymidine kinase or ribonucleotide reductase. Gene Ther 1994; 1:323–331.
27 Bradley JD, Kataoka Y, Advani S, Chung SM, Arani RB, Gillespie GY, Whitley RJ, Markert JM, Roizman B, Weichselbaum RR: Ionizing radiation improves survival in mice bearing intracranial high-grade gliomas injected with genetically modified herpes simplex virus. Clin Cancer Res 1999;5:1517–1522.
28 Brandt CR, Imesch P, Spencer B, Eliassi-Rad B, Syed NA, Untawale S, Robinson NL, Albert DM: The herpes simplex virus type 1 ribonucleotide reductase is required for acute retinal disease. Arch Virol 1997;142:883–896.
29 Brandt CR, Imesch PD, Robinson NL, Syed NA, Untawale S, Darjatmoko SR, Chappell RJ, Heinzelman P, Albert DM: Treatment of spontaneously arising retinoblastoma tumors in transgenic mice with an attenuated herpes simplex virus mutant. Virology 1997;229:283–291.
30 Brown SM, MacLean AR, Aitken JD, Harland J: ICP34.5 influences herpes simplex virus type 1 maturation and egress from infected cells in vitro. J Gen Virol 1994;75:3679–3686.
31 Brown SM, Ritchie DA, Subak-Sharpe JH: Genetic studies with herpes simplex virus type 1. The isolation of temperature-sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. J Gen Virol 1973;18:329–346.
32 Browne H, Bell S, Minson T, Wilson DW: An endoplasmic reticulum-retained herpes simplex virus glycoprotein H is absent from secreted virions: Evidence for reenvelopment during egress. J Virol 1996;70:4311–4316.
33 Bzik DJ, Fox BA, DeLuca NA, Person S: Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. Virology 1984;137:185–190.
34 Cabrera CV, Wohlenberg C, Openshaw H, Rey-Mendez M, Puga A, Notkins AL: Herpes simplex virus DNA sequences in the CNS of latently infected mice. Nature 1980;288:288–290.
35 Cai W, Schaffer PA: Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J Virol 1992;66:2904–2915.

36 Cai WH, Gu B, Person S: Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J Virol 1988;62:2596–2604.
37 Cameron JM, McDougall I, Marsden HS, Preston VG, Ryan DM, Subak-Sharpe JH: Ribonucleotide reductase encoded by herpes simplex virus is a determinant of the pathogenicity of the virus in mice and a valid antiviral target. J Gen Virol 1988;69:2607–2612.
38 Campadelli G, Brandimarti R, Di Lazzaro C, Ward PL, Roizman B, Torrisi MR: Fragmentation and dispersal of Golgi proteins and redistribution of glycoproteins and glycolipids processed through the Golgi apparatus after infection with herpes simplex virus 1. Proc Natl Acad Sci USA 1993;90:2798–2802.
39 Campbell ME, Palfreyman JW, Preston CM: Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. J Mol Biol 1984;180:1–19.
40 Caradonna SJ, Cheng YC: Induction of uracil-DNA glycosylase and dUTP nucleotidohydrolase activity in herpes simplex virus-infected human cells. J Biol Chem 1981;256:9834–9837.
41 Carew JF, Federoff H, Halterman M, Kraus DH, Savage H, Sacks PG, Schantz SP, Shah JP, Fong Y: Efficient gene transfer to human squamous cell carcinomas by the herpes simplex virus type 1 amplicon vector. Am J Surg 1998;176:404–408.
42 Carew JF, Kooby DA, Halterman MW, Federoff HJ, Fong Y: Selective infection and cytolysis of human head and neck squamous cell carcinoma with sparing of normal mucosa by a cytotoxic herpes simplex virus type 1 (G207). Hum Gene Ther 1999;10:1599–1606.
43 Carroll NM, Chiocca E, Takahashi K, Tanabe KK: Enhancement of gene therapy specificity for diffuse colon carcinoma liver metastases with recombinant herpes simplex virus. Ann Surg 1996;224:323–330.
44 Cassady KA, Gross M, Roizman B: The second-site mutation in the herpes simplex virus recombinants lacking the gamma134.5 genes precludes shutoff of protein synthesis by blocking the phosphorylation of eIF-2alpha. J Virol 1998;72:7005–7011.
45 Cassel WA, Murray DR, Phillips HS: A phase II study on the post surgical management of stage II malignant melanoma with a Newcastle disease virus oncolysate. Cancer 1983;52:856–860.
46 Chahlavi A, Rabkin S, Todo T, Sundaresan P, Martuza R: Effect of prior exposure to herpes simplex virus 1 on viral vector-mediated tumor therapy in immunocompetent mice. Gene Ther 1999;6:1751–1758.
47 Chahlavi A, Todo T, Martuza RL, Rabkin SD: Replication-competent herpes simplex virus vector G207 and cisplatin combination therapy for head and neck squamous cell carcinoma. Neoplasia 1999;1:162–169.
48 Chambers R, Gillespie GY, Soroceanu L, Adreansky S, Chatterjee S, Chou J, Roizman B, Whitley RJ: Comparison of genetically engineered herpes simplex viruses for the treatment of brain tumors in a scid mouse model of human malignant glioma. Proc Natl Acad Sci USA 1995;92:1411–1415.
49 Chase M, Chung RY, Chiocca EA: An oncolytic viral mutant that delivers the CYP2B1 transgene and augments cyclophosphamide chemotherapy. Nature Biotechnol 1998;16:444–448.
50 Chen MS, Prusoff WH: Association of thymidylate kinase activity with pyrimidine deoxyribonucleoside kinase induced by herpes simplex virus. J Biol Chem 1978;253:1325–1327.
51 Cheng YC, Grill SP, Dutschman GE, Nakayama K, Bastow KF: Metabolism of 9-(1,3-dihydroxy-2-propoxymethyl)guanine, a new anti-herpes virus compound, in herpes simplex virus-infected cells. J Biol Chem 1983;258:12460–12464.
52 Chou J, Kern ER, Whitley RJ, Roizman B: Mapping of herpes simplex virus-1 neurovirulence to gamma 34.5, a gene nonessential for growth in culture. Science 1990;250:1262–1266.
53 Chou J, Roizman B: The gamma 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programed cell death in neuronal cells. Proc Natl Acad Sci USA 1992;89:3266–3270.
54 Chou J, Roizman B: Herpes simplex virus 1 gamma-(1)34.5 gene function, which blocks the host response to infection, maps in the homologous domain of the genes expressed during growth arrest and DNA damage. Proc Natl Acad Sci USA 1994;91:5247–5251.
55 Chung RY, Saeki Y, Chiocca EA: B-myb promoter retargeting of herpes simplex virus gamma34.5 gene-mediated virulence toward tumor and cycling cells. J Virol 1999;73:7556–7564.

56 Clarke L, Waxman DJ: Oxidative metabolism of cyclophosphamide: Identification of the hepatic monooxygenase catalysts of drug activation. Cancer Res 1989;49:2344–2350.
57 Cocchi F, Lopez M, Menotti L, Aoubala M, Dubreuil P, Campadelli-Fiume G: The V domain of herpesvirus Ig-like receptor (HIgR) contains a major functional region in herpes simplex virus-1 entry into cells and interacts physically with the viral glycoprotein D. Proc Natl Acad Sci USA 1998;95:15700–15705.
58 Cocchi F, Menotti L, Mirandola P, Lopez M, Campadelli-Fiume G: The ectodomain of a novel member of the immunoglobulin subfamily related to the poliovirus receptor has the attributes of a bona fide receptor for herpes simplex virus types 1 and 2 in human cells. J Virol 1998;72: 9992–10002.
59 Coen DM, Fleming HE, Leslie LK, Retondo MJ: Sensitivity of arabinosyladenine-resistant mutants of herpes simplex virus to other antiviral drugs and mapping of drug hypersensitivity mutations to the DNA polymerase locus. J Virol 1985;53:477–488.
60 Coen DM, Furman PA, Gelep PT, Schaffer PA: Mutations in the herpes simplex virus DNA polymerase gene can confer resistance to 9-beta-D-arabinofuranosyladenine. J Virol 1982;41: 909–918.
61 Coen DM, Goldstein DJ, Weller SK: Herpes simplex virus ribonucleotide reductase mutants are hypersensitive to acyclovir. Antimicrob Agents Chemother 1989;33:1395–1399.
62 Coen DM, Kosz-Vnenchak M, Jacobson JG, Leib DA, Bogard CL, Schaffer PA, Tyler KL, Knipe DM: Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. Proc Natl Acad Sci USA 1989;86:4736–4740.
63 Coen DM, Schaffer PA: Two distinct loci confer resistance to acycloguanosine in herpes simplex virus type 1. Proc Natl Acad Sci USA 1980;77:2265–2269.
64 Cohen GH: Ribonucleotide reductase activity of synchronized KB cells infected with HSV. J Virol 1972;9:403–418.
65 Connors TA: The choice of prodrugs for gene directed enzyme prodrug therapy of cancer. Gene Ther 1995;2:702–709.
66 Corey L, Reeves WC, Holmes KK: Cellular immune response in genital herpes simplex virus infection. N Engl J Med 1978;299:986–991.
67 Coukos G, Makrigiannakis A, Kang EH, Caparelli D, Benjamin I, Kaiser LR, Rubin SC, Albelda SM, Molnar-Kimber KL: Use of carrier cells to deliver a replication-selective herpes simplex virus-1 mutant for the intraperitoneal therapy of epithelial ovarian cancer. Clin Cancer Res 1999;5:1523–1537.
68 Coukos G, Makrigiannakis A, Kang EH, Rubin SC, Albelda SM, Molnar-Kimber KL: Oncolytic herpes simplex virus-1 lacking ICP34.5 induces p53-independent death and is efficacious against chemotherapy-resistant ovarian cancer. Clin Cancer Res 2000;6:3342–3353.
69 Coukos G, Makrigiannakis A, Montas S, Kaiser LR, Toyozumi T, Benjamin I, Albelda SM, Rubin SC, Molnar-Kimber KL: Multi-attenuated herpes simplex virus-1 mutant G207 exerts cytotoxicity against epithelial ovarian cancer but not normal mesothelium and is suitable for intraperitoneal oncolytic therapy. Cancer Gene Ther 2000;7:275–283.
70 Cowan M, Davies J, Brookes K, Billstrom M, McLeish P, Buchan A, Skinner GR: Inhibition of rate of tumour growth in rodent species by inoculation of herpesviruses and encephalomyocarditis virus. J Med Virol 1990;30:211–215.
71 Croen KD, Ostrove JM, Dragovic LJ, Smialek JE, Straus SE: Latent herpes simplex virus in human trigeminal ganglia. Detection of an immediate early gene 'anti-sense' transcript by in situ hybridization. N Engl J Med 1987;317:1427–1432.
72 Crute JJ, Tsurumi T, Zhu L, Weller SK, Olivo PD, Challberg MD, Mocarski ES, Lehman IR: Herpes simplex virus 1 helicase-primase: A complex of three herpes-encoded gene products. Proc Natl Acad Sci USA 1989;86:2186–2189.
73 Culver KW, Ram Z, Walbridge S, Ishii H, Oldfield EH, Blaese RM: In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science 1992;256: 1550–1552.
74 Cupps TR, Fauci AS: Corticosteroid-mediated immunoregulation in man. Immunol Rev 1982; 65:133–155.

75 D'Angelica M, Karpoff H, Halterman M, Ellis J, Klimstra D, Edelstein D, Brownlee M, Federoff H, Fong Y: In vivo interleukin-2 gene therapy of established tumors with herpes simplex amplicon vectors. Cancer Immunol Immunother 1999;47:265–271.
76 D'Angelica M, Tung C, Allen P, Halterman M, Delman K, Delohery T, Klimstra D, Brownlee M, Federoff H, Fong Y: Herpes simplex virus (HSV)-mediated ICAM-1 gene transfer abrogates tumorigenicity and induces anti-tumor immunity. Mol Med 1999;5:606–616.
77 Darville JM, Blyth WA: Cell-mediated immunity in mice with primary, secondary and latent herpes simplex virus infection. Arch Virol 1982;74:135–144.
78 Deatly AM, Spivack JG, Lavi E, Fraser NW: RNA from an immediate early region of the type 1 herpes simplex virus genome is present in the trigeminal ganglia of latently infected mice. Proc Natl Acad Sci USA 1987;84:3204–3208.
79 Deatly AM, Spivack JG, Lavi E, O'Boyle DR 2nd, Fraser NW: Latent herpes simplex virus type 1 transcripts in peripheral and central nervous system tissues of mice map to similar regions of the viral genome. J Virol 1988;62:749–756.
80 Deiss LP, Chou J, Frenkel N: Functional domains within the a sequence involved in the cleavage-packaging of herpes simplex virus DNA. J Virol 1986;59:605–618.
81 DeLuca NA, McCarthy AM, Schaffer PA: Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J Virol 1985;56:558–570.
82 DeLuca NA, Schaffer PA: Activation of immediate-early, early and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. J Virol 1985;5: 1997–2008.
83 Deschatrette J, Fougere-Deschatrette C, Corcos L, Schimke RT: Expression of the mouse serum albumin gene introduced into differentiated and dedifferentiated rat hepatoma cells. Proc Natl Acad Sci USA 1985;82:765–769.
84 Deshmane SL, Fraser NW: During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. J Virol 1989;63:943–947.
85 Dienes HP, Knoblich A, Falke D: Loss of surface fibronectin after infection of cultured cells by HSV-1 and 2. Arch Virol 1985;86:223–237.
86 Dingwell KS, Brunetti CR, Hendricks RL, Tang Q, Tang M, Rainbow AJ, Johnson DC: Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. J Virol 1994;68:834–845.
87 Dix RD, McKendall RR, Baringer JR: Comparative neurovirulence of herpes simplex virus type 1 strains after peripheral or intracerebral inoculation of BALB/c mice. Infect Immun 1983;40:103–112.
88 Drummond CW, Eglin RP, Esiri MM: Herpes simplex virus encephalitis in a mouse model: PCR evidence for CNS latency following acute infection. J Neurol Sci 1994;127:159–163.
89 Dubbs DR, Kit S: Mutant strains of herpes simplex deficient in thymidine kinase-inducing activity. Virology 1964;22:493–502.
90 Dubin G, Frank I, Friedman HM: Herpes simplex virus type 1 encodes two Fc receptors which have different binding characteristics for monomeric immunoglobulin G (IgG) and IgG complexes. J Virol 1990;64:2725–2731.
91 Dubin G, Socolof E, Frank I, Friedman HM: Herpes simplex virus type 1 Fc receptor protects infected cells from antibody-dependent cellular cytotoxicity. J Virol 1991;65:7046–7050.
92 Efstathiou S, Kemp S, Darby G, Minson AC: The role of herpes simplex virus type 1 thymidine kinase in pathogenesis. J Gen Virol 1989;70:869–879.
93 Efstathiou S, Minson AC, Field HJ, Anderson JR, Wildy P: Detection of herpes simplex virus-specific DNA sequences in latently infected mice and in humans. J Virol 1986;57:446–455.
94 Elias P, O'Donnell ME, Mocarski ES, Lehman IR: A DNA binding protein specific for an origin of replication of herpes simplex virus type 1. Proc Natl Acad Sci USA 1986;83:6322–6326.
95 Everett RD: Analysis of the functional domains of herpes simplex virus type 1 immediate-early polypeptide Vmw110. J Mol Biol 1988;202:87–96.
96 Everett RD, Freemont P, Saitoh H, Dasso M, Orr A, Kathoria M, Parkinson J: The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. J Virol 1998;72:6581–6591.

97 Everett RD, Maul GG: HSV-1 IE protein Vmw110 causes redistribution of PML. EMBO J 1994; 13:5062–5069.
98 Farrell MJ, Dobson AT, Feldman LT: Herpes simplex virus latency-associated transcript is a stable intron. Proc Natl Acad Sci USA 1991;88:790–794.
99 Fenwick ML, McMenamin MM: Early virion-associated suppression of cellular protein synthesis by herpes simplex virus is accompanied by inactivation of mRNA. J Gen Virol 1984;65: 1225–1228.
100 Fenwick ML, Walker MJ: Suppression of the synthesis of cellular macromolecules by herpes simplex virus. J Gen Virol 1978;41:37–51.
101 Field HJ, Darby G, Wildy P: Isolation and characterization of acyclovir-resistant mutants of herpes simplex virus. J Gen Virol 1980;49:115–124.
102 Field HJ, Wildy P: The pathogenicity of thymidine kinase-deficient mutants of herpes simplex virus in mice. J Hyg (Lond) 1978;81:267–277.
103 Fine HA: The basis for current treatment recommendations for malignant gliomas. J Neurooncol 1994;20:111–120.
104 Fink DJ, DeLuca NA, Goins WF, Glorioso JC: Gene transfer to neurons using herpes simplex virus-based vectors. Annu Rev Neurosci 1996;19:265–287.
105 Forrester A, Farrell H, Wilkinson G, Kaye J, Davis-Poynter N, Minson T: Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. J Virol 1992;66:341–348.
106 Frame MC, Marsden HS, Dutia BM: The ribonucleotide reductase induced by herpes simplex virus type 1 involves minimally a complex of two polypeptides (136K and 38K). J Gen Virol 1985;66:1581–1587.
107 Friedman HM, Cohen GH, Eisenberg RJ, Seidel CA, Cines DB: Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. Nature 1984;309:633–635.
108 Friedman HM, Wang L, Fishman NO, Lambris JD, Eisenberg RJ, Cohen GH, Lubinski J: Immune evasion properties of herpes simplex virus type 1 glycoprotein gC. J Virol 1996; 70:4253–4260.
109 Fruh K, Ahn K, Djaballah H, Sempe P, van Endert PM, Tampe R, Peterson PA, Yang Y: A viral inhibitor of peptide transporters for antigen presentation. Nature 1995;375:415–418.
110 Fyfe JA, Keller PM, Furman PA, Miller RI, Elion GB: Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. J Biol Chem 1978;253:8721–8727.
111 Gaffney DF, McLauchlan J, Whitton JL, Clements JB: A modular system for the assay of transcription regulatory signals: the sequence TAATGARAT is required for herpes simplex virus immediate early gene activation. Nucleic Acids Res 1985;13:7847–7863.
112 Galea-Lauri J, Farzaneh F, Gäken J: Novel costimulators in the immune gene therapy of cancer. Cancer Gene Ther 1996;3:202–213.
113 Gallo ML, Dorsky DI, Crumpacker CS, Parris DS: The essential 65-kilodalton DNA-binding protein of herpes simplex virus stimulates the virus-encoded DNA polymerase. J Virol 1989; 63:5023–5029.
114 Geraghty RJ, Krummenacher C, Cohen GH, Eisenberg RJ, Spear PG: Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. Science 1998;280: 1618–1620.
115 Gerster T, Roeder RG: A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. Proc Natl Acad Sci USA 1988;85:6347–6351.
116 Gibson W, Roizman B: Proteins specified by herpes simplex virus. 8. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J Virol 1972;10:1044–1052.
117 Godowski PJ, Knipe DM: Transcriptional control of herpesvirus gene expression: Gene functions required for positive and negative regulation. Proc Natl Acad Sci USA 1986;83:256–260.
118 Goldsmith K, Chen W, Johnson DC, Hendricks RL: Infected cell protein (ICP)47 enhances herpes simplex virus neurovirulence by blocking the CD8+ T cell response. J Exp Med 1998;187: 341–348.

119 Goldstein DJ, Weller SK: Factor(s) present in herpes simplex virus type 1-infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: Characterization of an ICP6 deletion mutant. Virology 1988;166:41–51.
120 Goldstein DJ, Weller SK: Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensible for virus growth and DNA synthesis: Isolation and characterization of an ICP6 lacZ insertion mutant. J Virol 1988;62:196–205.
121 Goodman JL, Engel JP: Altered pathogenesis in herpes simplex virus type 1 infection due to a syncytial mutation mapping to the carboxy terminus of glycoprotein B. J Virol 1991;65:1770–1778.
122 Gordon YJ, Gilden DM, Becker Y: HSV-1 thymidine kinase promotes virulence and latency in the mouse. Invest Ophthalmol Vis Sci 1983;24:599–602.
123 Gottleib J, Marcy AI, Coen DM, Challberg MD: The herpes simplex virus type 1 UL42 gene product: A subunit of DNA polymerase that functions to increase processivity. J Virol 1990; 64:5976–5987.
124 Guyre PM, Girard MT, Morganelli PM, Manganiello PD: Glucocorticoid effects on the production and actions of immune cytokines. J Steroid Biochem 1988;30:89–93.
125 Haanes EJ, Nelson CM, Soule CL, Goodman JL: The UL45 gene product is required for herpes simplex virus type 1 glycoprotein B-induced fusion. J Virol 1994;68:5825–5834.
126 Hardy WR, Sandri-Goldin RM: Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. J Virol 1994;68:7790–7799.
127 Hay KA, Gaydos A, Tenser RB: The role of herpes simplex thymidine kinase expression in neurovirulence and latency in newborn vs. adult mice. J Neuroimmunol 1995;61:41–52.
128 Hayward GS, Jacob RJ, Wadsworth SC, Roizman B: Anatomy of herpes simplex virus DNA: Evidence for four populations of molecules that differ in the relative orientations of their long and short components. Proc Natl Acad Sci USA 1975;72:4243–4247.
129 He B, Chou J, Liebermann DA, Hoffman B, Roizman B: The carboxyl terminus of the murine MyD116 gene substitutes for the corresponding domain of the gamma(1)34.5 gene of herpes simplex virus to preclude the premature shutoff of total protein synthesis in infected human cells. J Virol 1996;70:84–90.
130 He B, Gross M, Roizman B: The gamma(1)34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1alpha to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. Proc Natl Acad Sci USA 1997;94:843–848.
131 Heeg U, Dienes HP, Muller S, Falke D: Involvement of actin-containing microfilaments in HSV-induced cytopathology and the influence of inhibitors of glycosylation. Arch Virol 1986; 91:257–270.
132 Heine JW, Honess RW, Cassai E, Roizman B: Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J Virol 1974;14:640–651.
133 Hengstler JG, Hengst A, Fuchs J, Tanner B, Pohl J, Oesch F: Induction of DNA crosslinks and DNA strand lesions by cyclophosphamide after activation by cytochrome P450 2B1. Mutat Res 1997;373:215–223.
134 Herrlinger U, Kramm CM, Aboody-Guterman KS, Silver JS, Ikeda K, Johnston KM, Pechan PA, Barth RF, Finkelstein D, Chiocca EA, Louis DN, Breakefield XO: Pre-existing herpes simplex virus 1 (HSV-1) immunity decreases, but does not abolish, gene transfer to experimental brain tumors by a HSV-1 vector. Gene Ther 1998;5:809–819.
135 Hill A, Jugovic P, York I, Russ G, Bennink J, Jewdell J, Ploegh H, Johnson D: Herpes simplex virus turns off the TAP to evade host immunity. Nature 1995;375:411–415.
136 Hill JM, Gebhardt BM, Wen R, Bouterie AM, Thompson HW, O'Callaghan RJ, Halford WP, Kaufman HE: Quantitation of herpes simplex virus type 1 DNA and latency-associated transcripts in rabbit trigeminal ganglia demonstrates a stable reservoir of viral nucleic acids during latency. J Virol 1996;70:3137–3141.
137 Hill JM, Sedarati F, Javier RT, Wagner EK, Stevens JG: Herpes simplex virus latent phase transcription facilitates in vivo reactivation. Virology 1990;174:117–125.
138 Ho DY, Mocarski ES: Beta-galactosidase as a marker in the peripheral and neural tissues of the herpes simplex virus-infected mouse. Virology 1988;167:279–283.

139 Hobbs WE 2nd, DeLuca NA: Perturbation of cell cycle progression and cellular gene expression as a function of herpes simplex virus ICP0. J Virol 1999;73:8245–8255.
140 Hoggan MD, Roizman B: The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. Am J Hyg 1959;70: 208–219.
141 Holland LE, Anderson KP, Shipman C Jr, Wagner EK: Viral DNA synthesis is required for the efficient expression of specific herpes simplex virus type 1 mRNA species. Virology 1980; 101:10–24.
142 Hollander MC, Zhan Q, Bae I, Fornace AJ Jr: Mammalian GADD34, an apoptosis- and DNA damage-inducible gene. J Biol Chem 1997;272:13731–13737.
143 Hollstein M, Sidransky D, Vogelstein B, Harris CC: p53 mutations in human cancers. Science 1991;253:49–53.
144 Homa FL, Brown JC: Capsid assembly and DNA packaging in herpes simplex virus. Rev Med Virol 1997;7:107–122.
145 Honess RW, Purifoy DJ, Young D, Gopal R, Cammack N, O'Hare P: Single mutations at many sites within the DNA polymerase locus of herpes simplex viruses can confer hypersensitivity to aphidicolin and resistance to phosphonoacetic acid. J Gen Virol 1984;65:1–17.
146 Honess RW, Roizman B: Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J Virol 1974;14:8–19.
147 Hunter WD, Martuza RL, Feigenbaum F, Todo T, Mineta T, Yazaki T, Toda M, Newsome JT, Platenberg RC, Manz HJ, Rabkin SD: Attenuated, replication-competent herpes simplex virus type 1 mutant G207: Safety evaluation of intracerebral injection in nonhuman primates. J Virol 1999;73:6319–6326.
148 Huszar D, Bacchetti S: Partial purification and characterization of the ribonucleotide reductase induced by herpes simplex virus infection of mammalian cells. J Virol 1981;37:580–588.
149 Hutchinson L, Goldsmith K, Snoddy D, Ghosh H, Graham FL, Johnson DC: Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. J Virol 1992;66:5603–5609.
150 Hutchinson L, Graham FL, Cai W, Debroy C, Person S, Johnson DC: Herpes simplex virus (HSV) glycoproteins B and K inhibit cell fusion induced by HSV syncytial mutants. Virology 1993; 196:514–531.
151 Hutchinson L, Johnson DC: Herpes simplex virus glycoprotein K promotes egress of virus particles. J Virol 1995;69:5401–5413.
152 Idowu AD, Fraser-Smith EB, Poffenberger KL, Herman RC: Deletion of the herpes simplex virus type 1 ribonucleotide reductase gene alters virulence and latency in vivo. Antiviral Res 1992; 17:145–156.
153 Izumi KM, Stevens JG: Molecular and biological characterization of a herpes simplex virus type 1 (HSV-1) neuroinvasiveness gene. J Exp Med 1990;172:487–496.
154 Jacob RJ, Morse LS, Roizman B: Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. J Virol 1979;29:448–457.
155 Jacobson JG, Leib DA, Goldstein DJ, Bogard CL, Schaffer PA, Weller SK, Coen DM: A herpes simplex virus ribonucleotide reductase deletion mutant is defective for productive acute and reactivatable latent infections of mice and for replication in mouse cells. Virology 1989;173:276–283.
156 Jamieson AT, Gentry GA, Subak-Sharpe JH: Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. J Gen Virol 1974;24:465–480.
157 Jarnagin WR, Delman K, Kooby D, Mastorides S, Zager J, Brennan MF, Brennan F, Blumgart LH, Federoff H, Fong Y: Neoadjuvant interleukin-12 immunogene therapy protects against cancer recurrence after liver resection in an animal model. Ann Surg 2000;231:762–771.
158 Javier RT, Stevens JG, Dissette VB, Wagner EK: A herpes simplex virus transcript abundant in latently infected neurons is dispensable for establishment of the latent state. Virology 1988;166:254–257.
159 Jia WW, McDermott M, Goldie J, Cyander M, Tan J, Tufaro F: Selective destruction of gliomas in immunocompetent rats by thymidine kinase-defective herpes simplex virus type 1. J Natl Cancer Inst 1994;86:1209–1215.

160 Johnson DC, Frame MC, Ligas MW, Cross AM, Stow ND: Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J Virol 1988; 62:1347–1354.
161 Johnson DC, Spear PG: O-linked oligosaccharides are acquired by herpes simplex virus glycoproteins in the Golgi apparatus. Cell 1983;32:987–997.
162 Jorgensen TJ, Katz S, Wittmack EK, Varghese S, Rabkin SD, Martuza RL: Ionizing radiation does not alter herpes simplex virus type 1 vector G207 antitumor activity in subcutaneous human and mouse prostate tumor models (unpublished data).
163 Jugovic PRL, Hill AM, Tomazin R, Ploegh H, Johnson DC: Inhibition of major histocompatibility complex class I antigen presentation in pig and primate cells by herpes simplex virus type 1 and 2 ICP47. J Virol 1998;72:5076–5084.
164 Kaiwar R, Kang T, Evans P, Spector F, Brown P, Spaete R: In vitro and in vivo characterization of RAV 9395, a live attenuated recombinant herpes simplex virus type 2. Abs Int Herpesvirus Work 1998;23:333.
165 Kaplitt MG, Tjuvajev JG, Leib DA, Berk J, Pettigrew KD, Posner JB, Pfaff DW, Rabkin SD, Blasberg RG: Mutant herpes simplex virus induced regression of tumors growing in immunocompetent rats. J Neurooncol 1994;19:137–147.
166 Karpoff HM, D'Angelica M, Blair S, Brownlee MD, Federoff H, Fong Y: Prevention of hepatic tumor metastases in rats with herpes viral vaccines and gamma-interferon. J Clin Invest 1997; 99:799–804.
167 Kastrukoff LF, Lau AS, Puterman ML: Genetics of natural resistance to herpes simplex virus type 1 latent infection of the peripheral nervous system in mice. J Gen Virol 1986;67:613–621.
168 Katzin DS, Connor JD, Wilson LA, Sexton RS: Experimental herpes simplex infection in the Owl Monkey. Proc Soc Exp Biol Med 1967;125:391–398.
169 Keir HM, Gold E: Deoxyribonucleic acid nucleotidyltransferase and deoxyribonuclease from cultured cells infected with herpes simplex virus. Biochim Biophys Acta 1963;72: 263–276.
170 Kesari S, Randazzo BP, Valyi-Nagy T, Huang QS, Brown SM, MacLean AR, Lee V, Trojanowski JQ, Fraser NW: Therapy of experimental human brain tumors using a neuroattenuated herpes simplex virus mutant. Lab Invest 1995;73:636–648.
171 Kieff ED, Bachenheimer SL, Roizman B: Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J Virol 1971;8:125–132.
172 Kirn DH: A tale of two trials: selectively replicating herpesviruses for brain tumors. Gene Ther 2000;7:815–816.
173 Koehler PJ: Use of corticosteroids in neuro-oncology. Anticancer Drugs 1995;6:19–33.
174 Koelle DM, Posavad CM, Barnum GR, Johnson ML, Frank JM, Corey L: Clearance of HSV-2 from recurrent genital lesions correlates with infiltration of HSV-specific cytotoxic T lymphocytes. J Clin Invest 1998;101:1500–1508.
175 Kogishi J, Miyatake S, Hangai M, Akimoto M, Okazaki K, Honda Y: Mutant herpes simplex virus-mediated suppression of retinoblastoma. Curr Eye Res 1999;18:321–326.
176 Kooby DA, Carew JF, Halterman MW, Mack JE, Bertino JR, Blumgart LH, Federoff HJ, Fong Y: Oncolytic viral therapy for human colorectal cancer and liver metastases using a multi-mutated herpes simplex virus type-1 (G207). FASEB J 1999;13:1325–1334.
177 Kramm CM, Chase M, Herrlinger U, Jacobs A, Pechan PA, Rainov NG, Sena-Esteves M, Aghi M, Barnett FH, Chiocca EA, Breakefield XO: Therapeutic efficiency and safety of a second-generation replication-conditional HSV1 vector for brain tumor gene therapy. Hum Gene Ther 1997;8:2057–2068.
178 Krisky DM, Marconi PC, Oligino TJ, Rouse RJ, Fink DJ, Cohen JB, Watkins SC, Glorioso JC: Development of herpes simplex virus replication-defective multigene vectors for combination gene therapy applications. Gene Ther 1998;5:1517–1530.
179 Kristensson K, Lycke E, Roytta M, Svennerholm B, Vahlne A: Neuritic transport of herpes simplex virus in rat sensory neurons in vitro. Effects of substances interacting with microtubular function and axonal flow [nocodazole, taxol and erythro-9-3-(2-hydroxynonyl)adenine]. J Gen Virol 1986;67:2023–2028.

180 Kristie TM, Roizman B: α4, the major regulatory protein of herpes simplex virus type 1, is stably and specifically associated with promoter-regulatory domains of a genes and of selected other viral genes. Proc Natl Acad Sci USA 1986;83:3218–3222.
181 Kristie TM, Sharp P A: Purification of the cellular C1 factor required for the stable recognition of the Oct-1 homeodomain by the herpes simplex virus alpha-trans-induction factor (VP16). J Biol Chem 1993;268:6525–6634.
182 Kucharczuk JC, Randazzo B, Chang MY, Amin KM, Elshami AA, Sterman DH, Rizk NP, Molnar-Kimber KL, Brown SM, MacLean AR, Litzky LA, Fraser NW, Albelda SM, Kaiser LR: Use of a 'replication-restricted' herpes virus to treat experimental human malignant mesothelioma. Cancer Res 1997;57:466–471.
183 Kutubuddin M, Federoff HJ, Challita-Eid PM, Halterman M, Day B, Atkinson M, Planelles V, Rosenblatt JD: Eradication of pre-established lymphoma using herpes simplex virus amplicon vectors. Blood 1999;93:643–654.
184 Kwon BS, Tan KB, Ni J, Oh KO, Lee ZH, Kim KK, Kim YJ, Wang S, Gentz R, Yu GL, Harrop J, Lyn SD, Silverman C, Porter TG, Truneh A, Young PR: A newly identified member of the tumor necrosis factor receptor superfamily with a wide tissue distribution and involvement in lymphocyte activation. J Biol Chem 1997;272:14272–14276.
185 Kwong AD, Frenkel N: Biology of herpes simplex virus (HSV) defective viruses and development of the amplicon system; in Kaplitt MG, Loewy AD (eds): Viral Vectors. Gene Therapy and Neuroscience Applications. San Diego, Academic Press, 1995, pp 25–42.
186 Kwong AD, Frenkel N: Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. Proc Natl Acad Sci USA 1987;84:1926–1930.
187 Lagunoff M, Roizman B: The regulation of synthesis and properties of the protein product of open reading frame P of the herpes simplex virus 1 genome. J Virol 1995;69:3615–3623.
188 Lam EW, Bennett JD, Watson RJ: Cell-cycle regulation of human B-myb transcription. Gene 1995; 160:277–281.
189 Lambright ES, Caparrelli DJ, Abbas AE, Toyoizumi T, Coukos G, Molnar-Kimber KL, Kaiser LR: Oncolytic therapy using a mutant type-1 herpes simplex virus and the role of the immune system. Ann Thorac Surg 1999;68:1756–1760.
190 Lambright ES, Kang EH, Force S, Lanuti M, Caparelli D, Kaiser LR, Albelda SM, Molnar-Kimber KL: Effect of pre-existing anti-herpes immunity on the efficacy of herpes simplex viral therapy in a murine intraperitoneal tumor model. Mol Ther 2000;2:387–393.
191 Langenberg AG, Corey L, Ashley RL, Leong WP, Straus SE: A prospective study of new infections with herpes simplex virus type 1 and type 2. Chiron HSV Vaccine Study Group. N Engl J Med 1999;341:1432–1438.
192 Larson DA, Wara WM: Radiotherapy of primary malignant brain tumors. Semin Surg Oncol 1998; 14:34–42.
193 Lasner TM, Kesari S, Brown SM, Lee MYV, Fraser NW, Trojanowski JQ: Therapy of a murine model of pediatric brain tumors using a herpes simplex virus type-1 ICP34.5 mutant and demonstration of viral replication within the CNS. J Neuropathol Exp Neurol 1996;55: 1259–1269.
194 Lee JM, Bernstein A: p53 mutations increase resistance to ionizing radiation. Proc Natl Acad Sci USA 1993;90:5742–5746.
195 Leib DA, Bogard CL, Kosz-Vnenchak M, Hicks KA, Coen DM, Knipe DM, Schaffer PA: A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent state with reduced frequency. J Virol 1989;63:2893–2900.
196 Leib DA, Machalek MA, Williams BR, Silverman RH, Virgin HW: Specific phenotypic restoration of an attenuated virus by knockout of a host resistance gene. Proc Natl Acad Sci USA 2000; 97:6097–6101.
197 Leib DA, Olivo PD: Gene delivery to neurons: Is herpes simplex virus the right tool for the job? Bioessays 1993;15:547–554.
198 Lekstrom-Himes JA, Pesnicak L, Straus SE: The quantity of latent viral DNA correlates with the relative rates at which herpes simplex virus types 1 and 2 cause recurrent genital herpes outbreaks. J Virol 1998;72:2760–2764.

199 Leopardi R, Van Sant C, Roizman B: The herpes simplex virus 1 protein kinase US3 is required for protection from apoptosis induced by the virus. Proc Natl Acad Sci USA 1997;94:7891–7896.
200 Liedtke W, Opalka B, Zimmermann CW, Lignitz E: Age distribution of latent herpes simplex virus 1 and varicella-zoster virus genome in human nervous tissue. J Neurol Sci 1993;116:6–11.
201 Ligas MW, Johnson DC: A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate into cells. J Virol 1988;62:1486–1494.
202 Littler E, Purifoy D, Minson A, Powell KL: Herpes simplex virus non-structural proteins. III. Function of the major DNA-binding protein. J Gen Virol 1983;64:983–995.
203 Lomonte P, Everett RD: Herpes simplex virus type 1 immediate-early protein Vmw110 inhibits progression of cells through mitosis and from G(1) into S phase of the cell cycle. J Virol 1999;73:9456–9467.
204 Lopez C: Genetics of natural resistance to herpesvirus infection in mice. Nature 1975;258:152–153.
205 Loutsch JM, Perng GC, Hill JM, Zheng X, Marquart ME, Block TM, Ghiasi H, Nesburn AB, Wechsler SL: Identical 371-base-pair deletion mutations in the LAT genes of herpes simplex virus type 1 McKrae and 17syn+ result in different in vivo reactivation phenotypes. J Virol 1999; 73:767–771.
206 Lowe SW, Bodis S, McClatchey A, Remington L, Ruley HE, Fisher DE, Housman DE, Jacks T: p53 status and the efficacy of cancer therapy in vivo. Science 1994;266:807–810.
207 Lubinski JM, Wang L, Soulika AM, Burger R, Wetsel RA, Colten H, Cohen GH, Eisenberg RJ, Lambris JD, Friedman HM: Herpes simplex virus type 1 glycoprotein gC mediates immune evasion in vivo. J Virol 1998;72:8257–8263.
208 Lycke E, Hamark B, Johansson M, Krotochwil A, Lycke J, Svennerholm B: Herpes simplex virus infection of the human sensory neuron. An electron microscopy study. Arch Virol 1988;101: 87–104.
209 Mackem S, Roizman B: Structural features of the herpes simplex virus alpha gene 4, 0, and 27 promoter-regulatory sequences which confer alpha regulation on chimeric thymidine kinase genes. J Virol 1982;44:939–949.
210 MacLean AR, Ul-Fareed M, Robertson L, Harland J, Brown SM: Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence-related sequences in Glasgow strain 17+ between immediate early gene 1 and the 'a' sequence. J Gen Virol 1991;72:631–639.
211 Maggioncalda J, Mehta A, Su YH, Fraser NW, Block TM: Correlation between herpes simplex virus type 1 rate of reactivation from latent infection and the number of infected neurons in trigeminal ganglia. Virology 1996;225:72–81.
212 Marconi P, Tamura M, Moriuchi S, Krisky DM, Niranjan A, Goins WF, Cohen JB, Glorioso JC: Connexin 43-enhanced suicide gene therapy using herpesviral vectors. Mol Ther 2000;1:71–81.
213 Margolis TP, Sedarati F, Dobson AT, Feldman LT, Stevens JG: Pathways of viral gene expression during acute neuronal infection with HSV-1. Virology 1992;189:150–160.
214 Markert JM, Coen DM, Malick A, Mineta T, Martuza RL: Expanded spectrum of viral therapy in the treatment of nervous system tumors. J Neurosurg 1992;77:590–594.
215 Markert JM, Malick A, Coen DM, Martuza RL: Reduction and elimination of encephalitis in an experimental glioma therapy model with attenuated herpes simplex mutants that retain susceptibility to acyclovir. Neurosurgery 1993;32:597–603.
216 Markert JM, Medlock MD, Rabkin SD, Gillespie GY, Todo T, Hunter WD, Palmer CA, Feigenbaum F, Tornatore C, Tufaro F, Martuza RL: Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: Results of a phase I trial. Gene Ther 2000; 7:867–874.
217 Martinez R, Sarisky RT, Weber PC, Weller SK: Herpes simplex virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication intermediates. J Virol 1996;70: 2075–2085.
218 Martuza RL: Conditionally replicating herpes vectors for cancer therapy. J Clin Invest 2000;105: 841–846.
219 Martuza RL, Malick A, Markert JM, Ruffner KL, Coen DM: Experimental therapy of human glioma by means of a genetically engineered virus mutant. Science 1991;252:854–856.

220 Maul GG, Ishov AM, Everett RD: Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. Virology 1996;217:67–75.
221 Mauri DN, Ebner R, Montgomery RI, Kochel KD, Cheung TC, Yu GL, Ruben S, Murphy M, Eisenberg RJ, Cohen GH, Spear PG, Ware CF: LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. Immunity 1998;8:21–30.
222 McGeoch DJ, Dalrymple MA, Davison AJ, Dolan A, Frame MC, McNab D, Perry LJ, Scott JE, Taylor P: The complete DNA sequence of the long unique region in the genome of herpes simpex virus type 1. J Gen Virol 1988;69:1531–1574.
223 McGregor F, Phelan A, Dunlop J, Clements JB: Regulation of herpes simplex virus poly (A) site usage and the action of immediate-early protein IE63 in the early-late switch. J Virol 1996;70:1931–1940.
224 McKie EA, Brown SM, MacLean AR, Graham DI: Histopathological responses in the CNS following inoculation with a non-neurovirulent mutant (1716) of herpes simplex virus type 1 (HSV 1): Relevance for gene and cancer therapy. Neuropathol Appl Neurobiol 1998;24:367–372.
225 McKie EA, MacLean AR, Lewis AD, Cruickshank G, Rampling R, Barnett SC, Kennedy PG, Brown SM: Selective in vitro replication of herpes simplex virus type 1 (HSV-1) ICP34.5 null mutants in primary human CNS tumours – Evaluation of a potentially effective clinical therapy. Br J Cancer 1996;74:745–752.
226 McKnight JL, Kristie TM, Roizman B: Binding of the virion protein mediating alpha gene induction in herpes simplex virus 1-infected cells to its cis site requires cellular proteins. Proc Natl Acad Sci USA 1987;84:7061–7065.
227 McLauchlan J, Phelan A, Loney C, Sandri-Goldin RM, Clements JB: Herpes simplex virus IE63 acts at the posttranscriptional level to stimulate viral mRNA 3′ processing. J Virol 1992;66:6939–6945.
228 McMenamin MM, Byrnes AP, Charlton HM, Coffin RS, Latchman DS, Wood MJ: A gamma34.5 mutant of herpes simplex 1 causes severe inflammation in the brain. Neuroscience 1998;83: 1225–1237.
229 McNearney TA, Odell C, Holers VM, Spear PG, Atkinson JP: Herpes simplex virus glycoproteins gC-1 and gC-2 bind to the third component of complement and provide protection against complement-mediated neutralization of viral infectivity. J Exp Med 1987;166:1525–1535.
230 Meignier B, Longnecker R, Mavromara-Nazos P, Sears AE, Roizman B: Virulence of and establishment of latency by genetically engineered deletion mutants of herpes simplex virus. Virology 1988;162:251–254.
231 Meignier B, Longnecker R, Roizman B: In vivo behavior of genetically engineered herpes simplex viruses R7017 and R7020: Construction and evaluation in rodents. J Infect Dis 1988;158:602–614.
232 Meignier B, Martin B, Whitley RJ, Roizman B: In vivo behavior of genetically engineered herpes simplex viruses R7017 and R7020. II. Studies in immunocompetent and immunosuppressed owl monkeys (*Aotus trivirgatus*). J Infect Dis 1990;162:313–321.
233 Melendez LV, Espana C, Hunt RD, Daniel MD, Garcia FG: Natural herpes simplex infection in the owl monkey (*Aotus trivirgatus*). Lab Anim Care 1969;19:38–45.
234 Mellerick DM, Fraser NW: Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. Virology 1987;158:265–275.
235 Mineta T, Rabkin SD, Martuza RL: Treatment of malignant gliomas using ganciclovir-hypersensitive, ribonucleotide reductase-deficient herpes simplex viral mutant. Cancer Res 1994; 54:3963–3966.
236 Mineta T, Rabkin SD, Yazaki T, Hunter WD, Martuza RL: Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. Nat Med 1995;1:938–943.
237 Miyatake S, Iyer A, Martuza RL, Rabkin SD: Transcriptional targeting of herpes simplex virus for cell-specific replication. J Virol 1997;71:5124–5132.
238 Miyatake S, Martuza RL, Rabkin SD: Defective herpes simplex virus vectors expressing thymidine kinase for the treatment of malignant glioma. Cancer Gene Ther 1997;4:222–228.
239 Miyatake SI, Tani S, Feigenbaum F, Sundaresan P, Toda H, Narumi O, Kikuchi H, Hashimoto N, Hangai M, Martuza RL, Rabkin SD: Hepatoma-specific antitumor activity of an albumin enhancer/promoter regulated herpes simplex virus in vivo. Gene Ther 1999;6:564–572.
240 Mohr I, Gluzman Y: A herpesvirus genetic element which affects translation in the absence of the viral GADD34 function. EMBO J 1996;15:4759–4766.

241 Montgomery RI, Warner MS, Lum BJ, Spear PG: Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell 1996;87:427–436.
242 Moolten FL: Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: Paradigm for a prospective cancer control strategy. Cancer Res 1986;46:5276–5281.
243 Moore AE: The oncolytic viruses. Prog Exp Tumor Res 1960;1:411–439.
244 Moriuchi S, Oligino T, Krisky D, Marconi P, Fink D, Cohen J, Glorioso JC: Enhanced tumor cell killing in the presence of ganciclovir by herpes simplex virus type 1 vector-directed coexpression of human tumor necrosis factor-alpha and herpes simplex virus thymidine kinase. Cancer Res 1998;58:5731–5737.
245 Moyal M, Berkowitz C, Rosen-Wolff A, Darai G, Becker Y: Mutations in the UL53 gene of HSV-1 abolish virus neurovirulence to mice by the intracerebral route of infection. Virus Res 1992;26:99–112.
246 Mueller DL, Jenkins MK, Schwartz RH: Clonal expansion versus functional clonal inactivation: A costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. Annu Rev Immunol 1989;7:445–480.
247 Mulvey M, Poppers J, Ladd A, Mohr I: A herpesvirus ribosome-associated, RNA-binding protein confers a growth advantage upon mutants deficient in a GADD34-related function. J Virol 1999; 73:3375–3385.
248 Nagashunmugam T, Lubinski J, Wang L, Goldstein LT, Weeks BS, Sundaresan P, Kang EH, Dubin G, Friedman HM: In vivo immune evasion mediated by the herpes simplex virus type 1 immunoglobulin G Fc receptor. J Virol 1998;72:5351–5359.
249 Nishiyama Y: Herpesvirus genes: Molecular basis of viral replication and pathogenicity. Nagoya J Med Sci 1996;59:107–119.
250 Nishiyama Y, Yamada Y, Kurachi R, Daikoku T: Construction of a US3 lacZ insertion mutant of herpes simplex virus type 2 and characterization of its phenotype in vitro and in vivo. Virology 1992;190:256–268.
251 Norrild B, Lehto VP, Virtanen I: Organization of cytoskeleton elements during herpes simplex virus type 1 infection of human fibroblasts: An immunofluorescence study. J Gen Virol 1986;67:97–105.
252 Oliveira AM, Erickson LA, Burgart LJ, Lloyd RV: Differentiation of primary and metastatic clear cell tumors in the liver by in situ hybridization for albumin messenger RNA. Am J Surg Pathol 2000;24:177–182.
253 Oliver L, Wald A, Kim M, Zeh J, Selke S, Ashley R, Corey L: Seroprevalence of herpes simplex virus infections in a family medicine clinic. Arch Fam Med 1995;4:228–232.
254 Olivo PD, Nelson NJ, Challberg MD: Herpes simplex virus DNA replication: The UL9 gene encodes an origin-binding protein. Proc Natl Acad Sci USA 1988;85:5415–5418.
255 Pardoll DM: Paracrine cytokine adjuvants in cancer immunotherapy. Annu Rev Immunol 1995; 13:399–415.
256 Parker JN, Gillespie GY, Love CE, Randall S, Whitley RJ, Markert JM: Engineered herpes simplex virus expressing IL-12 in the treatment of experimental murine brain tumors. Proc Natl Acad Sci USA 2000;97:2208–2013.
257 Parkinson J, Lees-Miller SP, Everett RD: Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. J Virol 1999;73:650–657.
258 Parris DS, Cross A, Haarr L, Orr A, Frame MC, Murphy M, McGeoch DJ, Marsden HS: Identification of the gene encoding the 65-kilodalton DNA-binding protein of herpes simplex virus type 1. J Virol 1988;62:818–825.
259 Pawlik TM, Nakamura H, Yoon SS, Mullen JT, Chandrasekhar S, Chiocca EA, Tanabe KK: Oncolysis of diffuse hepatocellular carcinoma by intravascular administration of a replication-competent, genetically engineered herpesvirus. Cancer Res 2000;60:2790–2795.
260 Penfold ME, Armati P, Cunningham AL: Axonal transport of herpes simplex virions to epidermal cells: evidence for a specialized mode of virus transport and assembly. Proc Natl Acad Sci USA 1994;91:6529–6533.
261 Pereira RA, Tscharke DC, Simmons A: Upregulation of class I major histocompatibility complex gene expression in primary sensory neurons, satellite cells, and Schwann cells of mice in response to acute but not latent herpes simplex virus infection in vivo. J Exp Med 1994;180:841–850.

262 Perng GC, Ghiasi H, Slanina SM, Nesburn AB, Wechsler SL: High-dose ocular infection with a herpes simplex virus type 1 ICP34.5 deletion mutant produces no corneal disease or neurovirulence yet results in wild-type levels of spontaneous reactivation. J Virol 1996;70:2883–2893.
263 Perng GC, Thompson RL, Sawtell NM, Taylor WE, Slanina SM, Ghiasi H, Kaiwar R, Nesburn AB, Wechsler SL: An avirulent ICP34.5 deletion mutant of herpes simplex virus type 1 is capable of in vivo spontaneous reactivation. J Virol 1995;69:3033–3041.
264 Peterson DL, Sheridan PJ, Brown WE: Animal models for brain tumors: Historical perspectives and future directions. J Neurosurg 1994;80:865–876.
265 Phelan A, Dunlop J, Clements JB: Herpes simplex virus type 1 protein IE63 affects the nuclear export of virus intron-containing transcripts. J Virol 1996;70:5255–5265.
266 Pinkert CA, Ornitz DM, Brinster RL, Palmiter RD: An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. Genes Dev 1987;1:268–276.
267 Pogue-Geile KL, Spear PG: The single base pair substitution responsible for the Syn phenotype of herpes simplex virus type 1, strain MP. Virology 1987;157:67–74.
268 Poliquin L, Levine G, Shore GC: Involvement of Golgi apparatus and a restructured nuclear envelope during biogenesis and transport of herpes simplex virus glycoproteins. J Histochem Cytochem 1985;33:875–883.
269 Post LE, Mackem S, Roizman B: Regulation of alpha genes of herpes simplex virus: Expression of chimeric genes produced by fusion of thymidine kinase with alpha gene promoters. Cell 1981;24:555–565.
270 Preston CM: Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant tsK. J Virol 1979;29:275–284.
271 Purifoy DJM, Lewis RB, Powell KL: Identification of the herpes simplex virus DNA polymerase gene. Nature 1977;269:621–623.
272 Pyles RB, Sawtell NM, Thompson RL: Herpes simplex virus type 1 dUTPase mutants are attenuated for neurovirulence, neuroinvasiveness, and reactivation from latency. J Virol 1992;66:6706–6713.
273 Pyles RB, Thompson RL: Evidence that the herpes simplex virus type 1 uracil DNA glycosylase is required for efficient viral replication and latency in the murine nervous system. J Virol 1994; 68:4963–4972.
274 Pyles RB, Warnick RE, Chalk CL, Szanti BE, Parysek LM: A novel multiply-mutated HSV-1 strain for the treatment of human brain tumors. Hum Gene Ther 1997;8:533–544.
275 Quinlan MP, Chen LB, Knipe DM: The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell 1984;36:857–868.
276 Rampling R, Cruickshank G, Papanastassiou V, Nicoll J, Hadley D, Brennan D, Petty R, MacLean A, Harland J, McKie E, Mabbs R, Brown M: Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma. Gene Ther 2000;7:859–866.
277 Randazzo BP, Bhat MG, Kesari S, Fraser NW, Brown SM: Treatment of experimental subcutaneous human melanoma with a replication-restricted herpes simplex virus mutant. J Invest Dermatol 1997;108:933–937.
278 Randazzo BP, Kesari S, Gesser RM, Alsop D, Ford JC, Brown SM, MacLean A, Fraser NW: Treatment of experimental intracranial murine melanoma with a neuroattenuated herpes simplex virus 1 mutant. Virology 1995;211:94–101.
279 Read GS, Frenkel N: Herpes simplex virus mutants defective in the virion-associated shutoff of host polypeptide synthesis and exhibiting abnormal synthesis of alpha (immediate early) viral polypeptides. J Virol 1983;46:498–512.
280 Read GS, Person S, Keller PM: Genetic studies of cell fusion induced by herpes simplex virus type 1. J Virol 1980;35:105–113.
281 Reeves WC, Corey L, Adams HG, Vontver LA, Holmes KK: Risk of recurrence after first episodes of genital herpes. Relation to HSV type and antibody response. N Engl J Med 1981;305:315–319.
282 Rice SA, Long MC, Lam V, Schaffer PA, Spencer CA: Herpes simplex virus immediate-early protein ICP22 is required for viral modification of host RNA polymerase II and establishment of the normal viral transcription program. J Virol 1995;69:5550–5559.

283 Richards JT, Kern ER, Overall JC Jr, Glasgow LA: Differences in neurovirulence among isolates of Herpes simplex virus types 1 and 2 in mice using four routes of infection. J Infect Dis 1981; 144:464–471.
284 Rixon FJ, Clements JB: Detailed structural analysis of two spliced HSV-1 immediate-early mRNAs. Nucleic Acids Res 1982;10:2241–2256.
285 Roberts MS, Boundy A, O'Hare P, Pizzorno MC, Ciufo DM, Hayward GS: Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (alpha 4) promoter and a specific binding site for the IE175 (ICP4) protein. J Virol 1988; 62:4307–4320.
286 Robertson LM, MacLean AR, Brown SM: Peripheral replication and latency reactivation kinetics of the non-neurovirulent herpes simplex virus type 1 variant 1716. J Gen Virol 1992;73:967–970.
287 Rock DL, Fraser NW: Detection of HSV-1 genome in central nervous system of latently infected mice. Nature 1983;302:523–525.
288 Rock DL, Nesburn AB, Ghiasi H, Ong J, Lewis TL, Lokensgard JR, Wechsler SL: Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. J Virol 1987;61:3820–3826.
289 Roizman B, Jenkins FJ: Genetic engineering of novel genomes of large DNA viruses. Science 1985;229:1208–1214.
290 Roizman B, Sears AE: Herpes simplex viruses and their replication; in Fields BN, Knipe DM, Howley PM (eds), Fields Virology, ed 3. Philadelphia, Lippincott-Raven, 1995, pp 2231–2296.
291 Roller RJ, Zhou Y, Schnetzer R, Ferguson J, DeSalvo D: Herpes simplex virus type 1 U(L)34 gene product is required for viral envelopment. J Virol 2000;74:117–129.
292 Romanelli MG, Cattozzo EM, Faggioli L, Tognon M: Fine mapping and characterization of the Syn 6 locus in the herpes simplex virus type 1 genome. J Gen Virol 1991;72:1991–1995.
293 Roop C, Hutchinson L, Johnson DC: A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. J Virol 1993;67:2285–2297.
294 Rosen A, Darai G: Mapping of the deletion in the genome of HSV-1 strain HFEM responsible for its avirulent phenotype. Med Microbiol Immunol (Berl) 1985;173:329–343.
295 Rosenthal SL, Stanberry LR, Biro FM, Slaoui M, Francotte M, Koutsoukos M, Hayes M, Bernstein DI: Seroprevalence of herpes simplex virus types 1 and 2 and cytomegalovirus in adolescents. Clin Infect Dis 1997;24:135–139.
296 Russell SJ: Replicating vectors for gene therapy of cancer: Risks, limitations and prospects. Eur J Cancer 1994;30:1165–1171.
297 Ruyechan WT, Morse LS, Knipe DM, Roizman B: Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J Virol 1979;29:677–697.
298 Sacks WR, Green CC, Aschaan DP, Schaffer PA: Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J Virol 1985;55:796–805.
299 Sanders VJ, Waddell AE, Felisan SL, Li XM, Conrad AJ, Tourtellotte WW: Herpes simplex virus in postmortem multiple sclerosis brain tissue. Arch Neurol 1996;53:125–133.
300 Sandri-Goldin RM: ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif. Genes Dev 1998;12:868–879.
301 Sawtell NM: Comprehensive quantification of herpes simplex virus latency at the single-cell level. J Virol 1997;71:5423–5431.
302 Sawtell NM, Poon DK, Tansky CS, Thompson RL: The latent herpes simplex virus type 1 genome copy number in individual neurons is virus strain specific and correlates with reactivation. J Virol 1998;72:5343–5350.
303 Schmidt OW, Fife KH, Corey L: Reinfection is an uncommon occurrence in patients with symptomatic recurrent genital herpes. J Infect Dis 1984;149:645–646.
304 Schnipper LS, Crumpacker CS: Resistance of herpes simplex virus to acyloguanosine: Role of the viral thymidine kinase and the polymerase loci. Proc Natl Acad Sci USA 1980;77:2270–2273.
305 Sears AE, Halliburton IW, Meignier B, Silver S, Roizman B: Herpes simplex virus 1 mutant deleted in the a22 gene: Growth and gene expression in permissive and restrictive cells and establishment of latency in mice. J Virol 1985;55:338–346.

306 Sedarati F, Izumi KM, Wagner EK, Stevens JG: Herpes simplex virus type 1 latency-associated transcription plays no role in establishment or maintenance of a latent infection in murine sensory neurons. J Virol 1989;63:4455–4458.
307 Sedarati F, Margolis TP, Stevens JG: Latent infection can be established with drastically restricted transcription and replication of the HSV-1 genome. Virology 1993;192:687–691.
308 Severini A, Scraba DG, Tyrrell DL: Branched structures in the intracellular DNA of herpes simplex virus type 1. J Virol 1996;70:3169–3175.
309 Sheldrick P, Berthelot N: Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harb Symp Quant Biol 1975;39:667–678.
310 Siegel D, Golden E, Washington AE, Morse SA, Fullilove MT, Catania JA, Marin B, Hulley SB: Prevalence and correlates of herpes simplex infections. The population-based AIDS in Multiethnic Neighborhoods Study. JAMA 1992;268:1702–1708.
311 Skinner GR, Cowan M, Davies J, Brookes K, Billstrom M, Buchan A: Reduced tumorigenicity of rodent tumour cells and tumour explants following infection with wild type and mutant herpes simplex virus, bovine mammillitis virus and encephalomyocarditis virus. Br J Exp Pathol 1988; 69:495–504.
312 Smee DF, Martin JC, Verheyden JP, Matthews TR: Anti-herpesvirus activity of the acyclic nucleoside 9-(1,3-dihydroxy-2-propoxymethyl)guanine. Antimicrob Agents Chemother 1983;23: 676–682.
313 Smiley JR, Smibert C, Everett RD: Expression of a cellular gene cloned in herpes simplex virus: Rabbit beta-globin is regulated as an early viral gene in infected fibroblasts. J Virol 1987; 61:2368–2377.
314 Smith IL, Hardwicke MA, Sandri-Goldin RM: Evidence that the herpes simplex virus immediate early protein ICP27 acts post-transcriptionally during infection to regulate gene expression. Virology 1992;186:74–86.
315 Smith RR, Huebner RJ, Rowe WP, Schatten WE, Thomas LB: Studies on the use of viruses in the treatment of carcinoma of the cervix. Cancer 1956;9:1211–1218.
316 Sodeik B, Ebersold MW, Helenius A: Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. J Cell Biol 1997;136:1007–1021.
317 Spaete RR, Frenkel N: The herpes simplex virus amplicon: A new eucaryotic defective-virus cloning-amplifying vector. Cell 1982;30:295–304.
318 Spear MA, Sun F, Eling DJ, Gilpin E, Kipps TJ, Chiocca EA, Bouvet M: Cytotoxicity, apoptosis, and viral replication in tumor cells treated with oncolytic ribonucleotide reductase-defective herpes simplex type 1 virus (hrR3) combined with ionizing radiation. Cancer Gene Ther 2000; 7:1051–1059.
319 Spear PG: Entry of alphaherpsviruses into cells. Semin Virol 1993;4:167–180.
320 Spector T, Averett DR, Nelson DJ, Lambe CU, Morrison WJR, St. Clair MH, Furman PA: Potentiation of antiherpetic activity of acyclovir by ribonucleotide reductase inhibition. Proc Natl Acad Sci USA 1985;82:4254–4257.
321 Spivack JG, Fareed MU, Valyi-Nagy T, Nash TC, O'Keefe JS, Gesser RM, McKie EA, MacLean AR, Fraser NW, Brown SM: Replication, establishment of latent infection, expression of the latency-associated transcripts and explant reactivation of herpes simplex virus type 1 gamma 34.5 mutants in a mouse eye model. J Gen Virol 1995;76:321–332.
322 Spivack JG, Fraser NW: Detection of herpes simplex virus type 1 transcripts during latent infection in mice. J Virol 1987;61:3841–3847.
323 Steiner I, Spivack JG, Deshmane SL, Ace CI, Preston CM, Fraser NW: A herpes simplex virus type 1 mutant containing a nontransinducing Vmw65 protein establishes latent infection in vivo in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia. J Virol 1990;64:1630–1638.
324 Steiner I, Spivack JG, Lirette RP, Brown SM, MacLean AR, Subak-Sharpe JH, Fraser NW: Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. EMBO J 1989;8:505–511.
325 Stern S, Tanaka M, Herr W: The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. Nature 1989;341:624–630.

326 Stevens JG, Wagner EK, Devi-Rao GB, Cook ML, Feldman LT: RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science 1987;235: 1056–1059.
327 Stow ND, Murray MD, Stow EC: Cis-acting signals involved in the replication and packaging of herpes simplex virus type-1 DNA; in Botchan M, Grodzicker T, Sharp PA (eds). Cancer Cells. Cold Spring Harbor, Cold Spring Harbor Laboratory, 1986, vol 4, pp 497–507.
328 Strelow LI, Leib DA: Analysis of conserved domains of UL41 of herpes simplex virus type 1 in virion host shutoff and pathogenesis. J Virol 1996;70:5665–5667.
329 Strelow LI, Leib DA: Role of the virion host shutoff (vhs) of herpes simplex virus type 1 in latency and pathogenesis. J Virol 1995;69:6779–6786.
330 Sundaresan P, Hunter WD, Martuza RL, Rabkin SD: Attenuated, replication-competent herpes simplex virus type 1 mutant G207: safety evaluation in mice. J Virol 2000;74: 3832–3841.
331 Suzutani T, Nagamine M, Shibaki T, Ogasawara M, Yoshida I, Daikoku T, Nishiyama Y, Azuma M: The role of the UL41 gene of herpes simplex virus type 1 in evasion of non-specific host defence mechanisms during primary infection. J Gen Virol 2000;81:1763–1771.
332 Tenser RB: Intracerebral inoculation of newborn and adult mice with thymidine kinase-deficient mutants of herpes simplex virus type 1. J Infect Dis 1983;147:956.
333 Tepper RI, Mule JJ: Experimental and clinical studies of cytokine gene-modified tumor cells. Hum Gene Ther 1994;5:153–164.
334 Terry-Allison T, Montgomery RI, Whitbeck JC, Xu R, Cohen GH, Eisenberg RJ, Spear PG: HveA (herpesvirus entry mediator A), a coreceptor for herpes simplex virus entry, also participates in virus-induced cell fusion. J Virol 1998;72:5802–5810.
335 Thompson RL, Sawtell NM: The herpes simplex virus type 1 latency-associated transcript gene regulates the establishment of latency. J Virol 1997;71:5432–5440.
336 Thompson RL, Wagner EK, Stevens JG: Physical location of a herpes simplex virus type-1 gene function(s) specifically associated with a 10 million-fold increase in HSV neurovirulence. Virology 1983;131:180–192.
337 Tigges MA, Leng S, Johnson DC, Burke RL: Human herpes simplex virus (HSV)-specific CD8+ CTL clones recognize HSV-2-infected fibroblasts after treatment with IFN-gamma or when virion host shutoff functions are disabled. J Immunol 1996;156:3901–3910.
338 Toda M, Martuza RL, Kojima H, Rabkin SD: In situ cancer vaccination: an IL-12 defective vector/replication-competent herpes simplex virus combination induces local and systemic anti-tumor activity. J Immunol 1998;160:4457–4464.
339 Toda M, Martuza RL, Rabkin SD: Tumor growth inhibition by intratumoral inoculation of defective herpes simplex virus vectors expressing granulocyte-macrophage colony-stimulating factor. Mol Ther 2000:2:324–329.
340 Toda M, Rabkin SD, Kojima H, Martuza RL: Herpes simplex virus as an in situ cancer vaccine for the induction of specific anti-tumor immunity. Hum Gene Ther 1999;10:385–393.
341 Toda M, Rabkin SD, Martuza RL: Treatment of human breast cancer in a brain metastatic model by G207, a replication-competent multimutated herpes simplex virus 1. Hum Gene Ther 1998; 9:2177–2185.
342 Todo T, Feigenbaum F, Rabkin SD, Lakeman F, Newsome JT, Johnson PA, Mitchell E, Belliveau D, Ostrove JM, Martuza RL: Viral shedding and biodistribution of G207, a multimutated conditionally replicating herpes simplex virus type 1, after intracerebral inoculation in aotus. Mol Ther 2000;2:588–595.
343 Todo T, Martuza RL, Dallman MJ, Rabkin SD: In situ expression of soluble B7–1 in the context of oncolytic herpes simplex virus induces a potent antitumor immune response. Cancer Res 2001; 61:153–161.
344 Todo T, Martuza RL, Rabkin SD, Johnson PA: Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing. Proc Natl Acad Sci USA 2001;98:6396–6401.
345 Todo T, Rabkin SD, Chahlavi A, Martuza RL: Corticosteroid administration does not affect viral oncolytic activity, but inhibits antitumor immunity in replication-competent herpes simplex virus tumor therapy. Hum Gene Ther 1999;10:2869–2878.

346 Todo T, Rabkin SD, Martuza RL: Evaluation of ganciclovir-mediated enhancement of the antitumoral effect in oncolytic, multimutated herpes simplex virus type 1 (G207) therapy of brain tumors. Cancer Gene Ther 2000;7:939–946.
347 Todo T, Rabkin SD, Sundaresan P, Wu A, Meehan KR, Herscowitz HB, Martuza RL: Systemic antitumor immunity in experimental brain tumor therapy using a multimutated, replication-competent herpes simplex virus. Hum Gene Ther 1999;10:2741–2755.
348 Tognon M, Guandalini R, Romanelli MG, Manservigi R, Trevisani B: Phenotypic and genotypic characterization of locus Syn 5 in herpes simplex virus 1. Virus Res 1991;8:135–150.
349 Topp KS, Meade LB, LaVail JH: Microtubule polarity in the peripheral processes of trigeminal ganglion cells: Relevance for the retrograde transport of herpes simplex virus. J Neurosci 1994; 14:318–325.
350 Torrisi MR, Di Lazzaro C, Pavan A, Pereira L, Campadelli-Fiume G: Herpes simplex virus envelopment and maturation studied by fracture label. J Virol 1992;66:554–561.
351 Toyoizumi T, Mick R, Abbas AE, Kang EH, Kaiser LR, Molnar-Kimber KL: Combined therapy with chemotherapeutic agents and herpes simplex virus type 1 ICP34.5 mutant (HSV-1716) in human non-small cell lung cancer. Hum Gene Ther 1999;10:3013–3029.
352 Trousdale MD, Steiner I, Spivack JG, Deshmane SL, Brown SM, MacLean AR, Subak-Sharpe JH, Fraser NW: In vivo and in vitro reactivation impairment of a herpes simplex virus type 1 latency-associated transcript variant in a rabbit eye model. J Virol 1991;65:6989–6993.
353 Tsutsumi H, Bernstein JM, Riepenhoff-Talty M, Cohen E, Orsini F, Ogra PL: Immune responses to herpes simplex virus in patients with recurrent herpes labialis: I. Development of cell-mediated cytotoxic responses. Clin Exp Immunol 1986;66:507–515.
354 Tuting T, Storkus WJ, Lotze MT: Gene-based strategies for the immunotherapy of cancer. J Mol Med 1997;75:478–491.
355 Vieweg J, Rosenthal FM, Bannerji R, Heston WD, Fair WR, Gansbacher B, Gilboa E: Immunotherapy of prostate cancer in the Dunning rat model: Use of cytokine gene modified tumor vaccines. Cancer Res 1994;54:1760–1765.
356 Visalli RJ, Courtney RJ, Meyers C: Infection and replication of herpes simplex virus type 1 in an organotypic epithelial culture system. Virology 1997;230:236–243.
357 Wadsworth S, Jacob RJ, Roizman B: Anatomy of herpes simplex virus DNA. II. Size, composition, and arrangement of inverted terminal repetitions. J Virol 1975;15:1487–1497.
358 Wagner EK, Bloom DC: Experimental investigation of herpes simplex virus latency. Clin Microbiol Rev 1997;10:419–443.
359 Wagner MJ, Summers WC: Structure of the joint region and the termini of the DNA of herpes simplex virus type 1. J Virol 1978;27:374–387.
360 Walker JR, McGeagh KG, Sundaresan P, Jorgensen TJ, Rabkin SD, Martuza RL: Local and systemic therapy of human prostate adenocarcinoma with the conditionally replicating herpes simplex virus vector G207. Hum Gene Ther 1999;10:2237–2243.
361 Walker MD, Green SB, Byar DP, Alexander E Jr, Batzdorf U, Brooks WH, MacCarty CS, Mahaley ISM Jr, Owens G, Ransohoff J, Robertson JT, Shapiro WR, Smith KR Jr, Wilson C, Strike TA: Randomized comparisons of radiotherapy and nitrosoureas for the treatment of malignant glioma after surgery. N Engl J Med 1980;303:1323–1329.
362 Wang F, Bade E, Kuniyoshi C, Spears L, Jeffery G, Marty V, Groshen S, Weber J: Phase I trial of a MART-1 peptide vaccine with incomplete Freund's adjuvant for resected high-risk melanoma. Clin Cancer Res 1999;5:2756–2765.
363 Ward PL, Roizman B: Herpes simplex genes: The blueprint of a successful human pathogen. Trends Genet 1994;10:267–274.
364 Warner MS, Geraghty RJ, Martinez WM, Montgomery RI, Whitbeck JC, Xu R, Eisenberg RJ, Cohen GH, Spear PG: A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus. Virology 1998;246:179–189.
365 Wei MX, Tamiya T, Rhee RJ, Breakefield XO, Chiocca EA: Diffusible cytotoxic metabolites contribute to the in vitro bystander effect associated with the cyclophosphamide/cytochrome P450 2B1 cancer gene therapy paradigm. Clin Cancer Res 1995;1:1171–1177.

366 Weise K, Kaerner HC, Glorioso J, Schroder CH: Replacement of glycoprotein B gene sequences in herpes simplex virus type 1 strain ANG by corresponding sequences of the strain KOS causes changes of plaque morphology and neuropathogenicity. J Gen Virol 1987;68:1909–1919.
367 Weissbach A, S.-Hong CL, Aucker J, Muller R: Characterization of herpes simplex virus-induced deoxyribonuclic acid polymerase. J Biol Chem 1973;248:6270–6277.
368 Weller SK, Lee KJ, Sabourin DJ, Schaffer PA: Genetic analysis of temperature-sensitive mutants which define the gene for the major herpes simplex virus type 1 DNA-binding protein. J Virol 1983;45:354–366.
369 Whiteley A, Bruun B, Minson T, Browne H: Effects of targeting herpes simplex virus type 1 gD to the endoplasmic reticulum and trans-Golgi network. J Virol 1999;73:9515–9520.
370 Whitley RJ, Kern ER, Chatterjee S, Chou J, Roizman B: Replication, establishment of latency, and induced reactivation of herpes simplex virus gamma 1 34.5 deletion mutants in rodent models. J Clin Invest 1993;91:2837–2843.
371 Wilson AC, LaMarco K, Peterson MG, Herr W: The VP16 accessory protein HCF is a family of polypeptides processed from a large precursor protein. Cell 1993;74:115–125.
372 Wittels M, Spear PG: Penetration of cells by herpes simplex virus does not require a low pH-dependent endocytic pathway. Virus Res 1991;18:271–290.
373 Wohlrab F, Francke B: Deoxyribopymidine triphosphatase activity specific for cells infected with HSV-1. Proc Natl Acad Sci USA 1980;77:1872–1876.
374 Yamada Y, Kimura H, Morishima T, Daikoku T, Maeno K, Nishiyama Y: The pathogenicity of ribonucleotide reductase-null mutants of herpes simplex virus type1 in mice. J Infect Dis 1991; 164:1091–1097.
375 Yazaki T, Manz HJ, Rabkin SD, Martuza RL: Treatment of human malignant meningiomas by G207, a replication-competent multimutated herpes simplex virus 1. Cancer Res 1995;55: 4752–4756.
376 Yoon SS, Carroll NM, Chiocca EA, Tanabe KK: Cancer gene therapy using a replication-competent herpes simplex virus type 1 vector. Ann Surg 1998;228:366–374.
377 Yoon SS, Carroll NM, Chiocca EA, Tanabe KK: Influence of p53 on herpes simplex virus type 1 vectors for cancer gene therapy. J Gastrointest Surg 1999;3:34–48.
378 Yoon SS, Nakamura H, Carroll NM, Bode BP, Chiocca EA, Tanabe KK: An oncolytic herpes simplex virus type 1 selectively destroys diffuse liver metastases from colon carcinoma. FASEB J 2000;14:301–311.
379 York IA, Roop C, Andrews DW, Riddell SR, Graham FL, Johnson DC: A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. Cell 1994;77:525–535.
380 Yoshikawa T, Hill JM, Stanberry LR, Bourne N, Kurawadwala JF, Krause PR: The characteristic site-specific reactivation phenotypes of HSV-1 and HSV-2 depend upon the latency-associated transcript region. J Exp Med 1996;184:659–664.
381 Zhan Q, Lord KA, Alamo I Jr, Hollander MC, Carrier F, Ron D, Kohn KW, Hoffman B, Liebermann DA, Fornace AJ Jr: The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. Mol Cell Biol 1994;14: 2361–2371.
382 Zweerink HJ, Corey L: Virus-specific antibodies in sera from patients with genital herpes simplex virus infection. Infect Immun 1982;37:413–421.
383 Varghese S, Newsome JT, Rabkin SD, McGeagh K, Mahoney D, Nielsen P, Todo T, Martuza RL: Preclinical safety evaluation of G207, a replication-competent herpes simplex virus type 1, inoculated intraprostatically in mice and nonhuman primates. Hum Gene Ther 2001;12:999–1010.

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dl1520 (ONYX-015) as an Antitumor Agent

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Introduction

The early region of the adenovirus genome transforms cells in tissue culture, and the gene products responsible for this effect have been well characterized. The best studied is the E1a protein, which binds to the cell cycle control protein Rb [1] and its relatives, and to the p300/CREB binding protein (CBP) [2]. By neutralizing Rb function, adenoviruses allow quiescent, differentiated cells to enter the S phase of the cell cycle and thus support efficient viral DNA replication. The precise effects of binding the transcriptional co-activator p300/CBP are not yet clear. The E1b proteins 19K and 55K suppress apoptosis during infection through bcl-2 like activity [3] and through inhibition of p53 [4], respectively. The early phase of lytic adenovirus infection is therefore characterized by abolition of a key cell cycle checkpoint (the Rb checkpoint), and suppression of apoptosis by p53-dependent and independent pathways. In these respects, the infected cell is remarkably similar to a tumor cell [5]. These cells generally lose the Rb checkpoint through mutations in Rb itself or indirectly through loss of p16 or overexpression of cyclin D1. In some tumor cells, most notably, those derived from cervical carcinoma, Rb and related proteins are neutralized by the human papillomavirus (HPV) protein E7 [6]. Tumor cells suppress p53-dependent apoptosis by mutating p53 itself, by increasing mdm-2 activity, or by expressing HPV E6, and they upregulate p53-independent anti-apoptotic pathways through overexpression of bcl-2 or components of the PI 3′ kinase pathway. In retrospect, this remarkable coincidence of functions explains why the early region should transform cells. However, it is unlikely that the biological pioneers who first conceived the use of DNA viruses as model systems

for studying the process of transformation would have expected the models to replicate the events involved in tumorigenesis so faithfully.

We have exploited this remarkable coincidence as the basis of a new strategy for killing cancer cells selectively. We have proposed that genes encoded by the adenovirus early region should be dispensable for replication in cancer cells, since the cancer cells provide the functions that these genes encode. Thus E1a should be dispensable in a cancer cell that already lacks Rb, and E1b 55K should be dispensable in tumor cells that already lack p53 [5]. Viruses lacking E1a or E1b 55K should therefore have host ranges restricted to cancer cells. These hypotheses make a number of assumptions and oversimplifications, but nevertheless they establish a potential means of achieving tumor specificity.

Biological Properties of dl1520

dl1520 lacks the E1b 55K gene, is defective for replication in certain cell types, and replicates efficiently in others as described below. The E1b 55K protein has several functions. The best characterized is its ability to bind p53, and to block its function, a function that is presumed necessary for replication in vivo. Cells that retain normal wild-type p53 are therefore expected to restrict replication of dl1520. Cells lacking p53 are expected to be permissive. This proposed selectivity suggested a role for dl1520 as an anticancer agent, since tumor cells generally lack functional p53 [7].

E1b 55K binds to p53 at a site that overlaps with the binding site of mdm-2, and this binding is thought to block transcriptional activation by p53 [8–10]. E1b 55K can also export p53 to the cytoplasm through a leptomycin-sensitive export system [11]. Efficient degradation of p53 depends on a second viral protein, E4orf6, that binds to the E1b 55K/p53 complex, but whose precise function remains unclear [12–15].

E1b 55K has additional functions that appear at first sight to be unrelated to p53 binding. Mutants in E1b 55K are deficient in selective export of viral late mRNAs and late viral protein synthesis is greatly reduced [16–18] E1b 55K and E4orf6 may interact directly with late viral mRNAs and export them through nuclear pores: it seems likely that this export function is related to export of p53 by the same complex, but this relationship is not clear. Unfortunately, much of the analysis of E1b 55K function has been performed in HeLa cells whose p53 status is hard to define. In uninfected HeLa cells, p53 is degraded by HPV E6, but E1a can induce p53, presumably by overwhelming HPV E6, so that p53 function is restored during infection. The level of functional p53 during infection of HeLa cells probably varies with time and multiplicity of infection, so that the precise state of p53 is impossible to measure in these experiments.

Construction of dl1520

dl1520 was created by Barker and Berk [19] in 1987 from plasmid pm2022 (derived from Ad2) which introduces a stop codon at amino acid 3 of the 495-amino acid E1b 55K protein (496 in Ad5), with an additional deletion of sequences between the *Pst*I site at base 2496 and the *Bgl*II site at 3323. This plasmid was cotransfected with dl309 (Ad5 derived) cleaved at *Xba*I and mutant viruses were the result of successful recombination. dl1502 is therefore a hybrid between Ad2 and Ad5. As a result of the construction of this hybrid, gene expression from the E3b region, which encodes the RID and 14.7-kD E3 proteins, is also disrupted.

Selectivity for Cancer Cells

Replication in Normal Cells

Expression of E1a leads to induction of p53 through a pathway shown in figure 1. E1a binds the Rb protein, releases E2F and this transcription factor turns on expression of p14ARF, a protein that inhibits mdm-2 [20]. This allows accumulation of p53, and subsequent activation of p53-responsive genes. These genes can, in principle, cause cell cycle arrest or apoptosis, and are therefore likely to restrict viral replication. Neutralization of p53 by E1b 55K is thought to prevent these inhibitory effects and allow efficient replication. However, the precise effects of inducing p53 during infection of normal human cells by dl1520, or related viruses, have not been measured. We must therefore consider the possibility that other effects of p53 that have not yet been described, could also affect virus replication and explain the need to neutralize p53 during lytic infection by wild-type virus. Nevertheless, p53 is indeed induced during infection of normal cells with dl1520 and similar adenovirus mutants [21], and it appears likely, though not formally proven, that this attenuates replication of this virus.

Table 1 summarizes published data regarding replication of dl1520 in normal human cells. Relative to wild-type adenovirus, dl1520 is attenuated for replication in many primary cells growing in tissue culture. The basis of this attenuation cannot be determined from these correlative data, but it is reasonable to assume that p53 plays a role, since p53 is likely to be induced by E1a expression in these cell types. Some caveats need to be considered. First, each of the references cited in table 1 compare dl1520 with wild-type adenovirus replication, either Ad2 or Ad5. However, dl1520 is a hybrid between Ad2 and Ad5, and contains a deletion in the E3b region in addition to the E1b 55K gene, as described above. While it is commonly assumed that the E3 region plays no role in replication in cell culture, this may not be the case in the cells

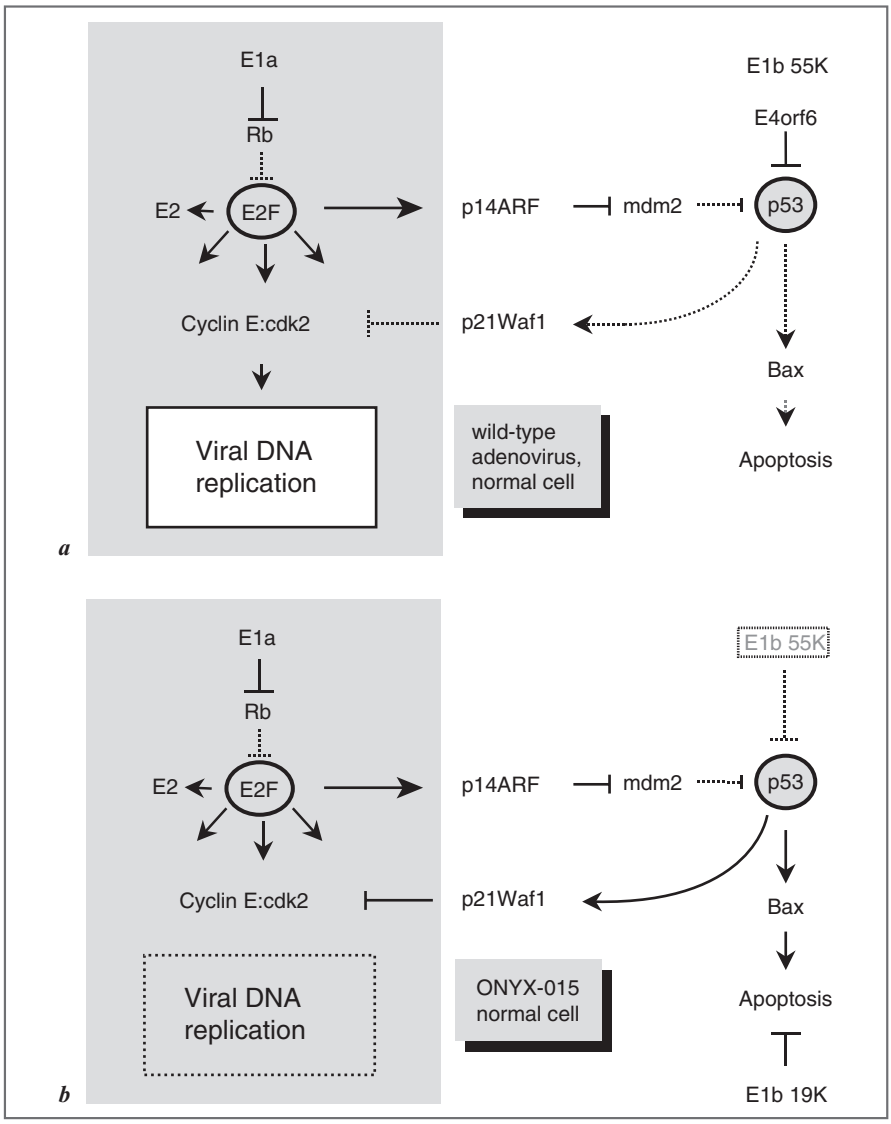

Fig. 1. (***a***) Pathways leading from E1a to p53 in normal cells infected with wild type adenovirus. E1a binds to Rb and related proteins, liberating E2F. This transcription factor activates expression of cellular genes involved in DNA synthesis, as well as the adenovirus E2 region. E2F also activates p14ARF, and inhibitor of mdm-2. In the absence of mdm-2, p53 accumulates. This accumulation is blocked by E1b 55K and E4orf6. (***b***) In normal cells infected with dl1520/ONYX-015, p53 accumulates but cannot be degraded efficiently because E1b 55K is not expressed. Downstream targets of p53, such as p21 and bax can now attenuate replication.

Table 1. Replication of dl1520 in normal cell lines, expressed as percent wild-type virus production per cell during a single burst of virus production

Cell type	% wt	Reference no.
IMR90	25	18
HNK	36	18
WI38	3.6	18
Fibroblasts	2–5	29
MEC	3	29
Fibroblasts	5	30
HUVECs	1	O'Shea and McCormick (unpubl. observ.)

IMR90 and WI38 are human diploid fibroblasts. HNK = Human neonatal kidney cells; MEC = mammary epithelial cells; HUVECs = Passage 2 human vascular endothelial cells.

described here. For example, E3 RID proteins could affect expression of growth factor receptors that are necessary for survival during infection. Furthermore, the deleted genome may replicate inefficiently due to structural features relating to replication and packaging, rather than specific gene expression. The correct comparison is a chimeric virus created by Barker and Berk [19] that is identical to dl1520 except that it expresses E1b 55K: it is referred to as AdD. Unfortunately this virus has not been used in any of the studies in which dl1520 is compared to wild-type virus.

Another caveat that needs to be considered is that primary cells in culture lose expression of p14ARF, a protein that regulates p53 through inhibition of mdm-2 activity. As a result, 'normal' cells may be defective in their ability to induce p53 during viral infection. It is therefore possible that certain primary cell lines that are relatively permissive for dl1520 may be defective in p53 induction. The connection between E2F, p14ARF and p53 has only recently been described, but demands a reexamination of some of the data summarized in table 1. Related to this caveat, it should be considered that human cells that grow rapidly in culture are the exception, not the rule, and the types of cell that are likely to be exposed to infection during clinical application of dl1520 may not be represented by cells like IMR-90 fibroblasts.

Despite these caveats, the possibility certainly exists that some normal cells in vivo may be susceptible to lytic productive replication by dl1520: no existing animal model can predict this possibility accurately. Clinical investigation of dl1520/ONYX-015 must clearly take this possibility into consideration.

The attenuation of dl1520 replication that we have observed in growth arrested, low passage epithelial cells (table 1) correlates with dramatic induction

Table 2. Replication of dl1520 in tumor cells with mutant p53 (expressed as in table 1)

Cell type	Mutation	% wt	Reference no.
C33A	R273C	50–100	7, 18, 29
H1299	null	15–40	18, 29, 30
Hep3B	null	14	18
OVCAR-3	R248Q	10	18
HacaT	H179R	10	30
SK-OV-3	del	6.3	18
Saos-2	null	5	18
JW2	null	5	30
PC-3	insertion	4.8	18
HT-29	R273H	2	30
U373	R273H	1	29

of p53, but may also be due to loss of other E1b 55K functions that are necessary for efficient replication, as discussed above. We are currently attempting to assess the contribution of p53 toward the attenuation of dl1520 replication in primary cells.

Replication of dl1520 in Tumor Cells with Mutant p53

Table 2 summarizes published data on dl1520 replication in tumor cells that express mutant p53, or fail to express p53 entirely. In C33a cells, replication is as efficient as wild-type virus, showing that all E1b 55k functions are dispensable in these cells. At the other extreme, JW2 cells fail to support replication efficiently even though p53 is not expressed. Presumably other functions of E1b 55K are essential in these cells. In cells expressing mutant p53, it is generally assumed that this protein is functionally inactive. Presumably, during the evolution of the tumor, a mutation occurred that inhibits p53 function sufficiently to overcome p53-dependent growth arrest or apoptosis. However, infection of such tumor cells with adenovirus results in p53 induction. Levels of p53 protein may now be achieved that have a biological function. Of particular note, the p53 mutation R273H that occurs in U373 and HT-29 has been shown to be a weak mutation that only impairs p53 function by a factor of 10 [22]. This may be sufficient to suppress p53 function during tumor development, but may not be sufficient during infection with dl1520. By analogy, E1a can overcome the effects of HPV E6 in HeLa cells [23], as described below. It is therefore insufficient to conclude that a cell line such as U373 and HT-29 is indeed p53-defective during the process of infection with dl1520.

Table 3. Replication of dl1520 in tumor cells with wild-type p53

Cell type	% wt	Reference no.
RKO	30–50	18, 29
A549	24–40	18, 29, 30
HCT116	10–50	30, Ries et al. (unpubl. observ.)
U87	28	29
U20S	1.2	5, 18, 29

Replication of dl1520 in Tumor Cells with Wild-Type p53

Table 3 shows that dl1520 replicates with varying degrees of efficiency in tumor cells with wild-type p53. As discussed by Harada and Berk [18] the decreased replication in these tumor cells relative to wild type is due to p53-dependent and independent mechanisms. We have recently investigated this issue in a model system using HCT116 colorectal cells. These express wild-type p53, but fail to express p14. We chose these cells because an isogenic derivative, in which the p53 gene was knocked out by homologous recombination, has been made available by Waldman et al. [24]. In $p53^{+/+}$ or $p53^{-/-}$ cells, dl1520 replicates at about 20–30% wild-type efficiency. This shows clearly that the attenuation of replication is due to p53-independent effects of E1b 55K. When p14ARF is expressed in $p53^{+/+}$ cells, p53 levels increase and dl1520 replication is selectively inhibited (Korn et al., submitted for publication), but in $p53^{-/-}$ cells, p14ARF has no effect. We conclude that loss of p14ARF facilitates efficient replication of dl1520 in these cells by suppressing p53 induction. However, complete loss of p53 does not restore full replication because dl1520 still lacks functions necessary for wild-type levels of replication.

Pre-Clinical Testing of dl1520

dl1520 was injected directly into human tumors grown as subcutaneous xenografts in nude mice [7, 25] or by intravenous injection of mice bearing these xenografts [26]. In the former case, complete destruction of tumor was seen in about 60% of injected tumors; in the latter case, complete responses were seen when virus was administered along with chemotherapy agents, even though virus replication within the distant tumor could be detected. Biopsy of tumors treated by either route revealed progressive spread of virus from throughout the tumor, suggesting that replication and infection of neighboring cells was responsible for tumor killing. The ability of dl1520 to destroy human

xenografts was enhanced by combination with chemotherapy agents such as 5-fluorouracil (5-FU) and cisplatin, for reasons that are not yet entirely clear [26]. One possible explanation is that host immune effector cells produce cytokines in response to infection, and these cytokines, such as TNF-α, kill uninfected tumor cells in the presence of chemotherapy agents. Other effects of these agents might also increase efficacy. For example, increases in vascular permeability or tumor architecture could increase viral spread during infection. It is also possible that the combined effects of virus and chemotherapy seen in these models, simply reflect the fractional killing of both regimes separately. However, the combination appears to be synergistic rather than additive, suggesting a more complex interaction, but this clearly merits further investigation.

Clinical Testing of dl1520, ONYX-015

dl1520 entered a phase-I trial for patients suffering from recurrent squamous cell carcinoma of the head and neck in April 1996. These patients had failed surgery, radiation and chemotherapy, and initially were selected based on the presence of mutant p53. A dose escalation was performed, from 10^7 to 10^{11} pfu of virus, purified from 293 cells, injected directly into tumors. In this study, no dose-limiting toxicity was reported, and it was concluded that dl1520 is safe.

While these studies were in progress, it was discovered that dl1520 is more effective in combination with approved chemotherapy protocols, at least in mouse xenograft studies [26], as discussed above. A phase-II study was therefore conducted in which 30 patients who had failed prior surgery or radiation therapy were treated by injection of 5 doses of virus at 10^{10} per dose, and tumor size was measured 4 weeks after injection. In this study, 8/30 showed a complete response (all injected tumor mass disappeared), and a further 11/30 showed more than 50% tumor reduction. The overall response rate was therefore 19/30 (63%), which compares favorably with historical rates of about 35% for this disease, with less than 10% expected complete responses [27]. A phase-III study is being planned based on these encouraging data. Other phase-I/II studies currently ongoing include treatment of oral leukoplakia, Barrett's esophageal cancer, pancreatic cancer, ovarian cancer and metastatic colorectal cancer. The latter study is of particular interest because it involves infusion of virus into the blood stream via the hepatic artery. To date, no dose-limiting toxicity has been reported, even after infusion of 10^{12} pfu virus, and patients are being monitored for signs of biological efficacy.

In conclusion, dl1520/ONYX-015 virus has been shown to be very well tolerated, and encouraging signs of efficacy have been reported. Based on these

data, it seems likely that dl1520/ONYX-015 will be approved for use as an anticancer agent. In the future, we anticipate that dl1520 will be the basis of another generation of viruses that replicate selectively in tumor cells, and also deliver genes selectively to tumor cells to further enhance biological efficacy. Indeed, the potential benefit of combining the suicide gene herpes simplex virus thymidine kinase into the dl1502 backbone has already been documented [28]. We also anticipate developments in viral delivery that might allow efficient killing of metastatic tumors, and that together these advances could provide a new approach towards safe and effective treatment of major forms of human cancer.

References

1 Dyson N, Bernards R, Friend SH, Gooding LR, Hassell JA, Major EO, Pipas JM, Vandyke T, Harlow E: Large T antigens of many polyomaviruses are able to form complexes with the retinoblastoma protein. J Virol 1990;64:1353–1356.

2 Lill NL, Grossman SR, Ginsberg D, DeCaprio J, Livingston DM: Binding and modulation of p53 by p300/CBP coactivators. Nature 1997;387:823–827.

3 Chiou SK, Rao L, White E: Bcl-2 blocks p53-dependent apoptosis (published erratum appears in Mol Cell Biol 1994;14:4333). Mol Cell Biol 1994;14:2556–2563.

4 White E, Chiou SK, Rao L, Sabbatini P, Lin HJ: Control of p53-dependent apoptosis by E1B, Bcl-2, and Ha-ras proteins. Cold Spring Harb Symp Quant Biol 1994;59:395–402.

5 McCormick F: Cancer therapy based on p53. Cancer J Sci Am 1999;5:139–144.

6 Sherr CJ: Cancer cell cycles. Science 1996;274:1672–1677.

7 Bischoff JR, Kirn DH, Williams A, Heise C, Horn S, Muna M, Ng L, Nye JA, Sampson-Johannes A, Fattaey A, McCormick F: An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science 1996;274:373–376.

8 Yew PR, Liu X, Berk AJ: Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. Genes Dev 1994;8:190–202.

9 Martin ME, Berk AJ: Adenovirus E1B 55K represses p53 activation in vitro. J Virol 1998; 72:3146–3154.

10 Yew PR, Berk AJ: Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. Nature 1992;357:82–85.

11 Dobbelstein M, Roth J, Kimberly WT, Levine AJ, Shenk T: Nuclear export of the E1B 55-kDa and E4 34-kDa adenoviral oncoproteins mediated by a rev-like signal sequence. EMBO J 1997;16: 4276–4284.

12 Roth J, Konig C, Wienzek S, Weigel S, Ristea S, Dobbelstein M: Inactivation of p53 but not p73 by adenovirus type 5 E1B 55-kilodalton and E4 34-kilodalton oncoproteins. J Virol 1998;72: 8510–8516.

13 Steegenga WT, Riteco N, Bos JL: Infectivity and expression of the early adenovirus proteins are important regulators of wild-type and DeltaE1B adenovirus replication in human cells. Oncogene 1999;18:5032–5043.

14 Steegenga WT, Shvarts A, Riteco N, Bos JL, Jochemsen AG: Distinct regulation of p53 and p73 activity by adenovirus E1A, E1B, and E4orf6 proteins. Mol Cell Biol 1999;19:3885–3894.

15 Steegenga WT, Riteco N, Jochemsen AG, Fallaux FJ, Bos JL: The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells. Oncogene 1998;16:349–357.

16 Babiss LE, Ginsberg HS, Darnell JE Jr: Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport. Mol Cell Biol 1985;5: 2552–2558.

17 Leppard KN, Shenk T: The adenovirus E1B 55 kd protein influences mRNA transport via an intranuclear effect on RNA metabolism. EMBO J 1989;8:2329–2336.
18 Harada JN, Berk AJ: p53-Independent and -dependent requirements for E1B-55K in adenovirus type 5 replication. J Virol 1999;73:5333–5344.
19 Barker DD, Berk AJ: Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection (published erratum appears in Virology 1987;158:263). Virology 1987;156:107–121.
20 Oren M: Regulation of the p53 tumor suppressor protein. J Biol Chem 1999;274:36031–36034.
21 Grand RJ, Owen D, Rookes SM, Gallimore PH: Control of p53 expression by adenovirus 12 early region 1A and early region 1B 54K proteins. Virology 1996;218:23–34.
22 Stahler F, Roemer K: Mutant p53 can provoke apoptosis in p53-deficient Hep3B cells with delayed kinetics relative to wild-type p53. Oncogene 1998;17:3507–3512.
23 Querido E, Marcellus RC, Lai A, Charbonneau R, Teodoro JG, Ketner G, Branton PE: Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. J Virol 1997;71:3788–3798.
24 Waldman T, Kinzler KW, Vogelstein B: p21 is necessary for the p53-mediated G1 arrest in human cancer cells. Cancer Res 1995;55:5187–5190.
25 Heise CC, Williams A, Olesch J, Kirn DH: Efficacy of a replication-competent adenovirus (ONYX-015) following intratumoral injection: Intratumoral spread and distribution effects. Cancer Gene Ther 1999;6:499–504.
26 Heise CC, Williams AM, Xue S, Propst M, Kirn DH: Intravenous administration of ONYX-015, a selectively replicating adenovirus, induces antitumoral efficacy. Cancer Res 1999;59:2623–2628.
27 Kirn D, Hermiston T, McCormick F: ONYX-015: Clinical data are encouraging. Nat Med 1998;4:1341–1342.
28 Wildner O, Morris JC, Vahanian NN, Ford H Jr, Ramsey WJ, Blaese RM: Adenoviral vectors capable of replication improve the efficacy of HSVtk/GCV suicide gene therapy of cancer. Gene Ther 1999;6:57–62.
29 Rothmann T, Hengstermann A, Whitaker NJ, Scheffner M, zur Hausen H: Replication of ONYX-015, a potential anticancer adenovirus is independent of p53 status in tumor cells. J Virol 1998;72:9470–9478.
30 Turnell AS, Grand RJ, Gallimore PH: The replicative capacities of large E1B-null group A and group C adenoviruses are independent of host cell p53 status. J Virol 1999;73:2074–2083.

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Attenuated Replication-Competent Adenoviruses (ARCA©) for Prostate Cancer: CV706 to CV787

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Introduction

The specificity, or therapeutic index, of anticancer chemotherapeutic agents has long been problematic. The majority of cancer chemotherapeutic agents, such as alkylating agents, antimetabolites, antibiotics, plant alkaloids, and other cytotoxic agents, nonspecifically injure or kill dividing cells [1]. These agents are noted for their poor specificity and low therapeutic ratio of toxicity towards target cancer cells compared to normal cells (e.g. therapeutic ratios of 2:1 to 6:1). In some instances, hormonal anticancer agents offer improved specificities [2], and the few biologic response modifiers [3], particularly humanized monoclonal antibodies, also offer greater anticancer specificity. However, cytotoxic agents remain the mainstay of cancer chemotherapy. The unwanted toxicity problems, most notably the myeloid stem cell suppression characteristic of these cytotoxic drugs, are so great that drugs designed to recover patients from the side effects of cytotoxic anticancer agents such as G-CSF, GM-CSF and erythopoietin [4] represent as significant a commercial market as the cytotoxic chemotherapy agents themselves (M. Simons, personal commun.).

Intense efforts to increase specific cancer cell cytotoxicity of new anticancer agents have frustrated researchers for decades and continue today. One such effort is gene therapy [5, 6]. In experimental models of gene therapy using replication defective adenoviruses, the use of prodrug converting enzymes such as herpes simplex virus thymidine kinase [7–11] and cytosine deaminase [10] under the control of transcriptional response elements (TREs) has shown

anticancer activity with significantly increased specificity. However, to destroy a solid tumor, replication-defective adenoviruses must deliver a therapeutic gene and precipitate a significant bystander effect, all before the host-immune response to the adenovirus coat proteins limits further treatment. The result is that despite the 61 (NIH ORDA October 1999) human clinical trials using replication-defective adenoviruses to treat cancer, to date none of these therapies have shown medical utility, progressed to phase-III trials, or become commercially successful. Unfortunately, even when gene transfer was successful, gene expression has been inadequate or too short-lived. The limiting issue has been inadequate efficacy.

To address some of these shortcomings we have focused on modifying the specificity and efficacy of replicating adenoviruses. We have tried to design therapeutics with sufficient specificity and efficacy so that the short-term expression of adenovirus will be successful in killing enough target cancer cells to be medically useful. Physically, replicating adenoviruses can infect a broad range of human cells and produce infectious progeny that could attack adjacent tumor cells leading to destruction of a solid tumor within a short period of time from a single virus treatment. In human patients, replicating adenoviruses would be expected to induce a strong cytotoxic T-cell response that could help eliminate productively infected cells.

Replicating viruses have been proposed to treat cancer for nearly a century [12]. The first sustained attempts to treat tumors in animal models occurred in the late 1940s and early 1950s when infections were induced with viruses such as avian pest, Russian Far East encephalitis, St. Louis encephalitis, Coxsackie, foot and mouth, Herpes simplex, influenzae, West Nile, dengue, Newcastle disease, vaccinia, and rabies [13]. A significant early attempt was made to explore the cell-killing properties of replicating cytolytic adenoviruses for the treatment of cancer in humans. The first isolates of adenovirus were shown to grow ‘luxuriantly’ on HeLa cells, cells originally derived from cervical cancer. It was proposed that perhaps adenoviruses would preferentially replicate in and destroy cervical cancers. In 1956 Smith et al. [14] tested ten different wild-type adenovirus serotypes, including adenovirus type 5 (Ad5), as a treatment for locally advanced cervical carcinoma. Virus was administered via intratumoral injection or intra-arterial injection. The virus stocks used were unpurified lysates of tissue culture cells; the number of infectious viral particles (plaque-forming units, pfu) and the total number of virus particles in the injected dose were not determined. Although long-term clinical benefit was not achieved, tumor necrosis and cavity formation was observed in 65% of treated patients, and these effects were limited to the carcinoma tissue. Side effects, detected primarily in patients receiving immunosuppression with cortisone, included febrile illness and malaise; in all cases, the symptoms resolved in 7–9 days.

Infectious virus was not recovered from any biopsy specimens or vaginal smears, but the titers of neutralizing antibodies were uniformly elevated by 5–7 days after injection [14]. This study is significant, for it illustrates the promise and limitations of oncolytic adenoviruses while describing the limited toxicity to be expected of replicating adenoviruses that do not contain transgenes encoding foreign proteins at these intermediate dose levels. However, adenoviruses were subsequently shown to replicate in many cell types and lacked the hoped-for preference for cervical cancer cells.

A resurgence of interest in replicating adenoviruses has occurred in the past decade due to the ability to genetically manipulate viruses. In 1996 Bischoff et al. [15] introduced the use of Ad5 deleted in the E1B 55-kD protein so that the virus (ONYX-015 = dl1520) preferentially replicates in $p53^-$ cells as compared to $p53^+$ cells by a factor of 100-fold. However, the mechanism of antitumor specificity of the ONYX-015 virus has come under criticism [16–19]. Also in 1996, Zhang et al. [20] showed that replicating wild-type adenovirus could eliminate tumor xenografts in nude mice. In 1997, Rodriguez et al. [21] introduced transcriptional targeting of adenovirus using the enhancer/promoter of the human prostate-specific antigen (PSA) gene to drive the Ad5 E1A gene.

We have focused on the use of TREs to control the expression of virus genes required for virus replication [21–23]. To test this idea we initially chose prostate cancer and the regulatory enhancer and promoter elements (prostate-specific enhancer, PSE) of PSA. PSA is the most widely used serum marker for the diagnosis and management of any form of cancer. It is produced in prostate cancer cells, and normal prostate ductal epithelia (which represents less than 5% of the cells of the prostate); it is also produced in much smaller amounts in the periurethral glands, and very rarely in tumors of the skin, salivary glands, and breast [24]. Since the prostate is an accessory organ, removal or ablation of the entire gland has no serious health repercussions [25–28]. Thus, the regulatory regions of the PSA gene are a reasonable choice for such an approach.

We reasoned that placing adenovirus genes under the control of the PSE would create a virus in which replication would be restricted primarily to PSA-producing (PSA^+) ductal epithelial cells within the prostate, and PSA^+ prostate cancer (PCA) cells. We describe CV706 (PSE driving the Ad5 E1A genes and deleted in the Ad5 E3 region) which is currently in clinical trials at the Brady Urological Institute of the Johns Hopkins Oncology Center under the direction of Jonathan Simons, MD, and Ted DeWeese, MD. We refer to the genetically engineered viruses using TREs as attenuated replication-competent adenoviruses (ARCA©). Since taking CV706 to clinic, we have focused on improving the specificity and efficacy of the ARCA© platform. Below we describe additional prostate-specific viruses on the developmental pathway from CV706

leading to CV787 (probasin, PB, driving the E1A gene, PSE driving the E1B gene and reintroduction of the Ad5 E3 region).

Adenovirus: Gene Expression and Regulation

Members of the Adenoviridae family of human adenoviruses were first cultured from the tonsils and adenoids of children in 1953 [29]. They represent 47 different serotypes which are distinguishable by antibody reactivity to epitopes on the virion surface. Each serotype is assigned to one of five subgroups (A–E). Members of a subgroup can exchange genetic material (recombine) efficiently, but they do not recombine with members of a different subgroup. Adenovirus types 1, 2, 5, and 6 are members of subgroup C. Ad5 is associated with a self-limiting, febrile respiratory illness and ocular disease in humans; infectious virus can be recovered from the throat, sputum, urine, and rectum. Ad5 is also associated with renal impairment, hepatic necrosis, and gastric erosions in immunosuppressed individuals [30, 31]. Ad5 and the other subgroup C viruses have little or no oncogenic potential in mammals [32]. A recent serological survey indicates that 57% of the adult population in the US has neutralizing antibodies to Ad5 with titers ranging from 1 : 2 to 1 : 512 [33].

The Ad5 genome is a double-stranded DNA molecule of 35,935 base pairs [34] containing short inverted terminal repeats [35]. Expression of the genome is a regulated cascade which is arbitrarily divided into early (E) and late (L) phases, with viral DNA replication required for maximal L gene expression (fig. 1). Related RNA transcripts are grouped according to the region of the genome from which they are transcribed as well as by the timing (E or L) of their expression. Viral gene expression is regulated at the levels of transcription, post-transcriptional modification (splicing), translation, and post-translational modification.

Products of the E1 region are essential for efficient expression of the other regions of the adenovirus genome. The E1A transcription unit is the first adenovirus sequence to be expressed during viral infection. E1A plays a role in a number of important biological functions in adenovirus-infected cells. E1A proteins are capable of immortalizing primary cells and of cooperating with E1B or H-ras to induce complete transformation [36] and are also involved in the regulation of gene expression. The E1A gene products can induce transcriptional activation of E1A and other early viral promoters, as well as of a number of cellular promoters [37]. The E1A region encodes at least six polypeptides [32]. The 289-amino acid E1A (289E1A) product is a multifunctional phosphoprotein that transactivates both viral and cellular genes [38–40]. The 289E1A interacts with both viral and host proteins including components of the cellular

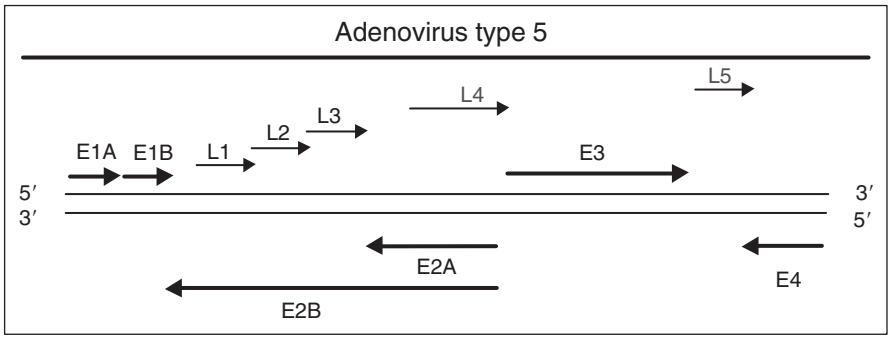

Fig. 1. Transcription map of adenovirus type 5. In the main, transcription of the early (E) genes is separated from transcription of the late (L) genes by the onset of virus DNA replication. E1A is the only gene transcribed during the first 2.5 h of infection.

transcription apparatus. The 289E1A protein can also induce host DNA synthesis in quiescent cells by two independent mechanisms [41–43]. The 243-amino acid E1A (243E1A) causes increased expression of the host cellular p53 protein and apoptosis (see below). The E1B region encodes three proteins with diverse functions [44] that modulate the activity of the 289E1A [45], regulate the levels of the host cellular p53 protein [46], block the tumor necrosis factor cytolysis of infected cells [47, 48], regulate viral E2, E3, and E4 promoters, and transactivate cellular promoters such as that for the heat-shock protein hsp70 [49, 50].

The E2 region encodes several proteins that are required for viral DNA replication. These include a DNA-binding protein [51], the viral DNA-dependent DNA polymerase, and the DNA terminal protein that are required for DNA replication [52–54].

The E3 region is not essential for replication in tissue culture and this region is deleted from most first-generation therapeutic adenoviruses [55, 56]. Proteins encoded by the E3 region modulate host-immune responses to infection by inhibiting transport of the major histocompatibility complex (MHC) class I protein to the cell surface, thereby impairing the cytotoxic T lymphocyte (CTL) response [57–59], and by blocking TNFα-induced cytolysis of infected cells [47, 48]. This defect in E3-deficient viruses may contribute to rapid loss of transgene expression in vivo in trials using first-generation therapeutic adenoviruses. The E3 region also encodes the adenovirus death protein. Although not essential in tissue culture, the death protein enhances cell killing and the release of progeny virions from the infected cells [60, 61].

Six transcripts of the E4 region have been identified. Some of the encoded proteins interact with and/or modulate the activity of E1 region proteins. For example, E4orf4 indirectly affects phosphorylation of 289E1A [62], and

E4orf6 interacts with the E1B 55-kD protein to modulate the level of cellular p53 protein [46].

The onset of viral DNA replication signals the switch from E to L gene expression. Although the precise mechanisms are not fully understood, this transition requires both *cis*- and *trans*-acting factors [63–65]. Late genes primarily encode the structural components of the virion and the nonstructural scaffolding proteins that are essential for the assembly of infectious virus. It is estimated that up to 10,000 adenovirus virions are accumulated per cell and most remain cell-associated [66]. The entire adenovirus replication cycle is completed in approximately 32–36 h [67].

All early regions except E3 are essential for adenovirus propagation. The virus replication can then be restricted to a cell type when one or two of these genes are under the control of tissue-specific genes, or transcriptional regulatory elements. The cytotoxicity associated with virus replication could also be limited to a certain type of cell.

Specificity: Attenuated Replication-Competent Adenovirus

We hypothesized that tropism of a virus could be redirected if expression of an essential viral gene could be controlled. Viruses generated from this approach would have the same capsid as its parental virus and they should be able to penetrate all cell types that express the CAR receptor [68, 69]. Presumably, in all cells containing the CAR receptor, these viruses would follow the normal cell entry process: they would penetrate the endosome, fuse with the endosome membrane, reach the cytoplasm, find transport to the nucleus, and uncoat the viral DNA. In a normal adenovirus replication cycle the E1A gene is the only gene expressed during the first 2.5 h of infection [37, 40, 70, 71]. In turn, the E1A proteins as transcription factors upregulate expression of the impending cascade of viral genes. However, we have genetically engineered prostate tissue-specific promoters and enhancers so as to drive the E1A genes. Thus, the E1A proteins should be preferentially expressed in prostate cells. Additionally, viral replication should preferentially take place in cells that express transcription factors, thus enabling activation of the tissue or tumor-specific transcription regulatory elements, prostate cells.

There are several criteria important in regard to the TRE necessary for the successful engineering of a therapeutic adenovirus: (1) the tissue-specific regulatory specificity should be tightly regulated; (2) the TRE should regulate the initiation of transcription of the adjacent gene; (3) transcription should be limited to tumor cells, or accessory cells with as few other sites of expression as medically tolerable, and (4) the promoter should be strong enough to drive

Table 1. ARCA©s for prostate cancer

Virus	E1A driven by	E1B driven by	E3 region	E4 driven by	Targeting cell
CV702	wt	wt	Deleted	wt	N/A
CV706	PSE	wt	Deleted	wt	Prostate cancer
CV711	wt	PSA	Deleted	wt	Prostate cancer
CV716	PSE	PSE	Deleted	wt	Prostate cancer
CV737	PB	wt	Deleted	wt	Prostate cancer
CV738	wt	PB	Deleted	wt	Prostate cancer
CV739	PB	PSE	Deleted	wt	Prostate cancer
CV740	PB	PB	Deleted	wt	Prostate cancer
CV757	wt	wt	Deleted	PSE	Prostate cancer
CV763	HK2	wt	Deleted	wt	Prostate cancer
CV764	PSE	HK2	Deleted	wt	Prostate cancer
CV787	PB	PSE	Full-length	wt	Prostate cancer
CV802	wt	wt	Full-length	wt	N/A

sufficient expression of essential viral genes. We chose prostate cancer and the TREs of PSA as our initial target.

Expression of the PSA gene is modulated by the PSE element that is located several thousand nucleotides upstream of the PSA promoter [72]. When fused to a fragment (position −230 to +7, relative to the start of transcription) containing the PSA promoter, the PSE (position −5322 to −3875, relative to the start of transcription) confers tissue-specific expression on a reporter gene such as that for chloramphenicol acetyltransferase (CAT) [72]. Sequence analysis of the PSE reveals the presence of regions with homology to steroid-response elements and to binding sites for several cellular transcription factors including c-Fos and AP-1 [23, 72, 73]. A functional androgen-response element within the PSE increases expression up to 100-fold in the presence of testosterone or the non-metabolized testosterone analog R1881.

Viruses Containing One Prostate-Specific Transcriptional Response Element

To test the feasibility of the ARCA© technology, we engineered the PSE fragment into the adenovirus genome and generated a first-generation virus, CV706. CV706 contains the PSE fragment (PSA promoter and enhancer) inserted immediately upstream of the E1A region and transcription of the E1A region is regulated by the PSE. The viruses described in this chapter are summarized in table 1. Virus characterization showed that CV706 was able to efficiently replicate in PSA^+ prostate carcinoma cell lines but not in the other

PSA^- human cell lines HBL-100, MCF-7, PANC-1, and OVCAR-3. CV706 also does not replicate efficiently in DU-145, a prostate cancer cell line which does not express PSA and does not contain the androgen receptor [21]. Further study indicated that the transcription of the E1A mRNA was regulated by the PSE. E1A mRNA was detectable in PSA^+ LNCaP cells, but was not detectable in PSA^- cells (data not shown). E1A protein was also reduced by 99% in PSA^- cells, compared to that in the PSA^+ LNCaP cells [21]. This indicates that the inserted PSE has successfully controlled expression of the E1A gene and the host range of this adenovirus mutant has been confined to a particular cell type.

We also showed that the tropism of adenovirus could be changed when the E1B gene or the E4 gene was placed under the control of the PSE TRE. CV711, whose E1B gene is placed under the control of PSE, and CV757, whose E4 genes are driven by PSE, both replicate similarly to wild-type adenovirus in PSA^+ cells but are highly attenuated in PSA^- cells. Cell specificity of CV711 viruses is similar to CV706 and replicates similarly to wild-type virus in PSA^+ cells. In contrast, CV757, a virus that contains the PSE driving the E4 region, shows significantly greater specificity for PSA^+ cells but suffers a significant reduction in the ability to replicate (data not shown). Thus, adenovirus mutants can be generated to target PSA^+ cells when any one of the E1A, E1B, of E4 genes are driven by the PSE.

This observation has been confirmed with other prostate-specific TREs. The rat PB gene is developmentally regulated in the prostate by androgens. Induction of the rat PB gene by androgens was shown to involve the participation of two different *cis*-acting DNA elements that bind the androgen receptor. An expression cassette carrying 426 bp of the PB gene promoter and 28 bp of 5′-untranslated region was found to be sufficient to target expression of a bacterial CAT reporter gene specifically to the prostate epithelium [74]. It was also shown that the same 5′-flanking region of PB gene promoter fragment fused to the SV40 Tag gene could lead to the development of progressive forms of prostate disease that histologically resemble human prostate cancer in transgenic animals [74]. The promoter of the rat PB gene was engineered into adenovirus to drive the expression of either the E1A gene or the E1B gene to generate CV737 and CV738, respectively. Both CV737 and CV738 showed significant specificity to PSA^+prostate carcinoma cells.

We also recently cloned the TRE of the hK2 gene. The hK2 gene is located 12 kb downstream from the PSA gene in a head-to-tail fashion, whereas the hK1 gene is located 30 kb upstream of the PSA gene in head-to-head fashion [75]. The PSA and hK2 gene share DNA (80%) and amino acid (78%) sequence homologies that suggests that they evolved by gene duplication from the same ancestral gene [76, 77]. Interestingly, the hK2 protein was recently shown to be

expressed in every prostate cancer, and the expression of hK2 protein incrementally increased from benign epithelium, to high-grade prostatic intraepithelial neoplasia, to adenocarcinoma. We recently described CV763 containing the hK2 promoter and enhancer driving the Ad5 E1 gene. CV763 behaved identically to CV706 [23].

Thus, the replication of adenovirus can be restricted to prostate cancer cells when one of the essential adenovirus genes E1A, E1B, or E4 is placed under the control of any one of three different prostate-specific TREs.

Viruses Containing Two Prostate-Specific Transcriptional Response Elements

Since both the E1A and E1B genes are essential for adenovirus replication, we reasoned that it was possible to create a virus with significantly higher specificity if both the E1A and E1B genes were under independent control of two TREs. To test this hypothesis, we generated an adenovirus mutant CV716, in which both the E1A gene and the E1B gene were under the control of PSE. In vitro study showed that CV716 replicated well in the PSA-producing prostate cancer cells. However, replication of CV716 was highly attenuated in nonprostate human cell lines. Compared to CV706, the efficiency of CV716 replication in nonprostate cancer cells has been further reduced by more than 100-fold giving a specificity for PSA^+ cells compared to PSA^- cells of nearly 10,000 : 1 (data not shown). The high degree of specificity for PSA^+ cells of CV716 as compared to PSA^- cells was found to be universally true [22, 23]. CV740, containing duplicate copies of the rat PB promoter, also showed this high level of specificity. However, CV716 and CV740 are genetically unstable resulting in self-inactivation of the virus. The E1A gene and one copy of the tissue-specific TRE inserts are deleted during replication. Southern blot analysis (fig. 2) of stocks of CV716 indicated a new band when annealed with an E1B-labeled probe. DNA sequence analysis of the cloned deletion mutant indicated that self-inactivation is due to homologous recombination between two identical inserted TREs.

In order to make a stable tissue-specific adenovirus we employed two different TREs to drive expression of viral early essential genes. In CV739 the E1A gene and the E1B genes are under the control of the TRE of the rat PB gene and PSA gene, respectively. CV739 replicates well in PSA^+ prostate cancer cells, but poorly in nonprostate human cancer cell lines. The cell specificity of CV739 was similar to that of CV716, again showing the roughly 10,000 : 1 selectivity for PSA^+ cells as compared to PSA^- cells. However, CV739 is stable. No replication-defective mutants with deleted genomes were found after extensive passages. The same is true for other CV739-like viruses including CV764. CV764 is a stable ARCA© variant containing the PSE driving the E1A genes

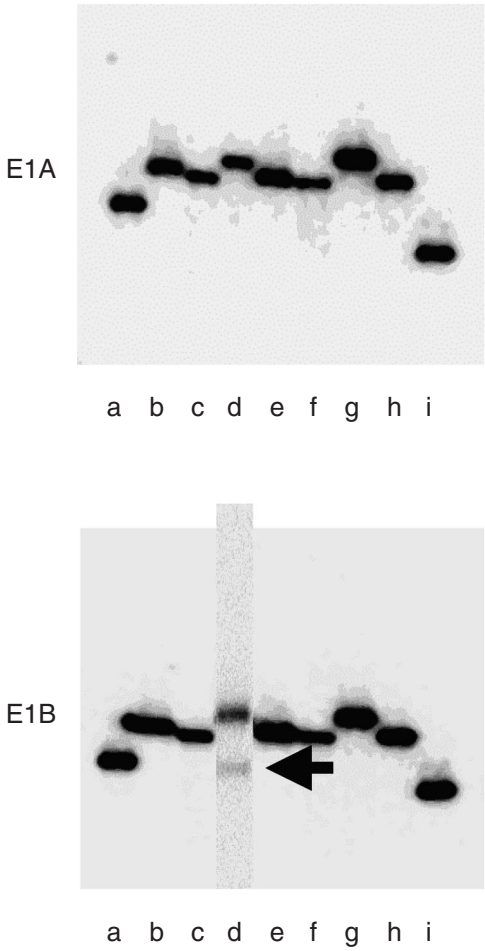

Fig. 2. Southern blots of CV716. The structure of ARCA variants was confirmed by Southern blot analysis of viral DNA. 10 ng of viral DNA (QIAmp blood kit, Qiagen) was digested with *Afl*II: lane a, CV702; lane b, CV706; lane c, CV711; lane d, CV716; lane e, CV739; lane f, CV763; lane g, CV764; lane h, CV787; lane i, CV802. DNA was fractionated through a 1% agarose gel and transferred by capillary transfer to a Nytran nylon membrane (Schleicher and Schuell). Viral DNA was probed with [α-^{32}P]dCTP-labeled PCR products specific for E1A or E1B sequences. The E1A probe was made by PCR from CV706 DNA amplifying a 938-bp fragment of Ad5 DNA, and the E1B probe was made by PCR from CV706 DNA amplifying an 881-bp product. Blots were hybridized overnight at 45 °C in Zip Hyb solution (Ambion), and washed two times in 2 × SSC, 0.15 SDS at room temperature and two times in 0.1 × SSC, 0.1% SDS at 65 °C. Blots were visualized by exposure in a GS-525 Molecular Imager (BioRad Laboratories).

and hK2 promoter and enhancer driving the E1B gene. The sequences of the PSE and hK2 promoter and enhancer are 80% identical yet the virus is genetically stable. Again, CV764 has the high therapeutic index of the other viruses containing two prostate-specific TREs with a cell specificity of 10,000 : 1 for PSA^+ cells compared to PSA^- cells [22, 23].

Taken together, these adenovirus variants show that tropism of adenovirus can be redirected by placing viral essential genes under the control of tissue-specific regulators. The cell selectivity of a stable oncolytic virus can be over 10,000 : 1 when the expression of more than one viral gene is driven by two different tissue-specific TREs.

Efficacy: Attenuated Replication-Competent Adenovirus

Viruses with the Addition of the E3 Region (CV787)

The E3 region has long been considered unnecessary for replication of adenovirus in vitro. It has been universally deleted from Ad5 gene therapy constructs until recent efforts to prolong transgene expression from replication-defective Ad5 gene therapy constructs [55, 56, 78–81]. The E3 region is believed to encode proteins that play a role in evading the host immune system prolonging virus infection [82]. Seven proteins encoded by the Ad E3 region have been identified and characterized: (1) a 19-kD glycoprotein (gp19k) known to inhibit transport of the MHC class I molecules to the cell surface thus impairing both peptide recognition and clearance of Ad-infected cells by CTLs, and one of the most abundant adenovirus early proteins [83, 84]; (2) the E3 14.7k protein and the E3 10.4/14.5k complex of proteins that inhibits the cytotoxic and inflammatory responses mediated by tumor necrosis factor [85–87]; (3) the E3 10.4/14.5k protein complex that downregulates the epidermal growth factor receptor which may inhibit inflammation and activate quiescent infected cells for efficient viral replication [88] as well as downregulate the apoptosis receptor CD95 [86], and (4) the E3 11.6 protein (adenoviral death protein) that promotes cell death and release of virus from infected cells [60, 61, 89]. The functions of three E3-encoded proteins, the 3.6k, 6.7k and 12.5k proteins are currently unknown [82]. Significantly, the E3 region has never been absent in clinical isolates.

The adenovirus E3 region was deleted from the tissue-specific ARCA©s described above. To test the possibility of increasing virus cytotoxicity, we created CV787 from its parent virus CV739 by engineering the full-length E3 region back into the viral genome. Thus CV739 contains the rat PB promoter driving the E1A gene and the PSE driving the E1B gene. Otherwise CV787 is identical to the recombinant wild-type adenovirus CV802. CV787 retained the

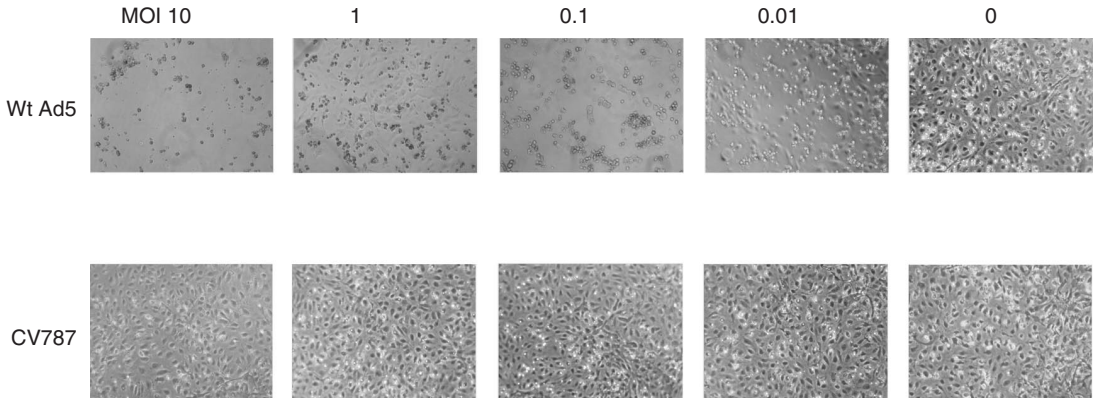

Fig. 3. Cytopathic effects of CV787 in primary human microvascular endothelial (hMVEC) cells. Primary hMVEC cells (Clonetics, San Diego, Calif.) were grown to 80% confluence and infected with either CV787 or CV702 for 2 h at increasing MOIs from 0.01 to 10. Plates were monitored daily for cytopathic effect, and the assay was terminated 10 days after infection.

high specificity of characteristics of two TRE-containing viruses driving the E1A and E1B genes. Cytopathic effect (CPE) assays and cell viability are shown in figures 3 and 4. Ten days after injection, the CPE assay of CV787 and the control wild-type virus CV802 on primary normal human microvascular endothelial cells shows that CV802 clears half the microscopic field at a multiplicity of infection (MOI) of 0.01 and yields a field of debris at higher MOIs. In contrast, CV787 produces no CPE even at MOIs of 10. In addition, MTT assays show that CV802 destroys all cells whereas CV787 only destroys PSA^+ cells (fig. 4). These data extend the high level of specificity of CV787 as shown by virus yield (pfu/cell) data previously described [22, 23].

The plaque assay showed that the E3-containing virus CV787 produced larger plaques that developed more rapidly compared to an E3-deleted counterpart virus, CV739 (fig. 5). Large, rapidly forming plaques could be caused by enhancing virion release from infected cells as suggested by the function of the E3 death protein, but could also be caused by large burst size as well. This hypothesis was supported by growth curve data in which CV787-infected LNCaP cells produced 10-fold more extracellular virus as well as 10-fold more total virus per cell compared to CV739-infected LNCaP cells (fig. 6). Thus, while the E3 region is not necessary for virus replication in vitro, it certainly aids virus replication.

To further characterize the effect of the addition of the Ad5 E3 region, cell viability and MTT assays were carried out to monitor mitochondrial activity in

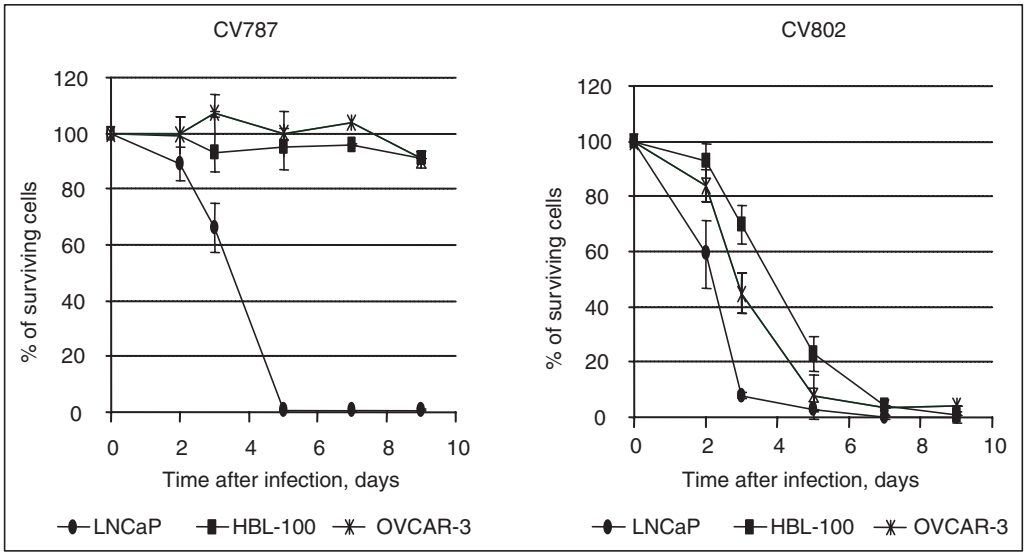

Fig. 4. Cell survival. Prostate cancer LNCaP PSA^{+}, breast epithelia HBL-100 PSA^{-}, and ovarian cancer OVCAR-3 PSA^{-} cells were infected with CV787 or CV802 at a MOI of 1. Cell survival was monitored daily by MTT assay.

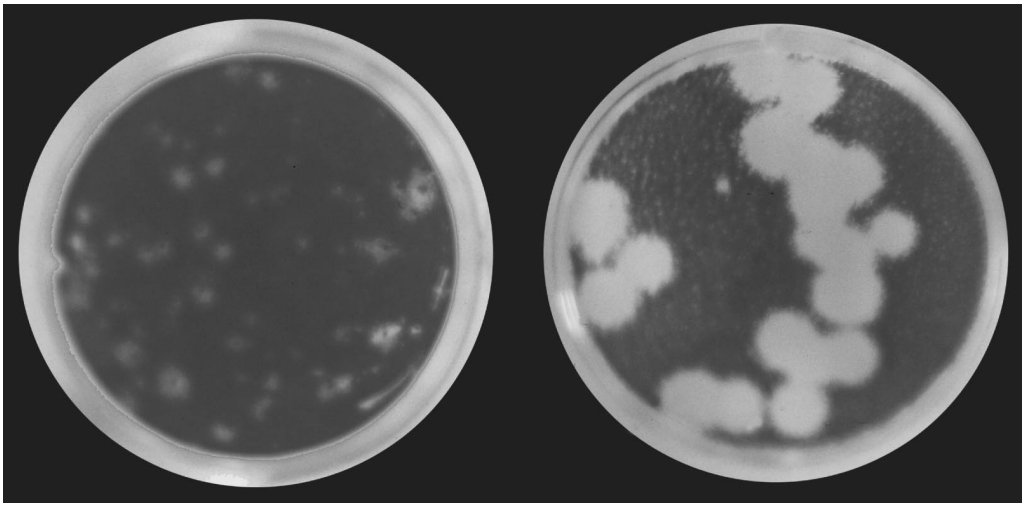

Fig. 5. Plaque morphology of CV739 and CV787. LNCaP cells were infected with CV739 and CV787. After a 1-hour adsorption period the plates were overlaid with agar and incubated for 10 days. After 10 days, the agar overlay was removed and the cells stained with crystal violet.

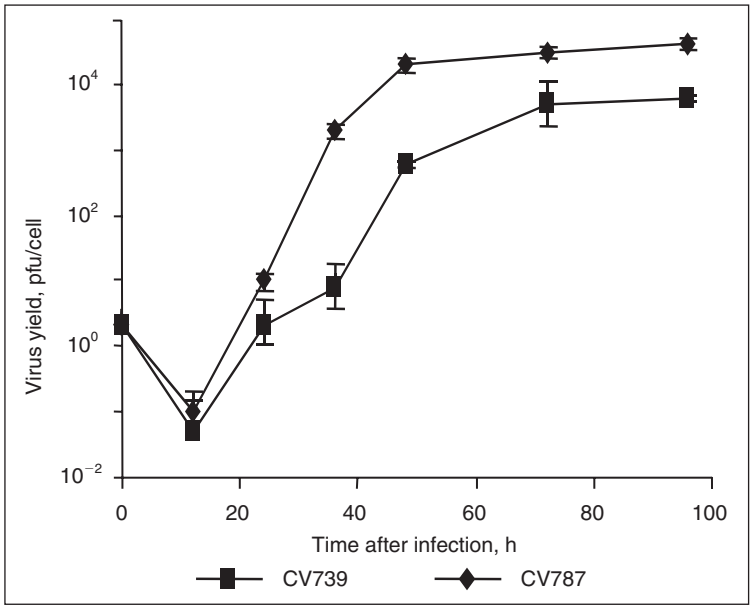

Fig. 6. One-step growth curves of CV739 and CV787. Monolayers of LNCaP cells were infected at a multiplicity of 2 pfu/cell with either CV39 or CV787. At the indicated times thereafter duplicate cell samples were harvested, lysed by three cycles of freeze-thawing, and the virus in the supernatants was assayed in triplicate in 293 cell monolayers.

CV739- and CV787-infected cells. In the trypan blue cell viability assay (fig. 7), LNCaP cells were infected with a MOI of 1 pfu/cell with CV739 and CV787. Both viruses killed all cells by 9 days after infection. However, 6 days after infection CV787 had killed 90% of the cells whereas CV739 had only killed 16% of the cells. In the mitochondrial MTT assay, cells infected with CV787 at multiplicity of 1 pfu/cell began to lose mitochondrial activity 3 days after infection and 90% of the cells were dead by 6 days. Cells infected with CV739 did not begin to lose mitochondrial activity 4 days after infection and retained 90% of mitochondrial activity at day 6 after infection [22]. These results indicate that CV787 has a stronger cell-killing ability than CV739. These results were confirmed in vivo as seen in the next section.

In vivo Antitumoral Studies of CV706 and CV787

In vivo studies evaluating intratumoral and intravenous administration of prostate-specific adenoviruses were conducted in the *nu/nu* mouse containing human tumor xenografts. Tumors were produced by subcutaneous injection of PSA-producing prostate cancer LNCaP cells into each flank of each mouse,

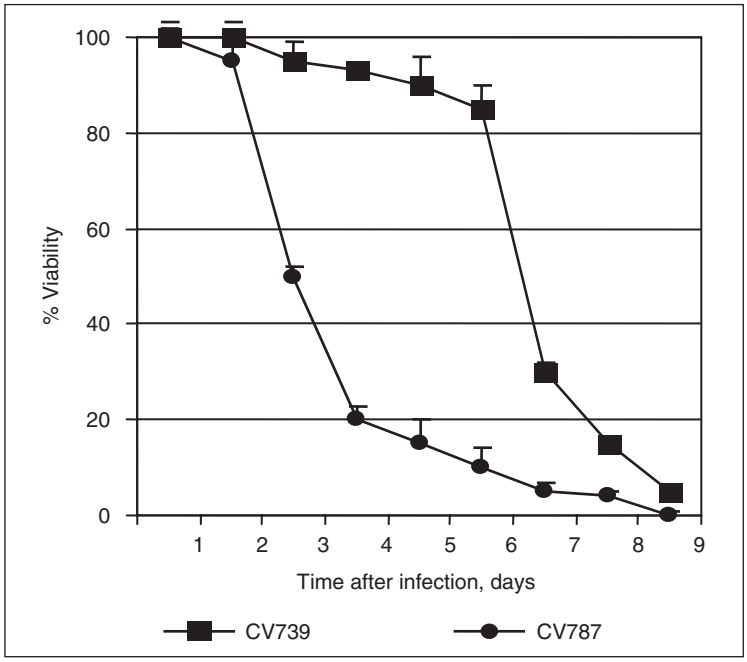

Fig. 7. Effect of Ad5 E3 region on cell survival. Prostate cancer LNCaP cells were infected with CV787 or CV802 at a MOI of 1. Cell survival was monitored daily by trypan blue exclusion.

and after establishment of palpable tumors (mean tumor volume 300 mm^3), the tumors were directly injected with purified virus or vehicle (PBS and 10% glycerol). Tumor growth was then followed for 6 weeks, at which time the mean tumor volume in each group was determined. A significant antitumoral activity was observed in the in vivo study for CV706. Tumor volume dropped by more than 80% in the animal group that was treated with CV706 by a single intratumoral injection. After 6 weeks, 5 of 10 mice were visually free of tumor [21].

In contrast, DU145 is a prostate cancer cell line that is PSA^- and does not produce the androgen receptor. Tumors of DU145 cells were induced in nude mice and challenged with buffer, wild-type Ad5 but E3 CV702, and CV706. The results showed that CV702 inhibited growth of DU145 tumors, whereas CV706 has no effect on tumor growth. Thus, the prostate-specific CV706 virus not only shows efficacy but also selectivity for PSA^+ cells in vivo [21].

The increased efficacy due to the Ad5 E3 region was also confirmed in vivo in the LNCaP xenograft animal model. A single intratumoral injection

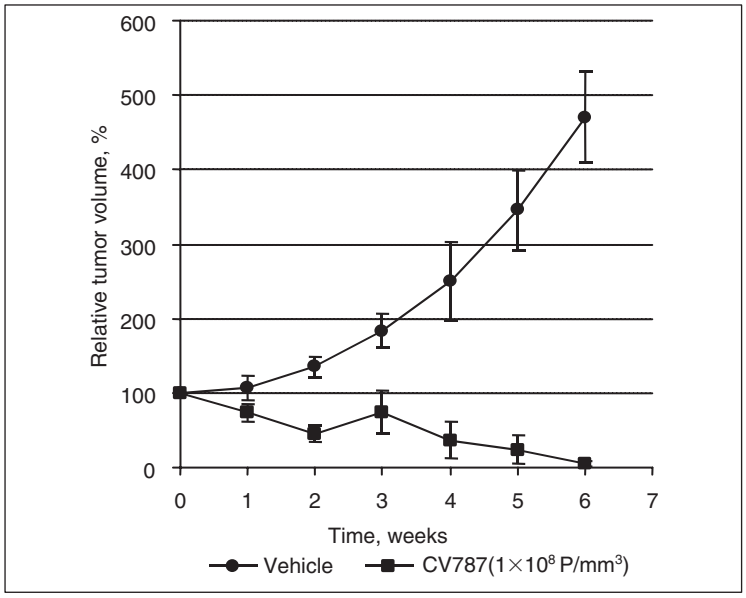

Fig. 8. Intratumoral injection activity of CV787 towards LNCaP xenografts. *nu/nu* mice with subcutaneous LNCaP tumors (average size 300 mm^3) were injected once into the tail vein with 1×10^9, 1×10^{10}, or 1×10^{11} particles of CV787. Tumor size was measured weekly.

of CV739 and CV787 yielded an identical reduction of LNCaP xenografts. However, CV739 required 100-fold more virus to achieve the same effect as CV787 [22]. A single intratumoral dose of 1×10^8 particles/mm^3 was curative for animals 6 weeks after treatment (n = 8; fig. 8). A single intravenous injection of CV739 at a dose of 5×10^{10} particles could stop tumor growth, whereas CV787 at this dose level caused a fourfold reduction in tumor volume [22]. Six weeks following a single intravenous injection of 1×10^{11} particles, the sizes of tumors were reduced to less than 5% of their original size, and 8 of 14 mice were visually free of tumors [22]. The residual tumors measurably present were immunohistologically devoid of PSA [22]. The serum PSA levels in mice injected intravenously with CV787 decreased to 5% of their starting values within 4 weeks. A dose–response curve of CV787 treating LNCaP xenografts is shown in figure 9. Whereas 1×10^9 and 1×10^{10} particles of CV787 administered as a single intravenous dose can control and regress tumors, 1×10^{11} particles can eliminate 300 mm^3 preexistent LNCaP xenografts. These data indicate that CV787 has significant antitumor activity and a single dose of

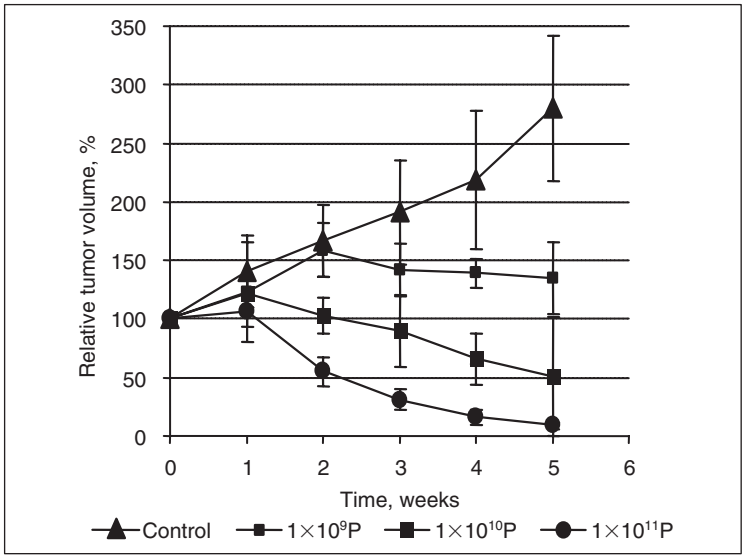

Fig. 9. Intravenous injection activity of CV787 towards LNCaP xenografts: a dose-ranging study. *nu/nu* mice with subcutaneous LNCaP tumors (average size 300 mm^3) were injected once into the tail vein with 1×10^9, 1×10^{10}, or 1×10^{11} particles of CV787. Tumor size was measured weekly.

intratumoral or intravenous administration can eliminate preexistent tumors in animal models.

Mechanism for Cell Killing of Oncolytic Virus

Infection with adenovirus causes profound changes in host-cell macromolecular synthesis that ultimately lead to cell death. Virion fiber protein inhibits macromolecular synthesis when applied directly to cells bearing the adenovirus receptor [90]; soluble penton protein causes CPEs in susceptible cells that are similar to those caused by infectious virus [91]. Cell-specific DNA synthesis, export of cellular mRNAs from the nucleus to the cytoplasm, and cell-specific translation are all inhibited after infection, but the precise mechanisms are not completely understood.

The 243E1a protein induces the full range of classical apoptotic events by increasing the level of the host cellular tumor-suppressor protein p53. The 289E1a protein induces apoptosis by a p53-independent mechanism that requires a product of the E4 region [92, 93]. The E1A-induced activation of the apoptosis pathway(s) must be modulated by E1B proteins to ensure efficient

virus replication prior to cell death [94]. Activation of the interferon-inducible RNase L pathway by the adenovirus-associated type I (VAI) RNA [95] may also contribute to the stimulation of apoptotic pathways in adenovirus-infected cells [96]. The E3 11.6-kD adenovirus death protein also has a role in cell killing and promotes the release of progeny virions from the cell [60, 61].

We have investigated how our oncolytic viruses kill tumors in the *nu/nu* mouse model. Immunohistochemical analyses were performed to assay for the de novo synthesis of CV787-encoded proteins in tumor xenografts and to examine the effects of treatment with CV787 on tumor morphology in vivo. Tumors were induced in 12-week-old athymic male mice by injection of 1×10^6 LNCaP cells in Matrigel and were allowed to develop for 4 weeks. The mice were injected intravenously on day 0 with 1×10^{11} particles of CV787 per animal. Tumors were excised from 2 animals on days 1, 3, 7, 14, 21, and 28. The tumors were cut into 6 pieces and each piece fixed, embedded in paraffin, and sectioned. Sections were stained for the presence of adenovirus protein by a double-antibody protocol with rabbit anti-adenovirus anti-bodies (Bethyl Laboratories, Inc.) and Fast Red stain followed by hematoxylin counter-stain.

On day 1, intracellular staining for adenovirus protein was detected in less than 1% of the tumor cells examined in 12 sections from 2 tumors. Occasional small clusters of stained cells, as well as dispersed single stained cells, were visible. By day 3, large clusters of cells expressing adenovirus proteins were detected in 1 of 2 tumors. In some instances, areas of tumor necrosis were adjacent to clusters of adenovirus protein-positive cells. On day 7, intracellular staining for adenovirus proteins was detected in $>10\%$ of the tumor cells examined in 12 sections from 2 tumors. Virus-infected cells within the tumor sections were prominent on day 21 and increased to more than 90% of the microscopic field of the section by day 28. These results demonstrated that CV787 replicated in and expressed virus-encoded gene products in the LNCaP xenografts. The increased distribution of virus protein-positive cells indicated that infectious progeny CV787 spread to adjacent cells within the tumor which was associated with progressive necrosis in vivo [22].

Adenovirus-induced apoptosis causes cell death in vitro, specifically at the late stage of infection [92], and this process may contribute to the therapeutic effect of oncolytic virus in vivo. LNCaP xenografts in athymic mice were treated on days 0–3 with vehicle alone or a total dose of 3.2×10^7 particles of CV706/mm^3 of tumor. Tumor biopsy specimens were taken on day 14 and 5-μm sections were prepared and examined for apoptosis. Extensive areas containing apoptotic nuclei were detected in sections of tumors treated with CV706. More than 25% of nuclei were apoptotic in some sections from CV706-treated tumors. In contrast, less than 2% of nuclei were apoptotic in sections from tumors treated with vehicle alone.

Clinical Development of CV706 and CV787

CV706 and CV787 are novel therapeutic agents with a novel mechanism of action. A phase-I trial of CV706 was initiated in 1998 at the Brady Urological Institute of the Johns Hopkins Oncology Center under the direction of Jonathan Simons, MD, and Ted DeWeese, MD. The patient population consists of men with locally recurrent prostate cancer with rising PSA levels following definitive external beam irradiation. Men in this category are usually left untreated or receive androgen ablation therapy as serum PSA levels rise significantly above 10 ng/ml. On average, these men have a life expectancy of 3 years. The virus was administered under spinal anesthesia using the brachytherapy template and ultrasound 3D imaging using the MMS Terapac Plus 6.6 B3DTUI (Charlottesville, Va.) treatment-planning software for implantation of radioactive seeds. Virus was initially administered with 20 0.1-ml aliquots from 10 brachytherapy needles. PSA levels were determined and biopsies obtained. As of this writing, 13 men have been treated and results have been encouraging. PSA levels initially dropped in all men treated yielding durable stabilization, as measured by PSA levels, of disease in a subset of these men. CV787 has entered a multicenter phase-I/II clinical trial in the same patient population with the intent of replacing CV706.

Factors Impacting Clinical Efficacy and Safety

The pathogenesis of adenoviral infections is influenced by a large number of factors, some pertaining to the virus and others pertaining to the host defenses of the virus. Important issues for the virus include: the route of infection; the size of the virus inoculum; the tropism of the virus for different cell types, and whether the virus spreads directly from cell to cell or through extracellular fluid. Clearly, the vascularization of tumors, the leakiness of capillaries to virus, and the physical size of the virus particle will affect intratumoral virus distribution. In the replication efficiency of the virus in prostate tumor cells, both the time of the replication cycle and the burst size are also important. Host defenses include: mechanical defenses (epithelia, mucosal, liver Kupffer cells, or the blood-brain barrier); nonspecific immune defenses (interferons, recognition of infected cells by natural killer cells, release of cytokines, macrophage recruitment and activation, and triggering of complement and kinin cascades), and specific immune defenses (humoral immunity, mostly IgM and IgG but also IgA, IgD and IgE, and finally cell-mediated immunity) [97].

In adenovirus-mediated prostate cancer therapy, the virus can be either injected directly into the tumor or administered by intravenous injection. In either case, the dose of virus is massive (10^{11}–10^{15} particles) compared to natural,

vaccine-induced adenovirus infections (10^0–10^6 particles) [98, 99], or the clinical trials with wild-type adenovirus (10^7–10^9 particles) [14]. Very little is known about the human host response to large doses of adenoviruses [14, 100] and nothing is known about the human host response to using the intravenous route of administration of large doses of replicating adenoviruses. Liver toxicity of virion proteins may be limiting at these high doses.

Therapeutic antibody studies have indicated that antibodies do not effectively penetrate the core of a solid tumor; extravasation is limited to the tumor periphery. This suggests that the accessibility of replicating virus to antibody binding should be minimal following direct intratumoral injection [33, 101]. Cell-mediated immunity directed toward infected tumor cells may actually enhance the efficacy of replicating viruses in cancer patients if enough replication and spread occur initially. However, a systemically delivered replicating adenovirus is going to face several potential hurdles: (1) the nonspecific removal of adenovirus by liver Kupffer cells; (2) the inactivation of virus by pre-existing circulating antibodies to adenovirus; (3) a limitation of viral replication mediated by a vigorous CTL response to virally infected cells, and (4) a limitation of the efficacy of repeat dosage by primary or secondary induction of humoral immunity.

Incorporation of the Ad5 genome into germ cells has been expressed as a concern but has not been found for any of the Ad5 gene therapy constructs. Indeed, adenovirus gene expression is characterized as transient in nature due to a lack of viral DNA integration. Virus shedding has been expressed as a concern but it has not been detected in any Ad5 clinical trial to date. In our clinical trial, virus replication was detected after 2–8 days but was undetectable after 2 weeks. It is difficult to estimate the increased cytolytic activity in humans of CV787 compared to CV706. However, replicating adenoviruses containing hepatitis B surface antigen (HBsAg), with and without the E3 region, have been tested in chimpanzees, a system permissive for infection by human adenoviruses [102]. In this study, the addition of the E3 region resulted in a 10- to 100-fold increase in virus shedding and a 10- to 100-fold increase in titer to HBsAg. However, one should not lose sight of the fact adenoviruses are ubiquitous. Twenty-three percent of normal healthy infants are seropositive for adenoviruses by 7 months of age [103] and CV787 is attenuated 10,000 : 1 compared to the wild-type virus. We believe the therapeutic use of CV787 will be safe; the major question is whether or not there is sufficient efficacy to be medically useful.

Conclusion

The safety of administering wild-type Ad5 either intratumorally and intravenously was demonstrated at intermediate doses (10^7–10^9 particles) over

40 years ago [14]. None of the treated patients had significant side effects. Safety and efficacy will be the major issues as adenovirus doses escalate from 10^{11} to 10^{15} particles. CV787 is a replication-competent adenovirus attenuated 10,000 : 1 compared to the wild-type virus in PSA^- cells, and is capable of eliminating distant mouse xenograft tumors with a single intravenous injection. This is an unprecedented therapeutic index for a cytotoxic agent as measured in vitro. The clinical target for CV787 is prostate cancer. Ongoing clinical trials of agents such as CV787 will resolve the issues of safety and efficacy and hopefully point to a new mode of cancer chemotherapy, one that includes the use of targeted cytolytic adenoviruses.

References

1 Donehoweer RC, Abeloff MD, Perry MC: Chemotherapy; in Abeloff MD, Armitage JO, Lichter AS, Niederhuber JE (eds): Clinical Oncology. New York, Churchill Livingston, 1995, pp 201–218.
2 Scher HI, Isaacs JT, Fuks Z, Walsh PC: Prostate; in Abeloff MD, Armitage JO, Lichter AS, Niederhuber JE (eds): Clinical Oncology. New York, Churchill Livingston, 1995, pp 1439–1472.
3 Golomb HM: Report of a multi-institutional study of 193 patients with hairy cell leukemia treated with interferon alpha2b. Semin Oncol 1988;5:7–9.
4 Demetri GD, Anderson KC: Bone marrow failure; in Abeloff MD, Armitage JO, Lichter AS, Niederhuber JE (eds): Clinical Oncology. New York, Churchill Livingston, 1995, pp 433–456.
5 Anderson WF: Gene therapy for cancer. Hum Gene Ther 1994;5:1–2.
6 Wilson JM: Adenoviruses as gene-delivery vehicles. N Engl J Med 1996;334:1185–1187.
7 Elshami AA, Kucharczuk JC, Sterman DH, Smythe WR, Hwang HC, Amin KM, Litzky LA, Albelda SM, Kaiser LR: The role of immunosuppression in the efficacy of cancer gene therapy using adenovirus transfer of the herpes simplex thymidine kinase gene. Ann Surg 1995;222: 298–307.
8 Kaneko S, Hallenbeck P, Kotane T, Nakabayashi H, McGarrity G, Tamaoki T, Anderson WF, Chiang YL: Adenovirus-mediated gene therapy of hepatocellular carcinoma using cancer-specific gene expression. Cancer Res 1995;55:5283–5287.
9 Kanai F, Shiratori Y, Yoshida Y, Wakimoto H, Hamada H, Kanegae Y, Saito I, Nakabayashi H, Tamaoki T, Tanaka T, Lan K, Kato N, Shina S, Omata M: Gene therapy for α-fetoprotein-producing human hepatoma cells by adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene. Hepatology 1996;23:1359–1368.
10 Kanai F, Lan K, Shiratori Y, Tanaka T, Ohashi M, Okudaira T, Yoshida Y, Wakimoto H, Hamada H, Nakabayashi H, Tamaoki T, Omata M: In vivo gene therapy for α-fetoprotein-producing hepatocellular carcinoma by adenovirus-mediated transfer of cytosine deaminase gene. Cancer Res 1997; 57:461–465.
11 Su H, Lu R, Chang JC, Kan YW: Tissue-specific expression of herpes simplex virus thymidine kinase gene delivered by adeno-associated virus inhibits the growth of human hepatocellular carcinoma in athymic mice. Proc Natl Acad Sci USA 1997;94:13891–13896.
12 Dock G: Influence of complicating diseases upon leukemia. Am J Med Sci 1904;127:536–592.
13 Moore AE: Effects of viruses on tumors. Annu Rev Microbiol 1954;8:393–410.
14 Smith RR, Huebner RJ, Rowe WP, Schatten WF, Thomas LB: Studies on the use of viruses in the treatment of carcinoma of the cervix. Cancer 1956;9:1211–1218.
15 Bischoff JR, Kirn DH, Williams A, Heise C, Horn S, Muna M, Ng L, Nye JA, Sampson-Johannes A, Fattaey A, McCormick F: An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science 1996;274:373–376.

16 Hall AR, Dix BR, O'Carroll SJ, Braithwaite AW: p53-dependent cell death/apoptosis is required for a productive adenovirus infection. Nat Med 1998;4:1068–1072.
17 Goodrum FD, Ornelles DA: p53 status does not determine outcome of E1B 55-kilodalton mutant adenovirus lytic cycle. J Virol 1998;72:9479–9490.
18 Horridge JJ, Leppard KN: RNA-binding activity of the E1B 55 kilodalton protein from human adenovirus type 5. J Virol 1998;72:9374–9379.
19 Gabler S, Schutt H, Groitl P, Wolf H, Shenk T, Dobner T: E1B 55-kilodalton-associated protein: A cellular protein with RNA-binding activity implicated in nucleocytoplasmic transport of adenovirus and cellular mRNAs. J Virol 1998;72:7960–7971.
20 Zhang JF, Hu C, Geng Y, Selm J, Klein SB, Orazi A, Taylor MW: Treatment of a human breast cancer xenograft with an adenovirus vector containing an interferon gene results in rapid regression due to viral oncolysis and gene therapy. Proc Natl Acad Sci USA 1996;93: 4513–4518.
21 Rodriguez R, Schuur ER, Lim HY, Henderson GA, Simons JW, Henderson DR: Prostate-attenuated replication competent adenovirus (ARCA) CV706: A selective cytotoxic for prostate-specific antigen positive prostate cancer cells. Cancer Res 1997;57:2559–2563.
22 Yu D-C, Chen Y, Seng M, Dilley J, Henderson DR: The addition of adenovirus type 5 region E3 enables Calydon virus 787 to eliminate distant prostate tumor xenografts. Cancer Res 1999;59: 4200–4203.
23 Yu D-C, Sakamoto GT, Henderson DR: Identification of the transcriptional regulatory sequence of human kallikrein 2 and their use in the construction of Calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. Cancer Res 1999;59:1498–1504.
24 Aumuller G, Seitz J, Lilja H, Abrahamsson PA, von der Kammer H, Scheit KH: Species- and organ-specificity of secretory proteins derived from human prostate and seminal vesicles. Prostate 1990;17:31–40.
25 Scher HI, Fossa S: Prostate cancer in the era of prostate-specific antigen. Curr Opin Oncol 1995;7:281–291.
26 Wilding G: Endocrine control of prostate cancer. Cancer Surv 1995;23:42–62.
27 Smith PH: Hormone therapy: An overview. Cancer Surv 1995;23:171–181.
28 Walsh PC: Radical prostatectomy: A procedure in evolution. Semin Oncol 1994;21:662–671.
29 Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG: Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc Soc Exp Med 1953;84:570–573.
30 Zahradnik JM, Spencer MJ, Porter DD: Adenovirus infection in the immunocompromised patient. Am J Med 1980;68:725–732.
31 Shields AF, Hackman RC, Fife KH, Corey L, Meyers JD: Adenovirus infections in patients undergoing bone-marrow transplantation. N Engl J Med 1985;312:529–533.
32 Horwitz MS: Adenoviridae and Their Replication, ed 2. New York, Raven Press, 1990.
33 Schulick AH, Vassalli G, Dunn PF, Dong G, Rade JJ, Zamarron C, Dichek DA: Established immunity precludes adenovirus-mediated gene transfer in rat carotid arteries. J Clin Invest 1997;99: 209–219.
34 Chroboczek J, Bieber F, Jacrot B: The sequence of the genome of adenovirus type 5 and its comparison with the genome of adenovirus type 2. Virology 1992;186:280–285.
35 Garon CF, Berry KW, Rose JA: A unique form of terminal redundancy in adenovirus DNA molecules. Proc Natl Acad Sci USA 1972;69:2391–2395.
36 Graham FL, Smiley J, Russell WC, Nairn N: Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 1977;36:59–74.
37 Berk AJ: Adenovirus promoters and E1A transactivation. Annu Rev Genet 1986;20:45–79.
38 Flint J, Shenk T: Adenovirus E1A protein paradigm viral transactivator. Annu Rev Genet 1989;23:141–161.
39 Shenk T, Flint J: Transcriptional and transforming activities of the adenovirus E1A proteins. Adv Cancer Res 1991;57:47–85.
40 Nevins JR: Transcriptional activation by viral regulatory proteins. Trends Biochem Sci 1991; 16:435–439.

41 Bellett AJ, Jackson P, David ET, Bennett EJ, Cronin B: Functions of the two adenovirus early E1A proteins and their conserved domains in cell cycle alteration, actin reorganization, and gene activation in rat cells. J Virol 1989;63:303–310.
42 Howe JA, Mymryk JS, Egan C, Branton PE, Bayley ST: Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis. Proc Natl Acad Sci USA 1990;87:5883–5887.
43 Zerler B, Roberts RJ, Matthews MB, Moran E: Different functional domains of the adenovirus E1A gene are involved in regulation of host cell cycle products. Mol Cell Biol 1987;7:821–829.
44 Matsuo T, Wold WSM, Hashimoto S, Rankin A, Symington J, Green M: Polypeptides encoded by transforming region E1B of human adenovirus 2: Immunoprecipitation from transformed and infected cells and cell-free translation of E1B-specific mRNA. Virology 1982;118:456–465.
45 Rao L, Debbas M, Sabbatini P, Hockenberry D, Korsmeyer S, White E: The adenovirus E1A protein induces apoptosis, which is inhibited by the E1B 19 kDa and Bcl-2 proteins. Proc Natl Acad Sci USA 1992;89:7742–7746.
46 Querido E, Marcellus RC, Lai A, Charbonneau R, Teodoro JG, Ketner G, Branton PE: Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. J Virol 1997;71:3788–3798.
47 Gooding LR, Aquino L, Duerksen-Hughes PJ, Day D, Horton TM, Yei SP, Wold WSM: The E1B 19,000-molecular-weight protein of group C adenoviruses prevents tumor necrosis factor cytolysis of human cells but not of mouse cells. J Virol 1991;65:4114–4123.
48 Gooding LR, Ranheim TS, Tollefson AE, Aquino L, Duerksen-Hughes P, Horton TM, Wold WSM: The 10,400- and 14,500-dalton proteins encoded by region E3 of adenovirus function together to protect many but not all mouse cell lines against lysis by tumor necrosis factor. J Virol 1991;65:3083–3094.
49 McGlade CJ, Tremblay ML, Yee SP, Ross R, Branton PE: Acylation of the 176R (19-kilodalton) early region 1B protein of human adenovirus type 5. J Virol 1987;61:3227–3234.
50 Yoshida K, Venkatesh L, Kuppuswamy M, Chinnadurai G: Adenovirus transforming 19-kD T antigen has an enhancer-dependent trans-activation function and relieves enhancer repression mediated by viral and cellular genes. Genes Dev 1987;1:645–658.
51 Linné T, Jörnvall H, Phillipson L: Purification and characterization of the phosphorylated DNA-binding protein from adenovirus-type-2-infected cells. Eur J Biochem 1977;76:481–490.
52 Lichy JH, Enomoto T, Field J, et al: Isolation of proteins involved in the replication of adenovirus DNA in vitro. Cold Spring Harbor Symp Quant Biol 1983;47:731–740.
53 Lichy JH, Field J, Horwitz MS, Hurwitz J: Separation of the adenovirus terminal protein precursor from its associated DNA polymerase: Role of both proteins in the initiation of adenovirus DNA replication. Proc Natl Acad Sci USA 1982;79:5225–5229.
54 Friefeld BR, Lichy J, Hurwitz J, Horwitz MS: Evidence for an altered adenovirus DNA polymerase in cells infected with the mutant H5ts149. Proc Natl Acad Sci USA 1983;80:1589–1593.
55 Ghosh-Choudhury G, Haj-Ahmad Y, Brinkley P, Rudy J, Graham FL: Human adenovirus cloning vectors based on infectious bacterial plasmids. Gene 1986;50:161–171.
56 Graham FL: Adenoviruses as expression vectors and recombinant vaccines. Trends Biotechnol 1990;8:85–87.
57 Feuerbach D, Burgert HG: Novel proteins associated with MHC class I antigen in cells expressing the adenovirus protein E3/19K. EMBO J 1993;12:3153–3161.
58 Beier DC, Cox JH, Vining DR, Cresswell P, Engelhard VH: Association of human class I MHC alleles with the adenovirus E3/19K protein. J Immunol 1994;152:3862–3872.
59 Lee MG, Abina MA, Haddada H, Perricaudet M: The constitutive expression of the immunomodulatory gp19k protein in E1-, E3- adenoviral vectors strongly reduces the host cytotoxic T cell response against the vector. Gene Ther 1995;24:256–262.
60 Tollefson AE, Scaria A, Hermiston TW, Ryerse JS, Wold LJ, Wold WS: The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. J Virol 1996;70:2296–306.
61 Tollefson AE, Ryerse JS, Scaria A, Hermiston TW, Wold WS: The E3-11.6-kDa adenovirus death protein (ADP) is required for efficient cell death: Characterization of cells infected with adp mutants. Virology 1996;220:152–162.

62 Müller U, Kleinberger T, Shenk T: Adenovirus E4orf4 protein reduces phosphorylation of c-fos and E1A proteins while simultaneously reducing the level of AP-1. J Virol 1992;66: 5867–5878.
63 Thomas GP, Mathews MB: DNA replication and the early to late transition in adenovirus infection. Cell 1980;22:523–533.
64 Shaw AR, Ziff EB: Transcripts from the adenovirus-2 major late promoter yield a single early family of 3′ coterminal mRNAs and five late families. Cell 1980;22:905–916.
65 Shaw AR, Ziff EB: Selective inhibition of adenovirus type 2 early region II and III transcription by an anisomycin block of protein synthesis. Mol Cell Biochem 1982;2:789–799.
66 Green M, Daesch GE: Biochemical studies on adenovirus multiplication. I. Kinetics of nucleic acid and protein synthesis in suspension cultures. Virology 1961;13:169–176.
67 Wold SM, Green M, Buttner W: The Molecular Biology of Animal Viruses. New York, Dekker, 1978.
68 Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW: Isolation of a common receptor for Coxsackie B virus and adenoviruses 2 and 5. Science 1997;275:1320–1323.
69 Tomko RP, Xu R, Phillipson L: HCAR and MCAR: The human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc Natl Acad Sci USA 1997;94: 3352–3356.
70 Berk AJ, Sharp PA: Structure of adenovirus 2 early mRNAs. Cell 1978;14:695–711.
71 Nevins J, Ginsberg H, Blanchard J, Wilson M, Darnell JJ: Regulation of the primary expression of the early adenovirus transcription. J Virol 1979;32:727–733.
72 Schuur ER, Henderson GA, Kmetec LA, Miller JD, Lamparski HG, Henderson DR: Prostate-specific antigen expression in regulated by an upstream enhancer. J Biol Chem 1996;271: 7043–7051.
73 Brookes DE, Zandvliet D, Watt F, Russell PJ, Molloy PL: Relative activity and specificity of promoters from prostate-expressed genes. Prostate 1998;35:18–26.
74 Greenberg NM, DeMayo FJ, Sheppard PC, Barrios R, Lebovitz R, Finegold M, Angelopoulou R, Dodd JG, Duckworth ML, Rosen JM, et al: The rat probasin gene promoter directs hormonally and developmentally regulated expression of a heterologous gene specifically to the prostate in transgenic mice. Mol Endocrinol 1994;8:230–239.
75 Riegman P, Vlietstra R, Suurmeuer L, Cleutjens C, Trapman J: Characterization of the human kallikrein locus. Genomics 1992;14:6–11.
76 Schedlich L, Bennetts B, Morris B: Primary structure of a human glandular kallikrein gene. DNA 1987;6:429–437.
77 Morris B: HGK-1: A kallikrein gene expressed in human prostate. Clin Exp Pharmacol Physiol 1989;16:345–351.
78 Haj-Ahmad Y, Graham FL: Development of a helper-independent human adenovirus vector and its use in the transfer of the herpes simplex virus thymidine kinase gene. J Virol 1986;57:267–274.
79 Robbins PD, Tahara H, Ghivizzani SC: Viral vectors for gene therapy. Trends Biotechnol 1998;16: 35–40.
80 Wivel NA, Wilson JM: Methods of gene delivery. Hematol Oncol Clin North Am 1998;12:483–501.
81 Ilan Y, Droguett G, Chowdhury NR, Li Y, Sengupta K, Thummala NR, Davidson A, Chowdhury JR, Horwitz MS: Insertion of the adenoviral E3 region into a recombinant viral vector prevents antiviral humoral and cellular immune responses and permits long-term gene expression. Proc Natl Acad Sci USA 1997;94:2587–2592.
82 Wold WS, Tollefson AE, Hermiston TW: E3 transcription unit of adenovirus. Curr Top Microbiol Immunol 1995;199:237–274.
83 Korner H, Burgert HG: Down-regulation of HLA antigens by the adenovirus type 2 E3/19K protein in a T-lymphoma cell line. J Virol 1994;68:1442–1448.
84 Flomenberg P, Gutierrez E, Hogan KT: Identification of class I MHC regions which bind to the adenovirus E3-19k protein. Mol Immunol 1994;31:1277–1284.
85 Li Y, Kang J, Horwitz MS: Interaction of an adenovirus E3 14.7-kilodalton protein with a novel tumor necrosis factor alpha-inducible cellular protein containing leucine zipper domains. Mol Cell Biol 1998;18:1601–1610.

86 Elsing A, Burgert HG: The adenovirus E3/10.4K-14.5K proteins down-modulate the apoptosis receptor Fas/Apo-1 by inducing its internalization. Proc Natl Acad Sci USA 1998;95: 10072–10077.
87 Dimitrov T, Krajcsi P, Hermiston TW, Tollefson AE, Hannink M, Wold WS: Adenovirus E3-10.4K/14.5K protein complex inhibits tumor necrosis factor-induced translocation of cytosolic phospholipase A2 to membranes. J Virol 1997;71:2830–2837.
88 Vinogradova O, Carlin C, Sonnichsen FD, Sanders CR 2nd: A membrane setting for the sorting motifs present in the adenovirus E3-13.7 protein which down-regulates the epidermal growth factor receptor. J Biol Chem 1998;273:17343–17350.
89 Hausmann J, Ortmann D, Witt E, Veit M, Seidel W: Adenovirus death protein, a transmembrane protein encoded in the E3 region, is palmitoylated at the cytoplasmic tail. Virology 1998;244: 343–351.
90 Levine AJ, Ginsberg HS: Mechanism by which fiber antigen inhibits multiplication of type 5 adenovirus. J Virol 1967;1:747–757.
91 Valentine RC, Pereira HG: Antigens and structure of the adenovirus. J Mol Biol 1965;13:13–20.
92 Teodoro JG, Shore GC, Branton PE: Adenovirus E1A proteins induce apoptosis by both p53-dependent and p53-independent mechanisms. Oncogene 1995;11:467–474.
93 Marcellus RC, Teodoro JG, Wu T, Brough DE, Ketner G, Shore GC, Branton PE: Adenovirus type 5 early region 4 is responsible for E1A-induced p53-independent apoptosis. J Virol 1996;70: 6207–6215.
94 Han J, Sabbatini P, Perez D, Rao L, Modna D, White E: The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death promoting Bax protein. Genes Dev 1996;10:461–477.
95 Desai SY, Patel RC, Sen GC, Malhotra P, Ghadge GD, Thimmapaya B: Activation of interferon-inducible 2′–5′ oligoadenylate synthetase by adenoviral VAI RNA. J Biol Chem 1995;270: 3454–3461.
96 Diaz-Guerra M, Rivas C, Esteban M: Activation of the IFN-inducible RNase L causes apoptosis of animal cells. Virology 1997;236:354–363.
97 Cunningham T: Pathogenesis of viral infection; in Glasso J, Whitley RJ, Merigan TC (eds): Antiviral Agents and Human Viral Diseases, ed 4. Philadelphia, Lippincott-Raven, 1997, pp 45–78.
98 Gutekunst RR, White RJ, Edmondson WP, Chanock RM: Immunization with live type 4 adenovirus: Determination of infectious virus dose and protective effect of enteric infection. Am J Epidemiol 1967;86:341–349.
99 Top FHJ, Buescher EL, Bancroft WH, Russell PK: Immunization with live types 7 and 4 adenovirus vaccines. II. Antibody response and protective effect against acute respiratory disease due to adenovirus type 7. J Infect Dis 1971;124:155–160.
100 Russi TJ, Hirschowitz EA, Crystal RG: Delayed-type hypersensitivity response to high doses of adenoviral vectors. Hum Gene Ther 1997;8:323–330.
101 Bramson JL, Hitt M, Gauldie J, Graham FL: Pre-existing immunity to adenovirus does not prevent tumor regression following intratumoral administration of a vector expressing IL-12 but inhibits virus dissemination. Gene Ther 1997;4:1069–1076.
102 Chengalvala MV, Bhat BM, Bhat RA, Dheer SK, Lubeck MD, Purcell RH, Murthy KK: Replication and immunogenicity of Ad7-, Ad4-, and Ad5-hepatitis B virus surface antigen recombinants, with or without a portion of E3 region, in chimpanzees. Vaccine 1997;15:335–339.
103 Nelson JK, Shields MD, Stewart MC, Coyle PV: Investigation of seroprevalence of respiratory virus infections in an infant population with a multiantigen fluorescence immunoassay using heel-prick blood samples collected on filter paper. Pediatr Res 1999;45:799–802.

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Reovirus as a Potential Anticancer Therapeutic

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Introduction

The premise of reovirus as a potential oncolytic agent in humans has been realized through many years of basic research in reovirus biology and biochemistry. In fact, research leading to the elucidation of the mechanism of reovirus oncolysis originated with virus receptor studies. Based on unexpected findings from these studies, focus was shifted to the role of the intracellular environment in reovirus susceptibility; it was found that cells with an activated Ras signaling pathway supported reovirus replication. The implications for the use of reovirus in cancer therapy are apparent when one considers that mutations involving Ras occur in approximately 30% of all human cancers [1, 2] and that mutations in other elements in the Ras pathway can also lead to cancer development. Importantly, it was found that normal, untransformed cells were not infectable by reovirus [3]. This lack of pathogenicity towards normal cells in vitro is reflected by the fact that reovirus infection in humans is typically subclinical and thus usually goes by unnoticed [4]. Further work revealed that not only does reovirus selectively replicate in transformed cell lines in vitro, but also in vivo, in murine models of cancer [5]. Altogether, a story emerges whereby a relatively nonpathogenic virus, reovirus type 3 Dearing, specifically targets many cancer types, resulting in complete tumor regression in murine models. Given the performance of reovirus in a laboratory environment, its potential use as cancer therapy in a clinical setting may yield interesting results.

Overview of the Biology of Reovirus

Reovirus belongs to the family *Reoviridae*, which is currently composed of nine genera, whose members have been shown to infect a variety of plants, animals and insects. One important human pathogen belonging to this family is rotavirus, which is a major cause of diarrhea and enteritis [6]. While rotavirus infection can have serious clinical implications, infection by reovirus is often asymptomatic [4, 7].

The very name, reovirus, implies the nonpathogenicity of the virus. In 1959, Sabin [8] coined the descriptive acronym, 'reovirus', from respiratory enteric orphan virus. The name originated because these viruses were usually isolated from the respiratory and gastroenteric tracts. However, infection was not associated with a defined disease condition in humans and was hence, an 'orphan' virus [4]. Reovirus is ubiquitous, found in both stagnant and fresh water, and in sewage [9, 10]. Virus has also been isolated from a variety of natural hosts, including chimpanzees, monkeys, pigs, cattle, cats, sheep, mice, and humans [11]. In vitro, reovirus has also been found to infect a variety of cell types from many species; for example mouse L929 fibroblasts, many human cancer cell lines, and Madin-Darby canine kidney (MDCK) cells are all infectable by reovirus [6]. Although most infections in humans go unnoticed, because of the ubiquity of reovirus, greater than 50% of adults have had previous exposure and carry anti-reovirus antibodies by the age of 20–30 years [6, 11, 12]. Some studies have found up to 70–100% of adults carrying anti-reovirus antibodies [13, 14].

Reovirus belongs to the genus *Orthoreoviridae*. This genus is characterized by the presence of a segmented, double-stranded RNA genome encased in a protein core and outer capsid with icosahedral symmetry. There are three size classes of dsRNA segments in mammalian reoviruses, large (designated L1, L2, and L3), medium (M1, M2, and M3) and small (S1, S2, S3, and S4). Transcription of each segment generates messenger RNA containing one or two open reading frames that code for a total of three nonstructural proteins and eight structural proteins comprising the capsid. Proteins are designated λ, μ, or σ, depending on the gene segment from which they originated.

The protein encoded by the S1 gene segment, σ1, is the viral attachment protein and the determinant of hemagglutination activity of reovirus [6, 15, 16]. Hemagglutination-inhibition tests are used to distinguish the three serotypes of mammalian reovirus. Prototypes of each serotype were isolated from children's respiratory and enteric tracts, and they are type 1 Lang, type 2 Jones, type 3 Abney, and type 3 Dearing. All three serotypes have ten segments of

double-stranded RNA, and due to the segmented nature of the genome, they can undergo inter-serotypic reassortment upon coinfection. The use of inter-serotypic reassortant viruses has proven useful in the study of individual gene functions as they pertain to the reovirus lytic cycle, as well as to reovirus pathogenesis in mice.

The reovirus lytic cycle begins with attachment of a virion to sialic acid residues on the cell surface via the trimeric σ1 cell attachment protein, which protrudes from the 12 vertices of the icosahedral capsid [15, 17–19]. Following attachment, clathrin-coated pits form and the virus enters by receptor-mediated endocytosis. Within the resulting endosome/lysosomes, acid-dependent proteolysis of viral outer capsid proteins σ3 and μ1/μ1c begins, generating an intermediate, subviral particle, or ISVP. Infection can be halted at this point with weak bases such as ammonium chloride or E-64 to block lysosomal, acid-dependent proteolytic enzymes. This demonstrates the necessity of proteolysis for the lytic cycle [20, 21]. Proteolysis of σ3 and μ1/μ1c can also occur in vivo by proteases within the intestinal lumen after peroral inoculation, or in vitro digestion can be performed with trypsin and chymotrypsin [22]. The resulting preformed ISVPs are still capable of cellular penetration, and do not require any further proteolysis to undergo a productive infection. Studies using recombinant viruses show that σ3-deficient virions are still capable of infection in the presence of ammonium chloride, and that protease-resistant μ1/μ1c-containing particles are still capable of infecting cells [23, 24]. This suggests that the degradation of σ3 is important in the initial steps in viral infection, but that cleavage of μ1/μ1c to its fragments (designated ϕ and μ1/μ1δ) may be dispensable for a productive infection.

At this point in the reovirus infection cycle, loss and degradation of σ3 in the endosome/lysosome theoretically exposes μ1/μ1c, allowing for penetration of the ISVP across the lysosomal membrane. μ1/μ1c has been shown to be capable of disrupting membrane bilayers in vitro [25, 26]. μ1/μ1c is also myristoylated, which may aid in ISVP/membrane fusion [27]. Following this step, primary transcription of 10 capped, full-length transcripts takes place, mediated by the viruses' double-stranded RNA-dependent RNA polymerase. Primary transcripts are translated using host machinery and subsequently associate with primary translation products to form RNA assortment complexes.

Final synthesis of minus strand genomic RNA occurs within these nascent particles and secondary transcription of late viral mRNAs begins. Late viral protein synthesis from secondary transcripts often coincides with a decrease in host protein synthesis [28]. Final assembly of the outer capsid yields progeny reovirus particles, leading to cell lysis and death.

Antitumor Mechanism

Transformation and Susceptibility to Reovirus

In 1977, Hashiro et al. [29] reported that reovirus efficiently replicated in specific transformed cell lines, yet was unable to productively infect normal cells of various origins. Duncan et al. [30] had similar findings using SV-40-transformed and normal WI-38 cells. It was apparent that transformation was related to reovirus susceptibility, however the underlying molecular basis of this susceptibility was unclear.

It was interesting that, although many cells possess the reovirus receptor for attachment, sialic acid [18, 19, 31], normal cells were not capable of supporting a productive reovirus infection [3, 32]. Further studies revealed that, in addition to the untransformed cells mentioned above, 3T3 cells (derivatives of 3T3) lacking the epidermal growth factor (EGF) receptor (designated NR6) were also relatively resistant to reovirus type 3 Dearing. NR6 cells transfected with the EGF receptor (HER5 cells) were, however, very infectable [32]. It was also found that the EGF receptor was recognized by reovirus [33]. From these findings, one might have postulated that, in addition to sialic acid, reovirus binding to the EGF receptor was involved in the initial stages of infection. However, subsequent studies revealed that this was not the case.

In later studies, Strong et al. [90] found that, upon introduction of a truncated EGF receptor (v-*erbB*) into NIH-3T3 fibroblasts, these poorly infectable cells were rendered extremely susceptible to reovirus. Since the truncated receptor lacked an extracellular ligand-binding domain, this study dismissed the role of the receptor as an important mediator of reovirus attachment. Rather, it indicated that reovirus was taking advantage of the induction of intracellular phenomena, whereby cellular susceptibility was conferred by the activation of signaling pathways by the cytosolic domain of the EGF receptor. It is of note that, although this receptor is truncated, it does possess constitutively active tyrosine kinase activity that impinges on intracellular signaling pathways. Therefore, it became important to determine if constitutive activation of signal transduction pathways downstream of the EGF receptor rendered cells infectable.

Downstream of the Epidermal Growth Factor Receptor

Although many signaling cascades emanate from the EGF receptor, the main pathway that regulates cell growth and survival is the Ras pathway (fig. 1). Normal activation of the EGF receptor, leading to activation of downstream signaling pathways, is initiated by ligand binding and subsequent receptor dimerization. This stimulates receptor tyrosine kinase (RTK) activity, leading to autophosphorylation of the cytoplasmic tail of the receptor [34–36]. The resulting

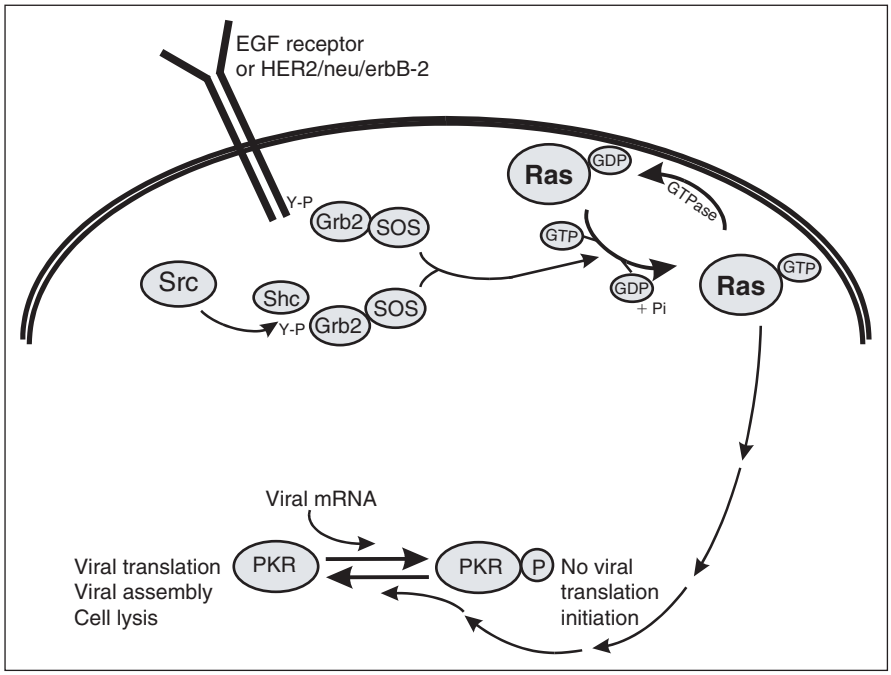

Fig. 1. Reovirus usurpation of the Ras pathway (see text). Reproduced with permission of the Journal of Clinical Investigation.

phosphotyrosine residues can then serve as docking sites for a variety of signaling proteins, including the adapter molecules, Shc, through its phosphotyrosine-binding domain (PTB), and Grb2, through its phosphotyrosine-binding Src-homology 2 (SH2) domain [37, 38]. Grb2 can also be recruited to the receptor indirectly through SH2 interaction with Shc phosphotyrosine residues [39, 40]. Recruitment of the GTP exchange factor SOS along with Grb2 to the plasma membrane allows SOS to activate GTP for GDP exchange on the small G protein Ras [41]. Formation of Ras-GTP can also be achieved through the activation of nonreceptor tyrosine kinases such as Src, which initiate the cascade through tyrosine phosphorylation of Shc and subsequent recruitment of Grb2 with SOS.

Once GDP is exchanged on Ras for GTP, the small G protein is capable of stimulating the activity of many cellular transduction pathways [35]. One well-studied pathway downstream of Ras is the mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) pathway. This pathway is stimulated by growth hormones such as EGF and insulin, as well as by chemical means such as treatment with phorbol esters. Ras-GTP is capable of

activating this pathway through binding and stimulation of Raf-1 kinase at the plasma membrane [42]. Raf-1 subsequently phosphorylates and stimulates MAPK kinase/ERK kinase (MEK), which is a dual specificity, serine/threonine and tyrosine kinase with an activity towards ERK [42–44]. Upon phosphorylation of ERK on specific threonine and tyrosine residues, it is activated to phosphorylate other enzymes such as p90 RSK, as well as transcription factors such as Elk-1.

Accumulation of Ras-GTP can also lead to activation of other signaling cascades [35]. For example, cellular stresses such as ultraviolet light, oxidative stress and nerve growth factor withdrawal from pheochromocytoma cells can stimulate the stress-activated protein kinase (SAPK) and the p38/HOG stress-activated kinase cascades [45, 46]. Activation of these pathways has been shown to be involved in both apoptosis [46, 47] and transformation [48, 49].

The myriad of signaling molecules downstream of the EGF receptor make for a daunting challenge when one is considering precisely which pathway(s) must be activated to permit reovirus replication. When cells with activated SOS or Ras were tested, it was found that they were also infectable [3]. This suggests that the Ras signaling pathway downstream of the EGF receptor plays an important role in host cell susceptibility to reovirus infection.

Ras and the Double-Stranded RNA-Activated Protein Kinase, PKR

Why were cells with activated Ras pathway members infectable, when untransformed NIH-3T3 cells were not? The answer may lie in the mechanisms of normal cellular defense against viral infection. Typically, upon initiation of viral replication within a cell, RNA replication intermediates by producing extensive double-stranded structures which activate the double-stranded RNA-activated protein kinase, PKR [6]. In the case of reovirus, PKR kinase activity is potently stimulated by double-stranded panhandle structures in S1 mRNA (fig. 1) [50]. Upon binding of two PKR molecules to double-stranded RNA, their kinase activity is stimulated and the molecules autophosphorylate in an intermolecular fashion [51]. Full enzymatic activation occurs via this transphosphorylation reaction, and PKR consequently develops activity towards other substrates, predominantly the α subunit of the translation initiation factor, eIF2. When eIF2-α is phosphorylated, it sequesters the initiation factor eIF2B. Sequestration inhibits the GDP/GTP exchange on eIF2 that is required for translation initiation, which halts viral translation initiation, and thus halts viral replication [52, 53]. A lack of PKR activity, achieved using the inhibitor 2-aminopurine, or using $PKR^{-/-}$ mouse embryo fibroblasts, allows reovirus to successfully infect a cell [3]. This verifies the importance of PKR in the cellular defense against reovirus infection.

Upon reovirus internalization by NIH-3T3 cells, primary transcription takes place (including S1 mRNA synthesis), and PKR autophosphorylates [3]. No viral proteins are synthesized and the infection is aborted at the stage of primary transcription. Presumably, PKR activation leads to phosphorylation of eIF2-α and selective inhibition of viral translation. In v-*erbB*-, *sos*- and *ras*-transformed cells, however, PKR activation was not observed [3]. Translation of viral protein could then ensue, leading to productive infection of the transformed cells. Somehow, PKR activity was inhibited in these cells, causing a lethal breakdown in the cellular defense against viral replication.

Previously, Mundschau and Faller [54] had also found an association between Ras transformation and inhibition of PKR activity. Their group found that transformation of BALB cells with K-*ras* lead to induction of an inhibitory activity that was capable of preventing PKR autophosphorylation and thus activation. This inducible inhibitor was not competitive double-stranded RNA, as none could be isolated from nucleic acid extracts of K-*ras*-transformed cells. It was not a small molecule either, for the inhibitory activity was heat-labile. Fractionation of K-*ras* extracts revealed a peak of PKR inhibitory activity at a M_r of approximately 100 kD, and dubbed this putative protein RIKI, for Ras-induced kinase inhibitor [55].

The question that remains is what lies downstream of Ras that actually signals to PKR to inhibit its typical kinase activity in response to reovirus infection. There is a good correlation between MAPK activity and cellular susceptibility to reovirus, however this association is not absolute (unpublished data). This may be explained by the activation of alternative pathways downstream of Ras, which would inhibit PKR and render cells susceptible.

Additional studies demonstrate the presence of MAPK-independent PKR inhibition, and furthermore suggest which alternative pathways may be involved. For example, abrogation of MAPK activity in Ras-transformed cells using the MEK inhibitor, PD98059, does not prevent Ras inhibition of PKR activity, and thus infection by reovirus proceeds unimpeded [3]. This implies that the signal transduced by Ras to inhibit PKR does not pass through MEK and MAPK. Interestingly, treatment of Ras-transformed cells with PD98059 actually *enhanced* the infectability of the cells [3]. It has been shown that blockage of one signaling pathway can enhance signaling through other axes, such as the stress-activated protein kinase cascades [44]. The involvement of JNK (c-Jun N-terminal kinase) and p38 stress-activated kinase cascades in reovirus replication is currently under investigation, and the link of cellular stress pathways to PKR activity is the topic of much research [56]. Studies are ongoing to determine the link between cellular transformation with Ras pathway members and inhibition of PKR activity.

Animal Tumor Models

Treatment of Tumor Xenografts in Immunocompromised Mice

The first studies conducted in vivo using reovirus type 3 Dearing as an oncolytic agent utilized v-*erbB*-transformed NIH-3T3 fibroblasts to establish tumors in severe combined immunodeficient (SCID) mice [5]. When hind flank subcutaneous tumors of approximately 0.5×0.5 cm had been established, they were injected once directly with 1×10^7 plaque forming units (pfu) of virus. After a period of 12 days, tumor growth had been restricted by 80% compared to control mice injected with UV-inactivated virus. This was repeated using the human glioma cell line, U87, in SCID mice. U87 cells are known to overexpress the platelet-derived growth factor receptor, and thus have high levels of Ras activity and are indeed very infectable by reovirus. After treatment with reovirus (as described above), tumor regression was achieved in 80% of the animals. From these studies it was apparent that reovirus was capable of killing murine and human tumor xenografts in an immune-compromised animal model.

Reovirus-Mediated Therapy in an Immune-Competent Organism

An important possibility to consider when using a virus as an oncolytic therapy is that the immune system may interfere with viral replication and prevent tumor killing. Studies have found that both the cellular and humoral arms of the immune response are important factors in reovirus infection and pathogenesis [57, 58]. The primary challenge associated with studying immunological effects on virus oncolysis, however, is that some oncolytic viruses replicate exclusively in human tissue. Consequently, only models utilizing human tumor xenografts in immunocompromised animals are possible, and the effect of an intact immune system cannot be studied. Because reovirus has a broad host range, it was possible to use a syngeneic mouse model to study reovirus oncolysis [5]. In this model, C3H mice were implantated on the hind flanks with *ras*-transformed C3H-10T1/2 fibroblasts. Tumors were injected six times with 1×10^8 pfu of reovirus over a period of 9 days, followed by treatment every 48 h with 1×10^7 pfu of reovirus for another 12 days. Both live-virus-treated and dead-virus-treated animals mounted similar anti-reovirus humoral immune responses. It was found that reovirus was still capable of eradicating tumors completely using this regimen, without any animal morbidity or mortality [5]. Additionally, no recurrence of tumor growth was seen in the animals that had exhibited complete regression. Thus, the presence of a competent immune system could not prevent the elimination of cancer in these mice.

Further studies were conducted which investigated the effect of the immune system on reovirus oncolysis in vivo. Due to the prevalence of reovirus exposure in the human population, there is a danger that patients will already

carry anti-reovirus antibodies and/or memory T cells specific for the virus. This 'priming' of the immune system could give rise to an enhanced immune response to reovirus administration, potentially leading to clearance of the virus before it can exert its oncolytic effects. Therefore, a model was established reflecting the typical immune scenario in adults. In this model, mice were exposed to reovirus 2 weeks prior to initiation of the experiment, and it was assured that the mice carried anti-reovirus antibodies. Implanted tumors were treated as before, and identical results of complete tumor regression were observed. This suggests that the presence of neutralizing antibodies had little effect on the capacity of reovirus to specifically target and destroy the tumors.

Use with Other Cancer Therapeutics

Reovirus has also been studied in conjunction with chemotherapeutic agents as an immunotherapy against cancer, rather than as a direct oncolytic agent. It has been found that treatment of L1210 leukemia ascites tumors with 1,3-bis(2-chloroethyl)-1-nitrosourea and reovirus results in increased survivability compared to treatment with only one therapy [59, 60]. This, combined with evidence that mice reject challenge with homologous tumor after the therapy, suggests that reovirus enhances recognition of the tumor cells by the immune system [61]. In contrast to these findings, rejection of homologous tumor challenge does not occur after reovirus therapy of the hind-flank tumor model in C3H mice, even though an immune response is mounted upon treatment with reovirus (as demonstrated by an increase in anti-reovirus antibody titer; unpublished data). Ultimately, the role of the immune system in tumor regression remains to be elucidated.

Alternative Delivery Mechanisms

Delivery of reovirus through routes alternative to intratumoral injection are currently under investigation. Current ongoing research shows that reovirus has the potential to kill tumors remote from the initial site of injection and replication in SCID mice; this raises the possibility of metastatic tumor treatment, concurrent with treatment of the primary tumor (unpublished data). If the virus is spreading systemically in this model, this also suggests that intravenous treatment of tumors could prove useful. As reovirus pathogenesis can vary with respect to the route of inoculation (see Safety Issues), the toxicity of such a delivery mechanism remains to be determined.

Safety Issues

Murine models of infection have been used extensively to study the pathogenesis of reovirus, especially as it applies to replication in the gastrointestinal

tract [22, 58, 62–65]. Some characteristics of murine models of reovirus infection seem to parallel those seen in humans, however other pathologies are not documented in humans, and are dependent on the use of singular strains of reovirus (for example serotype 3, clone 8B, a myocarditis-inducing form of reovirus [66]) or the use of immunocompromised or newborn animals. Reovirus type 3 has been linked to some illnesses in humans, for example biliary disease. The natural sites of replication of reovirus, the respiratory and enteric tracts, correlate with associations of infection with mild respiratory illnesses and diarrhea. However, an unequivocal etiological relationship with more serious disease is lacking. Thus, reovirus used for oncolytic studies was not modified in any way to alter its pathogenicity in humans.

Murine Models of Reovirus Infection

Many models of reovirus infection have been established in mice. Typically, the natural route of peroral inoculation allows the viral outer capsid to be proteolytically degraded within the intestinal lumen [22]. Reovirus uses the ileal microfold (M) cells to cross the intestinal wall, where it can subsequently be found in the underlying Peyer's patches and infecting gut epithelial cells from the basolateral side [65]. At this point, viral spread from the gastrointestinal tract is dependent on the serotype of virus: type 1 spreads to the mesenteric lymph nodes and spleen, whereas type 3 Dearing does not move beyond the Peyer's patches in adult mice [63]. Gastrointestinal infection is usually cleared without notable morbidity or mortality in this model.

There is a significant age- and inoculation route-dependence on the pathogenicity and virulence of reovirus infection in mice. For example, newborn mice inoculated perorally with type 3 reovirus show increased susceptibility to infection, for the virus spreads from the Peyer's patches to as far as the mesenteric lymph nodes [63]. Furthermore, upon intramuscular or footpad injection of newborn mice, type 3 Dearing was shown to enter the sciatic nerve and inferior spinal cord, cumulating in lethal meningoencephalitis [57, 67]. Although the severity of disease can range from mild to severe, reovirus pathogenesis can be restrained with the use of adoptive transfer of immunity. For example, it has been shown that adoptive transfer of reovirus-immune T lymphocytes protects against reovirus-induced meningoencephalitis [57, 68–70].

The potential to treat possible toxicity associated with reovirus replication through the administration of neutralizing anti-reovirus antibodies (both therapeutically versus reovirus infection and prophylactically) has also been investigated. Some animal toxicity did occur upon treatment of SCID mice in the flank tumor model described by Coffey et al. [5]. It was found that once complete tumor regression had been achieved mice would often develop necrosis of the extremities, including the hind limbs and ears [5] (unpublished data). SCID

mice have previously been shown to be susceptible to reovirus infection, however adoptive transfer of specific anti-reovirus antibodies confers protective immunity, enhancing clearance of the virus and survivability of the animal [58, 71]. This does not appear to be mediated through the complement system, however some studies have emphasized the importance of anti-reovirus antibodies in inhibiting reovirus replication at pre- and post-cellular attachment steps [70–72]. In addition, it has been found that anti-reovirus antibodies have a role in restricting viral spread from the primary site of replication [58, 68]. Such a treatment could prove useful in the immunocompromised animal tumor model to reduce morbidity and mortality.

In immunocompetent murine tumor models, as previously mentioned, reovirus was administered at high titers with multiple intratumoral injections. Mice were healthy for the duration of the experiment, and no morbidity or mortality was observed for 6 months following the experiment (unpublished data). Immunofluorescent analysis of paraffin-embedded sections revealed viral replication in tumors only, and did not uncover viral proteins in heart, liver, brain, or spleen tissue. The effect of alternate remote routes of administration on therapy toxicity is currently under investigation.

Association of Reovirus with Human Disease

Hepatobiliary disease, both in neonates and adults, has been extensively studied in relation to reovirus infection [reviewed in 73]. First reports measured levels of anti-reovirus antibodies in neonates with idiopathic, extrahepatic biliary atresia (EHBA) and neonatal hepatitis, compared to healthy controls and found conflicting results [74–77]. More recent studies using RT-PCR to detect viral RNA in affected tissue also offer conflicting results, which may be attributable to differing methodologies [78, 79]. One single case has been documented where virus-like particles were visualized by electron microscopy, however infectious virus has not been isolated [80]. It is difficult to confirm an etiologic role of reovirus in hepatobiliary disease, especially given the ubiquity of viral exposure in contrast to the relatively low incidence of EHBA and choledochal cysts. While many of these studies have found an association between what appears to be reovirus infection and hepatobiliary disease, the association may not be a causal one, and may simply reflect exposure to reovirus coinciding with presentation of the disease.

In adults, idiopathic liver and biliary disease has been examined as well with respect to reovirus infection. No correlation was found between idiopathic cholestatic liver disease and anti-reovirus antibody titers: 91% of cholestatic disease patients were seropositive, 70% of chronic liver disease patients, and 100% of healthy volunteers [13, 14]. Viral cultures were also negative, as was immunocytochemistry of reovirus antigens in sample biopsy tissues [14].

Again, the relationship between reovirus infection and adult hepatobiliary disease is not well established.

Outbreaks of reovirus infection among children in nurseries have been studied. Upon infection by reovirus, a rise in seropositivity is documented. In their investigation in 1960, Rosen et al. [7] observed a rise in titer in 76% of 34 children during an outbreak. Symptoms of children from whom virus could be isolated included coughs, diarrhea and coryza, which may have been a result of reovirus infection, or they may have had another etiology. The investigators concluded that, although the majority of children were exposed to reovirus and developed anti-reovirus antibodies, this particular outbreak 'was not recognis(able) clinically' [7].

Perhaps the most compelling evidence authenticating the nonpathogenicity of reovirus involves a study in 1963 by Rosen et al. [4]. In this study, male volunteers from American federal correctional institutions aged 21–38 years were inoculated intranasally with reovirus type 1, 2, or 3. Nine of twenty-seven individuals developed mild symptoms such as sneezing, malaise, and pharyngitis, but had no signs of fever. The mild illness observed was not necessarily due to reovirus infection, as some of the sick volunteers showed no evidence of productive infection by serologic or virologic tests. Of the 9 volunteers inoculated with reovirus type 3, 2 developed 'mild' rhinitis. Reovirus was able to replicate as evidenced by the presence of virus in anal specimens from 8 of the 9 volunteers, and by the presence of anti-reovirus antibodies. The ability of the intestinal tract to support reovirus replication, leading to the viral shedding noted in this paper could be due to the high levels of tyrosine kinase and MAPK activity in rapidly dividing, undifferentiated crypt cells [81]. These data indicate that, although reovirus is capable of undergoing a productive infection in adults, infection is benign in terms of significant pathology.

Applicability of Reovirus as an Oncolytic Therapy

Given that approximately 30% of all human cancers arise from or carry a mutation in one of the *ras* genes, reovirus has the potential to be effective in the treatment of a variety of human tumor types. There are three known *ras* genes K-, H-, and N-*ras*, all of which can be found mutated in human cancers. Mutation of residues 12, 13, and 61 are the most common, and such mutation leads to the inability of Ras-GTPase-activating proteins (GAPs) to stimulate Ras-GTPase activity [49, 82]. Since Ras-GTPase activity is intrinsically low without stimulation, overall Ras-GTP levels will rise without the catalytic influence of Ras-GAPs. Stimulation of mitogenic and anti-apoptotic pathways

by high levels of Ras-GTP contributes to uncontrolled cellular proliferation and establishment of a tumor.

A wide spectrum of tumors harbor these activating *ras* mutations. For example, 40–50% of colorectal cancers habor mutations in *ras* [83–85] in addition to many lung adenocarcinomas [86], thyroid neoplasms, seminomas, and acute myelogenous, chronic myelomonocytic and acute lymphoblastic leukemias [87]. Perhaps the most prevalent incidence of *ras* mutation in a form of human cancer is seen in adenocarcinoma of the exocrine pancreas, where up to 95% of tumors carry a mutation at codon 12 in the K-*ras* gene [88]. A small proportion of tumors arise from amplification of *ras* protooncogenes, and are potential targets for reovirus oncolytic therapy as well. These include 4–8% of ovarian, 3% of breast, and 4% of lung cancers [89]. There is also applicability to benign premalignant hyperplasia, as groups have found K-*ras* mutations in adenomatous tissue of the colon [83–85]. It is evident that *ras* mutation and amplification contribute to a significant proportion of human malignancies and premalignancies, and thus reovirus has the potential to target a wide array of tumor species.

The scope of tumors theoretically treatable by reovirus expands further when one considers that activation of the Ras signal transduction pathway can occur through more means than simple amplification or mutation of Ras. As mentioned above, initial work conducted on reovirus biology demonstrated that cells transformed with v-*erbB*, a truncated EGF receptor mutant with constitutive tyrosine kinase activity, are infectable by reovirus [90]. Cells transformed with other members of the EGF receptor family also are infectable by reovirus, including HER2/*neu*/c-*erbB2*-expressing cells (unpublished data). Finally, as previously mentioned, nonreceptor tyrosine kinase activation can lead to Ras activation, as seen in v-*src*-transformed cells. These cells have overactive signaling through the Ras pathway, as indicated by MAPK activation, and are indeed infectable by reovirus (unpublished data).

When one contemplates the reported incidences of receptor and nonreceptor tyrosine kinase activation in human cancers, it becomes apparent that reovirus treatment may be applicable to a broad spectrum of tumors. Twenty-five to thirty percent of breast cancers have an amplification of HER2/*neu*/c-*erbB2*, resulting in overexpression of the protein, high levels of receptor tyrosine kinase activity, and high levels of Ras activation. The same has been found for ovarian malignancies. In some cases of breast and ovarian cancers, overexpression of the gene product can occur in the absence of extra copies of the gene [91]. Furthermore, amplification and/or overexpression of this gene are poorly prognostic in terms of cancer recurrence. In fact, its overexpression has been reported to be a more accurate predictor of recurrence than any other determinant, save node-positive findings. Although the prognosis associated

with these genetic lesions is often poor, the environment created by these lesions should favor reovirus oncolysis.

Amplification of receptor tyrosine kinases is also documented in a variety of other neoplasms. Amplification of the EGF receptor gene, c-*erbB* has been found in 38–50% of glioblastomas, and to a lesser extent in head and neck, gastric, and esophageal carcinomas [89, 92]. Supporting the theoretical incidences of Ras pathway activation in these tumor types which overexpress receptor tyrosine kinases, many breast carcinoma and glioblastoma cell lines have been found infectable by reovirus, as well as primary human tumor tissues of these types (unpublished data).

Of the nonreceptor tyrosine kinases, Src and Bcr-Abl are clinically important in oncogenesis. Src has been found highly active in most isolates of breast cancer and mutated in advanced colon cancer [93–96]. *src*-transformed cells have been found to be infectable by reovirus (unpublished data). It is also well known that the Philadelphia chromosome product, Bcr-Abl (found in 95% of chronic myelogenous leukemia and 10% of acute lymphocytic leukemia) possesses constitutive nonreceptor-tyrosine kinase activity [97]. Since Bcr-Abl-mediated transformation is dependent on Ras activation, reovirus could theoretically replicate in these cells as well. Elevated levels of tyrosine kinase activity in neoplasms, whether it originates from receptor or nonreceptor tyrosine kinases, favor the use of reovirus as an effective oncolytic agent.

Overactivation of the Ras pathway can occur through many means, and this is reflected in the range of possible genetic abnormalities leading to transformation and cancer. Optimistically, although there exists such a potential for uncontrolled cellular proliferation, this potential is matched by the capacity of reovirus to replicate in a broad variety of tumor backgrounds.

Human Trials

Phase I clinical trials are currently underway.

Conclusions

Given the effectiveness of reovirus as an oncolytic agent in the laboratory environment, reovirus will hopefully have a significant impact in a clinical setting. The knowledge accumulated on viral biology, both of reovirus and of other viruses described within this book, has permitted researchers and clinicians to broach a new field in cancer therapy research: the use of viral-based

‘magic bullets’ to target cancer. The hope persists that such therapies might be able to successfully overcome the final, most formidable challenge of treating cancer in the human organism.

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References

1 Lowe PN, Skinner RH: Regulation of Ras signal transduction in normal and transformed cells. Cell Signal 1994;6:109–123.
2 Levitzki A: Signal-transduction therapy. A novel approach to disease management. Eur J Biochem 1994;226:1–13.
3 Strong JE, Coffey MC, Tang D, Sabinin P, Lee PW: The molecular basis of viral oncolysis: Usurpation of the Ras signaling pathway by reovirus. EMBO J 1998;17:3351–3362.
4 Rosen L, Evans HE, Spickard A: Reovirus infections in human volunteers. Am J Hyg 1963; 77:29–37.
5 Coffey MC, Strong JE, Forsyth PA, Lee PW: Reovirus therapy of tumors with activated Ras pathway. Science 1998;282:1332–1334.
6 Tyler KL, Fields BN: Reoviruses; in Fields BN, Knipe DM, Howley PM (eds): Fields Virology. Philadelphia, Lippincott-Raven, 1996, pp 1597–1623.
7 Rosen L, Hovis J, Mastrota FM, Bell JA, Huebner RJ: Observations on a newly recognized virus (Abney) of the reovirus family. Am J Hyg 1960;71:258–265.
8 Sabin AB: Reoviruses: A new group of respiratory and enteric viruses formerly classified as ECHO type 10 is described. Science 1959;130:1387–1389.
9 Ridinger DN, Spendlove RS, Barnett BB, George DB, Roth JC: Evaluation of cell lines and immunofluorescence and plaque assay procedures for quantifying reoviruses in sewage. Appl Environ Microbiol 1982;43:740–766.
10 Stanley NF: Reoviruses. Br Med Bull 1967;23:150–154.
11 Jackson GG, Muldoon RL: Viruses causing common respiratory infection in man. IV. Reoviruses and adenoviruses. J Infect Dis 1973;128:811–833.
12 Stanley NF: The reovirus murine models. Prog Med Virol 1974;18:257–272.
13 Minuk GY, Paul RW, Lee PW: The prevalence of antibodies to reovirus type 3 in adults with idiopathic cholestatic liver disease. J Med Virol 1985;16:55–60.
14 Minuk GY, Rascanin N, Paul RW, Lee PW, Buchan K, Kelly JK: Reovirus type 3 infection in patients with primary biliary cirrhosis and primary sclerosing cholangitis. J Hepatol 1987;5:8–13.
15 Lee PWK, Hayes EC, Joklik WK: Protein sigma 1 is the reovirus cell attachment protein. Virology 1981;108:156–163.
16 Weiner HL, Fields BN: Neutralization of reovirus: the gene responsible for the neutralization antigen. J Exp Med 1977;146:1305–1310.
17 Strong JE, Leone G, Duncan R, Sharma RK, Lee PW: Biochemical and biophysical characterization of the reovirus cell attachment protein sigma 1: Evidence that it is a homotrimer. Virology 1991;184:23–32.
18 Paul RW, Choi AH, Lee PW: The alpha-anomeric form of sialic acid is the minimal receptor determinant recognized by reovirus. Virology 1989;172:382–385.
19 Choi AH, Paul RW, Lee PW: Reovirus binds to multiple plasma membrane proteins of mouse L fibroblasts. Virology 1990;178:316–320.

20 Sturzenbecker LJ, Nibert M, Furlong D, Fields BN: Intracellular digestion of reovirus particles requires a low pH and is an essential step in the viral infectious cycle. J Virol 1987;61:2351–2361.
21 Canning WM, Fields BN: Ammonium chloride prevents lytic growth of reovirus and helps to establish persistent infection in mouse L cells. Science 1983;219:987–988.
22 Bodkin DK, Nibert ML, Fields BN: Proteolytic digestion of reovirus in the intestinal lumens of neonatal mice. J Virol 1989;63:4676–4681.
23 Chandran K, Nibert ML: Protease cleavage of reovirus capsid protein mu1/mu1C is blocked by alkyl sulfate detergents, yielding a new type of infectious subvirion particle. J Virol 1998;72: 467–475.
24 Chandran K, Walker SB, Chen Y, et al: In vitro recoating of reovirus cores with baculovirus-expressed outer-capsid proteins mu1 and sigma3. J Virol 1999;73:3941–3950.
25 Lucia-Jandris P, Hooper JW, Fields BN: Reovirus M2 gene is associated with chromium release from mouse L cells. J Virol 1993;67:5339–5345.
26 Hooper JW, Fields BN: Monoclonal antibodies to reovirus sigma 1 and mu 1 proteins inhibit chromium release from mouse L cells. J Virol 1996;70:672–677.
27 Nibert ML, Schiff LA, Fields BN: Mammalian reoviruses contain a myristoylated structural protein. J Virol 1991;65:1960–1967.
28 Zweerink HJ, Joklik WK: Studies on the intracellular synthesis of reovirus-specified proteins. Virology 1970;41:501–518.
29 Hashiro G, Loh PC, Yau JT: The preferential cytotoxicity of reovirus for certain transformed cell lines. Arch Virol 1977;54:307–315.
30 Duncan MR, Stanish SM, Cox DC: Differential sensitivity of normal and transformed human cells to reovirus infection. J Virol 1978;28:444–449.
31 Gentsch JR, Pacitti AF: Effect of neuraminidase treatment of cells and effect of soluble glycoproteins on type 3 reovirus attachment to murine L cells. J Virol 1985;56:356–364.
32 Strong JE, Tang D, Lee PW: Evidence that the epidermal growth factor receptor on host cells confers reovirus infection efficiency. Virology 1993;197:405–411.
33 Tang D, Strong JE, Lee PW: Recognition of the epidermal growth factor receptor by reovirus. Virology 1993;197:412–414.
34 Schlessinger J: How receptor tyrosine kinases activate Ras. Trends Biochem Sci 1993;18:273–275.
35 Campbell SL, Khosravi-Far R, Rossman KL, Clark GJ, Der CJ: Increasing complexity of Ras signaling. Oncogene 1998;17:1395–1413.
36 Vojtek AB, Der CJ: Increasing complexity of the Ras signaling pathway. J Biol Chem 1998;273: 19925–19928.
37 Kavanaugh WM, Williams LT: An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. Science 1994;266:1862–1865.
38 Batzer AG, Blaikie P, Nelson K, Schlessinger J, Margolis B: The phosphotyrosine interaction domain of Shc binds an LXNPXY motif on the epidermal growth factor receptor. Mol Cell Biol 1995;15:4403–4409.
39 Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA: Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature 1993;363:45–51.
40 Pelicci G, Lanfrancone L, Grignani F, et al: A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. Cell 1992;70:93–104.
41 Buday L, Downward J: Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell 1993;73: 611–620.
42 van Aelst L, Barr M, Marcus S, Polverino A, Wigler M: Complex formation between RAS and RAF and other protein kinases. Proc Natl Acad Sci USA 1993;90:6213–6217.
43 Kyriakis JM, App H, Zhang XF, et al: Raf-1 activates MAP kinase-kinase. Nature 1992;358: 417–421.
44 Jarvis WD, Fornari FAJ, Auer KL, et al: Coordinate regulation of stress- and mitogen-activated protein kinases in the apoptotic actions of ceramide and sphingosine. Mol Pharmacol 1997;52: 935–947.

45 Derijard B, Hibi M, Wu IH, et al: JNK1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 1994;76:1025–1037.
46 Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME: Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326–1331.
47 Verheij M, Bose R, Lin XH, et al: Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 1996;380:75–79.
48 Rodrigues GA, Park M, Schlessinger J: Activation of the JNK pathway is essential for transformation by the Met oncogene. EMBO J 1997;16:2634–2645.
49 Clark GJ, Westwick JK: Der CJ: p120 GAP modulates Ras activation of Jun kinases and transformation. J Biol Chem 1997;272:1677–1681.
50 Bischoff JR, Samuel CE: Mechanism of interferon action. Activation of the human P1/eIF-2 alpha protein kinase by individual reovirus s-class mRNAs: s1 mRNA is a potent activator relative to s4 mRNA. Virology 1989;172:106–115.
51 Thomis DC, Samuel CE: Mechanism of interferon action: Evidence for intermolecular autophosphorylation and autoactivation of the interferon-induced, RNA dependent protein kinase PKR. J Virol 1993;67:7695–7700.
52 Levin DH, Petryshyn R, London IM: Characterization of double-stranded-RNA-activated kinase that phosphorylates alpha subunit of eukaryotic initiation factor 2 (eIF-2 alpha) in reticulocyte lysates. Proc Natl Acad Sci USA 1980;77:832–836.
53 Panniers R, Henshaw EC: A GDP/GTP exchange factor essential for eukaryotic initiation factor 2 cycling in Ehrlich ascites tumor cells and its regulation by eukaryotic initiation factor 2 phosphorylation. J Biol Chem 1983;258:7928–7934.
54 Mundschau LJ, Faller DV: Oncogenic ras induces an inhibitor of double-stranded RNA-dependent eukaryotic initiation factor 2 alpha-kinase activation. J Biol Chem 1992;267: 23092–23098.
55 Mundschau LJ, Faller DV: Endogenous inhibitors of the dsRNA-dependent eIF-2 alpha protein kinase PKR in normal and ras-transformed cells. Biochimie 1994;76:792–800.
56 Williams BR: PKR; a sentinel kinase for cellular stress. Oncogene 1999;18:6112–6120.
57 Virgin HW, Tyler KL: Role of immune cells in protection against and control of reovirus infection in neonatal mice. J Virol 1991;65:5157–5164.
58 Barkon ML, Haller BL, Virgin HW: Circulating immunoglobulin G can play a critical role in clearance of intestinal reovirus infection. J Virol 1996;70:1109–1116.
59 Bryson JS, Cox DC: Characteristics of reovirus-mediated chemoimmunotherapy of murine L1210 leukemia. Cancer Immunol Immunother 1988;26:132–138.
60 Steele TA, Cox DC: Reovirus type 3 chemoimmunotherapy of murine lymphoma is abrogated by cyclosporine. Cancer Biother 1995;10:307–315.
61 Williams ME, Cox DC, Stevenson JR: Rejection of reovirus-treated L1210 leukemia cells by mice. Cancer Immunol Immunother 1986;23:87–92.
62 Derrien M, Fields BN: Reovirus type 3 clone 9 increases interleukin-1alpha level in the brain of neonatal, but not adult, mice. Virology 1999;257:35–44.
63 Kauffman RS, Wolf JL, Finberg R, Trier JS, Fields BN: The σ1 protein determines the extent of spread of reovirus from the gastrointestinal tract of mice. Virology 1982;124:403–410.
64 Wolf JL, Kauffman RS, Finberg R, Dambrauskas R, Fields BN, Trier JS: Determinants of reovirus interaction with the intestinal M cells and absorptive cells of murine intestine. Gastroenterology 1983;85:291–300.
65 Wolf JL, Rubin DH, Finberg R, et al: Intestinal M cells: a pathway for entry of reovirus into the host. Science 1981;212:471–472.
66 Sherry B, Li XY, Tyler KL, Cullen JM, Virgin HW: Lymphocytes protect against and are not required for reovirus-induced myocarditis. J Virol 1993;67:6119–6124.
67 Tyler KL, McPhee DA, Fields BN: Distinct pathways of viral spread in the host determined by reovirus S1 gene segment. Science 1986;233:4765–4770.
68 Tyler KL, Virgin HW, Bassel-Duby R, Fields BN: Antibody inhibits defined stages in the pathogenesis of reovirus serotype 3 infection of the central nervous system. J Exp Med 1989; 170:887–900.

69 George A, Kost SI, Witzleben CL, Cebra JJ, Rubin DH: Reovirus-induced liver disease in severe combined immunodeficient (SCID) mice. A model for the study of viral infection, pathogenesis, and clearance. J Exp Med 1990;171:929–934.
70 Virgin HW, Bassel-Duby R, Fields BN, Tyler KL: Antibody protects against lethal infection with the neurally spreading reovirus type 3 (Dearing). J Virol 1988;62:4594–4604.
71 Tyler KL, Mann MA, Fields BN, Virgin HW: Protective anti-reovirus monoclonal antibodies and their effects on viral pathogenesis. J Virol 1993;67:3446–3453.
72 Virgin HW, Dermody TS, Tyler KL: Cellular and humoral immunity to reovirus infection; in Tyler KL, Oldstone MBA (eds): Reoviruses II. New York, Springer, 1998, pp 147–162.
73 Organ EL, Rubin DH: Pathogenesis of reovirus gastrointestinal and hepatobiliary disease; in Tyler KL, Oldstone MBA (eds): Reoviruses II. New York, Springer, 1998, pp 67–83.
74 Morecki R, Glaser JH, Cho S, Balistreri WF, Horwitz MS: Biliary atresia and reovirus type 3 infection. N Engl J Med 1982;307:481–484.
75 Glaser JH, Balistreri WF, Morecki R: Role of reovirus type 3 in persistent infantile cholestasis. J Pediatr 1984;105:912–915.
76 Brown WR, Sokol RJ, Levin MJ, et al: Lack of correlation between infection with reovirus 3 and extrahepatic biliary atresia or neonatal hepatitis. J Pediatr 1988;113:670–676.
77 Dussaix E, Hadchouel M, Tardieu M, Alagille D: Biliary atresia and reovirus type 3 infection. N Engl J Med 1984;310:658.
78 Tyler KL, Sokol RJ, Oberhaus SM, et al: Detection of reovirus RNA in hepatobiliary tissues from patients with extrahepatic biliary atresia and choledochal cysts. Hepatology 1998;27:1475–1482.
79 Steele MI, Marshall CM, Lloyd RE, Randolph VE: Reovirus 3 not detected by reverse transcriptase-mediated polymerase chain reaction analysis of preserved tissue from infants with cholestatic liver disease. Hepatology 1995;21:697–702.
80 Glaser JH, Morecki R: Reovirus type 3 and neonatal cholestasis. Semin Liver Dis 1987;7:100–107.
81 Mamajiwalla SN, Burgess DR: Differential regulation of the activity of the 42 kD mitogen activated protein kinase (p42mapk) during enterocyte differentiation in vivo. Oncogene 1995; 11:377–386.
82 Bos JL: ras oncogenes in human cancer: A review (published erratum appears in Cancer Res 1990; 50:1352). Cancer Res 1989;49:4682–4689.
83 Forrester K, Almoguera C, Han K, Grizzle WE, Perucho M: Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. Nature 1987;327:298–303.
84 Vogelstein B, Fearon ER, Hamilton SR, et al: Genetic alterations during colorectal-tumor development. N Engl J Med 1988;319:525–532.
85 Bos JL, Fearon ER, Hamilton SR, et al: Prevalence of ras gene mutations in human colorectal cancers. Nature 1987;327:293–297.
86 Slebos RJ, Kibbelaar RE, Dalesio O, et al: K-ras oncogene activation as a prognostic marker in adenocarcinoma of the lung. N Engl J Med 1990;323:561–565.
87 Beaupre DM, Kurzrock R: *RAS* and leukemia: From basic mechanisms to gene-directed therapy. J Clin Oncol 1999;17:1071–1079.
88 Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N, Perucho M: Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. Cell 1988;53:549–554.
89 Brison O: Gene amplification and tumor progression. Biochim Biophys Acta 1993;1155:25–41.
90 Strong JE, Lee PW: The v-erbB oncogene confers enhanced cellular susceptibility to reovirus infection. J Virol 1996;70:612–616.
91 Slamon DJ, Godolphin W, Jones LA, et al: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989;244:707–712.
92 Bredel M, Pollack IF: The p21-*Ras* signal transduction pathway and growth regulation in human high-grade gliomas. Brain Res Brain Res Rev 1999;29:232–249.
93 Verbeek BS, Vroom TM, Adriaansen-Slot SS, et al: c-Src protein expression is increased in human breast cancer. An immunohistochemical and biochemical analysis. J Pathol 1996;180:383–388.
94 Ottenhoff-Kalff AE, Rijksen G, van Beurden EACM, Hennipman A, Michels AA, Staal GEJ: Characterization of protein tyrosine kinases from human breast cancer: Involvement of the *c-src* oncogene product. Cancer Res 1992;52:4773–4778.

95 Jacobs C, Rubsamen H: Expression of pp60c-src protein kinase in adult and fetal human tissue: High activities in some sarcomas and mammary carcinomas. Cancer Res 1983;43:1696–1702.
96 Irby RB, Mao W, Coppola D, et al: Activating SRC mutation in a subset of advanced human colon cancers. Nat Genet 1999;21:187–190.
97 Zou X, Calame K: Signaling pathways activated by oncogenic forms of Abl tyrosine kinase. J Biol Chem 1999;274:18141–18144.

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Autonomous Parvoviruses

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Introduction

The family of Parvoviridae is comprised of non-enveloped nuclear-replicating DNA viruses that owe their name to their small size (approximately 20–25 nm in diameter) [1]. Parvoviridae members that are infectious for vertebrates are subdivided into the so-called adeno-associated viruses (AAVs; genus Dependovirus) and autonomous parvoviruses (genera Parvovirus and Erythrovirus). AAVs typically require a helper virus for their efficient replication, while the lytic life cycle of autonomous parvoviruses relies on cellular factors whose expression is associated with proliferation (the S phase of the cell cycle) and differentiation [1–4]. This chapter is devoted to the autonomous parvoviruses, with special emphasis being given to two members of the genus Parvovirus, namely the H-1 virus and the minute virus of mice (MVM). Although the natural hosts of these agents are rodents, H-1 virus, MVM and the related parvoviruses LuIII and rat virus (RV) are able to grow in a variety of human cells and thus have potential for human applications. In keeping with this view, the H-1 virus was able to induce a viremia in infected patients, while having no pathological effects [5].

Several rodent parvoviruses, including H-1 virus and MVM, have been shown to exert an oncosuppressive effect, i.e. they are able to inhibit the formation of spontaneous as well as chemically or virally induced tumors in laboratory animals [6]. Implants of tumor cells, including human neoplastic cells, can also be targets for the parvoviral oncosuppressive activity in recipient animals [6–8]. The mechanism of parvovirus-induced tumor suppression is unclear to date and is likely to involve several components. Besides modulating immune or inflammatory responses that may contribute to tumor rejection [9–11],

parvoviruses can directly and preferentially kill some neoplastic cells. For instance, a number of in vitro transformed or tumor-derived human and rodent cell lines of fibroblastic and epithelial origin appear significantly more sensitive to the cytocidal action of these viruses than the corresponding untransformed parental cells [7, 12–19]. Though also observed in a short-term tissue culture system [20], this oncolytic effect needs to be evaluated under in vivo conditions for its overall contribution to the antineoplastic activity of parvoviruses.

The predilection of several rodent parvoviruses for neoplastic cells has been known for a long time, as these agents were isolated from tumor implants or tumor cell filtrates under conditions in which they could not be detected in normal tissues [21, 22]. This oncotropism was recently exploited to isolate a new rat parvovirus from a tumor implanted in an animal that was suspected of being parvovirus-infected [23]. Some tumor cells appear to provide these viruses with an environment beneficial to their amplification and expression. Indeed, the in vitro transformation of a number of human and rodent cells by various treatments (radiation, chemicals, oncogenes) was found to correlate not only with their sensitization to parvovirus-induced killing, as stated above, but also with their increased capacity for sustaining certain steps of the viral life cycle. Transformation-enhanced events during the parvovirus growth cycle include in particular DNA amplification and gene expression, while no significant change in virus uptake has so far been reported [7, 12–17, 19]. Interestingly, the production and toxic activity of the nonstructural polypeptide NS1, the main viral effector of parvovirus replication and cytolysis, are both stimulated in oncogene-transformed cells [14–15, 17, 24–26]. It should be stated, however, that transformed cells showing an increased permissiveness for parvovirus DNA replication, gene expression and resultant cytopathic effect are not always able to support the full infection that would lead to the release of progeny particles [13, 18]. The intrinsic propensity of various rodent parvoviruses for performing at least part of their lytic life cycle in (pre)neoplastic cells makes them appealing anticancer tools. However, the mere fact that these agents were often isolated from growing tumors shows that the natural viruses are not always successful in eradicating infected tumors. Yet, the oncotropic and oncolytic properties of these parvoviruses may be exploited by using them as DNA-replication-competent vectors to achieve a targeted co-expression of viral and heterologous genes with therapeutic potential in tumors.

Biology of the Autonomous Parvoviruses

Genome Organization

Parvoviruses contain an approximately 5,150-nucleotide-long linear single-stranded DNA genome flanked by short inverted repeats [1, 27]. The genomes

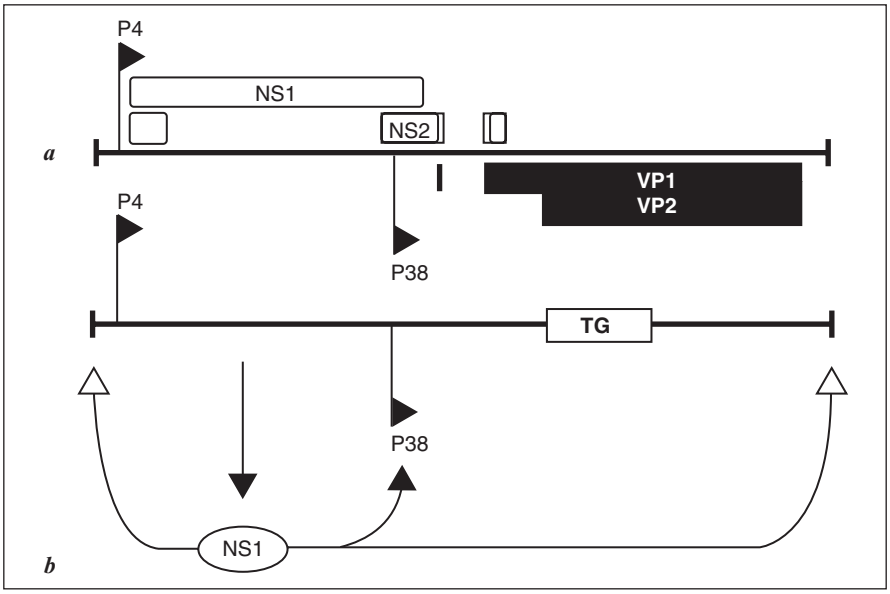

Fig. 1. Schematic representation of the genome organization of autonomous rodent parvoviruses (***a***) and derived recombinant viruses (***b***). The viral genome flanked by palindromic sequences at both ends are depicted by solid lines and standing bars, respectively. The positions of the transcription start sites of the P4 and P38 promoters are indicated by flags. ***a*** The NS- and VP-coding regions are indicated by open and filled boxes, respectively. ***b*** The coding region of the transgene is indicated by the open box (labeled TG). The translation of the transgene product starts at the VP2 initiation site. The length of the residual nontranslated VP sequence may vary depending from the size of the transgene. The replicative and transactivating functions of the NS1 polypeptide are indicated by open and closed arrow heads, respectively.

of the H-1 and MVM viruses consist of two large open reading frames (ORF) whose expression is directed by the P4 and P38 promoters, respectively (fig. 1). The P4 promoter is located on the left-hand side of the genome and controls the transcription unit encoding the regulatory nonstructural proteins (NS1 and NS2), whilst the P38 promoter is located in the middle of the genome and controls expression of the gene encoding the capsid proteins VP1 and VP2. All transcripts terminate close to the right end of the genome. Due to the differential splicing of P4- and P38-directed precursor mRNAs, NS1 and the smaller NS2 polypeptides overlap in their N-terminal regions, and VP2 is entirely contained within VP1 [1, 27]. Besides playing essential roles in viral DNA replication and cytotoxicity (see below), NS1 is a transcription factor capable of strongly transactivating the internal P38 promoter [28, 29]. The NS2 product appears to

regulate several steps in the viral life cycle [30, 31], and is required for the MVM parvovirus to exert pathological effects in its natural host [32]. To form the parvoviral capsids, VP1 and VP2 associate in a defined stoichiometry [33]. As a result of proteolytic VP2 cleavage, full but not empty particles may contain copies of a third capsid protein (VP3).

Replication, Transcription and Oncotropism

Both ends of the genome possess palindromic sequences that can form hairpin structures and serve as self-priming origins of DNA replication [1, 27, 34]. H-1 virus and MVM DNA replicate in infected cell nuclei through a continuous elongation mechanism resulting in the formation of double-stranded (duplex) monomeric and multimeric intermediates [27, 34]. In contrast to AAV, autonomous parvovirus DNA has so far not been found to integrate into the host genome under natural conditions [35]. The input single-stranded viral DNA is first converted into a duplex form in an S-phase-dependent fashion, through a process that does not rely on viral proteins. A strict correlation was observed between the occurrence of conversion and the S-phase-associated appearance of cyclin A/cdk2 kinase activity [36]. Conversion is followed by expression of the duplex monomeric replicative intermediate and by its amplification via multimeric forms in an NS1-dependent manner [27, 34]. NS1 displays helicase, DNA-nicking, ATPase, and sequence-specific DNA-binding activities which, together with functions supplied by specific host factors, are required for the initiation of strand-displacement synthesis from terminal and internal origins located in monomeric and multimeric replicative forms, respectively [1, 34, 37, 38]. NS1 becomes covalently attached to the DNA 5′ ends as a result of its site-specific single-strand nicking activity [34]. As the production of single-stranded progeny genomes of parvovirus MVM from duplex replicative intermediates by a displacement synthesis reaction does not take place in the absence of capsids, replication and encapsidation appear to be tightly coupled processes [39, 40]. It is worth mentioning that oncogenic transformation results in an enhanced capacity of human fibroblasts for parvoviral DNA amplification [12, 13]. In particular, neoplastic transformation was shown to correlate with a striking stimulation of the resolution of MVM DNA concatamers into monomer-length replication intermediates [41].

It has recently been shown that the parvoviral promoter P4 is induced at the G1/S phase transition. This induction results from both P4 release from a cell confluence-associated repression involving in cis cyclic AMP response elements and in the trans cyclin-dependent kinase inhibitor p27, and P4 activation mediated by the cellular transcription factor E2F [42]. The S-coupled induction of the P4 promoter is necessary for production of the viral replicative protein NS1 in amounts high enough to initiate viral DNA amplification [2]. Several

oncoproteins can further contribute to transcriptional stimulation from the pivotal P4 promoter, leading to higher levels of NS proteins in oncogene-transformed versus nontransformed cells [14, 15, 24]. Transcription factors of the ATF and Ets families mediate the increased P4 promoter activity in *ras*-transformed rat fibroblasts compared to nontransformed parental cells [43, 44]. The transformation of human fibroblasts with simian virus 40 correlates with a stimulation of the P4 promoter via the transcription factor NF-Y [45, 46]. In the parvoviral DNA context, the promoter P4 can thus be considered responsive to various cell cycle regulators and oncoproteins. It should be stated that the induction of the P4 promoter in proliferating and transformed cells gives an underestimation of the overall stimulation of parvoviral gene (or transgene) expression. Indeed, the amplification of viral duplex DNA intermediates, i.e. transcription templates, can also be enhanced in these cells as a result of both an increased production of the viral replicative protein NS1 and apparent changes in cellular replication factors, as discussed above.

It follows from these different regulations that parvovirus growth shows an absolute requirement for cell proliferation [47], whilst further enhancement of the parvoviral life cycle in transformed versus nontransformed cells varies both qualitatively and quantitatively depending on the system being considered. Transformation of proliferating cells may result in an increase in their full permissiveness, i.e. competence for sustaining a productive parvovirus infection [12, 13, 18], or only in their ability to replicate and/or express parvoviral DNA in the absence of virus production [13–19]. An enhanced capacity for parvovirus growth is, however, not an obligatory consequence of neoplastic transformation, since a few exceptions to the general correlation between these two features have also been observed [17, 19]. It is worth noting that, except for one study [20], the analysis of parvovirus oncotropism was carried out with established cell lines and therefore needs to be extended to short-term cultures of tumor versus normal cells.

Cytotoxicity and Oncolysis

The viral NS1 protein is endowed with a cytotoxic function and is considered to be a major mediator of parvovirus-induced cytotoxicity [48–54], although other viral effectors may also play a role in cell killing [50]. The NS1 nucleoside triphosphate-binding domain of the MVMp, H-1 and B19 viruses is required for cytotoxicity in the full protein context [49, 52, 54]. Furthermore, cell survival can be jeopardized as a result of the mere expression of the NS1 polypeptide ends, in particular the C-terminal region which contains a transcription-activating domain [52]. H-1 parvovirus induces apoptosis in rat glioblastoma cells and in the rat cerebellum [55]. The expression of NS proteins by the B19 and H-1 viruses correlates with the induction of apoptosis in human erythroid progenitors [4, 53, 56] and leukemic promonocytic cells [57], respectively. These observations do not

rule out, however, that necrotic cell death occurs concomitantly or prevails in other cell types [58–60]. MVM-induced cytotoxicity requires cell proliferation and correlates with cell cycle perturbations, in particular S/G2 arrest [58, 59].

The mechanism(s) through which NS1 exerts its cytopathic effect remain elusive to date. Possible clues can be found in the molecular alterations induced by NS1 in target cells at the level of transcription [61], chromatin integrity [59] and phosphorylation or synthesis of specific proteins [26]. Most interestingly, the expression of similar amounts of NS1 protein in pairs of normal and ras-transformed cell cultures was found only to cause the death of the latter [25]. This observation suggests that cell transformation is associated with NS1 modifications and/or changes in cellular targets or regulators of the viral product, making NS1 more toxic for the transformants compared to the parental cells. It follows that the above-mentioned sensitization of many transformed cells to the cytopathic effect of parvoviruses may be traced back not only to the enhanced production of NS1 proteins (see previous section) but also to the reduced tolerance of these cells to a given amount of the viral toxin. Should the latter effect also occur in vivo, NS1 may qualify as an oncolytic effector.

Altogether, the data reviewed in this and the previous section indicate that oncoproteins can aggravate the outcome of a parvovirus infection by upregulating both the replication and intrinsic cytopathic effect of these viruses. It should also be stated that, conversely, tumor suppressor proteins may restrain the parvoviral life cycle in normal cells. Indeed, the resistance of certain human erythroleukemic cell clones to H-1 virus-induced killing was found to correlate with the reactivation of p53 expression, while the functional inactivation of endogenous p53 led to the sensitization of rat embryo fibroblasts to this virus [62]. Since the NS1 protein is essential for both parvovirus replication and cytotoxicity, it is tempting to hypothesize that transformation-associated post-translational modifications of the viral product may contribute to both the oncotropic and oncolytic properties of parvoviruses. This is presently a matter of speculation but would be in agreement with recent findings which showed that NS1 phosphorylation is necessary for the replicative functions of this protein [63] and may also modulate its cytopathic effect [64]. It is noteworthy that NS1 is more particularly a target substrate for atypical isoforms of the protein kinase C (PKC) family [65]. Given their involvement in the regulation of cell growth, differentiation and transformation, PKCs may constitute one of the elements interconnecting the parvoviral life cycle with the cell's progression into the neoplastic state. Finally, it should be stated that the MVMp and H-1 virus-derived vectors developed so far for gene therapy purposes retain the viral elements (replication origins, promoters and NS transcription unit) which are involved in the oncotropic and oncolytic properties of these viruses, pointing to their special suitability for anticancer applications (see below).

Host Range

Some autonomous parvoviruses exhibit a very specific host range with respect both to the animal species and cell types that they are able to infect. This specificity can be exemplified by B19 which deserves to be designated as a human erythrotropic parvovirus [4]. The narrow host range of B19 can to a large extent be explained by the fact that the erythrocyte P antigen globoside serves as the virus receptor [66], although restrictions at other steps of the virus life cycle may also contribute to the B19 tropism [67, 68]. Other parvoviruses display a broader host range. This is in particular the case for the rat virus H-1 and MVM, which form the subject of the present review and proved able to replicate notably in human cells, and for which a number of cell types, including fibroblasts, epithelial and hematopoietic cells, were found to be targets [6, 7, 12–14, 16, 57, 62, 69–72].

Even the less specific parvoviruses do not replicate in all cell types. As stated above, parvovirus replication requires host cells to enter the S-phase of the mitotic cycle. Yet, S-phase functions are not enough to fulfill the cellular requirements of the parvoviral life cycle. Some tissues in parvovirus-infected susceptible hosts are not destroyed although they contain actively dividing cells [21, 22]. The differentiation state of the cells also appears to determine their susceptibility to parvovirus infection [3, 72–75]. In a few cases, this S-phase-independent restriction appears to be due to the absence of specific receptors for the virus on the cell surface. Such a surface barrier is, however, not the general rule. Although they have not yet been identified, the cellular receptor(s) of H-1 virus and MVM are likely to involve ubiquitous N-acetylneuraminic acid (sialyl)-containing glycoproteins [27]. In most cases, the differentiation-associated restriction to parvovirus replication takes place at step(s) following adsorption onto the cell surface. An allotropic determinant distinguishing the fibrotropic prototype virus MVMp from the lymphotropic strain MVMi was found to be encoded by the capsid protein gene in a region which also appears to determine the tissue and species tropism of canine, porcine, feline and mink parvoviruses [76–78]. Yet, MVMp and MVMi are bound and taken up regardless of whether the cells are permissive or not [3]. MVMp appears to be unable to initiate transcription in mouse T lymphocytes due to an early block in the viral life cycle at the level of uncoating [79]. It should be stated in this respect that internalization, nuclear transport and the subsequent uncoating of virions remain ill-defined processes. For cell entry, the canine parvovirus uses the endocytic pathway, a route that may be necessary for its ability to achieve a productive infection [80]. Some of these prereplicative steps of the parvoviral life cycle appear to be blocked in nonpermissive cells, although restrictions at later stages may also be involved [17, 27, 79]. The cellular factors that limit viral replication in nonpermissive cells are largely

unknown. Identification of these factors represents an interesting issue, given their association with the differentiation state of the host cells.

Pathology

Given that some rodent parvoviruses are being considered as vectors for human applications (see above), the pathology of these viruses in their natural host deserves attention. The diseases induced by rodent parvoviruses depend on the age of the animal at the time of infection as well as the virus strain used. Infection of naive fetuses and neonates with some of these viruses (in particular RV, H-1 virus and MVMi) often terminates fatally [21, 22, 74, 75, 81, 82]. Enteritis, hepatitis, cerebellar hypoplasia and ataxia, stunted growth, renal infarcts, hemorrhages of various organs (such as intestine, lung, liver and brain) and leukopenia are hallmarks of deleterious parvovirus infections [21–23, 74, 75, 81]. Cell populations with a high mitotic activity are typical targets for such infections. In contrast, the infection remains clinically nonapparent when these same viruses are used to infect juveniles or adult animals [21, 22]. Other rodent parvoviruses, such as MVMp and the recently isolated mouse parvovirus type 1 (MPV-1) and rat parvovirus type 1 (RPV-1), seem to be devoid of pathogenic activity even if infection takes place at the neonatal stage [22, 23, 83]. Asymptomatic parvovirus infections still deserve to be monitored, especially in laboratory animals. Indeed, the virus often establishes a persistent infection which can last for several months or years in the absence of clinical symptoms, despite the presence of circulating antibodies [21, 22], which may interfere with immune responses [84–86] and tumorigenesis (see below). Potential reservoirs for long-term virus maintenance and production may exist in some of the tissues which are special targets for parvovirus replication at early developmental stages and whose proliferation keeps on going or is occasionally reactivated. It is worth noting in this regard that endothelial, lymphoid and erythroid tissues are more affected by certain rodent parvoviruses as is apparent from consequent clinical symptoms and confirmed by in situ detection of viral DNA replication intermediates [21–23, 75, 81, 85]. Interestingly, the endotheliotropism of rodent parvoviruses might contribute to the oncosuppressive activity of these agents (or their recombinant derivatives, see below) through the inhibition of tumor neoangiogenesis.

Potential risks and benefits need to be weighed up when considering the use of rodent parvoviruses as anticancer agents. The balance is tipped in favor of the use of such viruses by the fact that animals have already become resistant to the pathological effects of even the most virulent rodent parvoviruses before the onset of tumor growth for the majority of tumors. The question then arises as to what extent these animal data can be extrapolated to the human situation. As stated above, parvovirus H-1 proved to be able to infect humans, leading to

viremia followed by sustained seroconversion [5, 87]. Retrospective studies failed to reveal any consistent link between human diseases and possible prior infections with the H-1 virus [21]. The injection of H-1 virus was also well tolerated by cancer patients, as shown initially by Toolan et al. [5] and confirmed in a more recent phase-I clinical trial [87].

Immunological Responses

Besides eliciting an antiviral immune response which can have various outcomes (virus eradication, establishment of a persistent infection coexisting with the immune response, potentiation of virus infectivity and pathogenesis), parvoviruses may also interfere with the host immune system.

Humoral Antiviral Responses

The formation of infectious immune complexes is a typical feature of Aleutian mink disease parvovirus (ADV) and is responsible for the antibody-dependent enhancement of macrophage infection with ADV and the consequent induction of a cytokine disorder characterized by hypergammaglobulinemia and related pathological lesions [88]. Rodent parvoviruses are highly immunogenic often inducing life-time immunity. A humoral neutralizing response takes place within 2 weeks of experimental animal infection which limits virus spread in the contaminated organisms as well as viral excretion and risk of reinfection at a later time [21, 22]. Maternally acquired passive immunity protects the young against early infection. Parvoviruses which do not cause an immune suppression in vivo, such as MVMp, are efficiently counteracted by the immune system and fail to establish a chronic infection, becoming undetectable in adult animals [22, 32]. Yet, seemingly normal hosts may show coexistence of virus and specific antibodies for quite some time [21–23], and infection may persist in a non-apparent form and become reactivated under special conditions. For example, the induction of tissue regeneration was reported to allow the reactivation of self-limited infections with the rat parvoviruses H-1 and RV [89]. Other rodent parvoviruses, such as the mouse parvovirus MPV-1, cause immunosuppression in vivo (see below) and, as a probable consequence of this property, are not fully eradicated by the immune response. MPV-1 establishes persistent infections and shedding in adult mice, despite the presence of circulating antibodies [83]. Virus persistence may account for continued antigenic stimulation and maintenance of high antibody titers. To sum up, positive antibody titers to rodent parvoviruses are consistent indicators of a previous infection with these agents and of a state of at least partial protection against reinfection. Antibodies directed against rodent parvoviruses are detected in the sera of the respective natural hosts with a frequency of between 60 and 100% [21]. It is noteworthy in this respect that the frequency of positive antibody titers to rodent parvoviruses in

human populations has been reported to be extremely low [21]. Thus, there is little evidence for rodent parvoviruses infecting humans by any natural route, although cross-contamination may take place occasionally. This lack of natural immunity is a definite advantage when considering the use of rodent parvoviruses (or their recombinant derivatives) for human applications based on their ability to replicate in some human cells after experimental infection. Seroconversion was found to occur in patients injected with parvovirus H-1, concurrently with the termination of the viremic phase [5].

Cellular Antiviral Responses

The role of the cellular immune response in controlling parvovirus infections is less clear. Yet T cells have been implicated as critical elements in host immunity to rat parvovirus infections, given the fact that natural killer (NK) cells and humoral immunity appear to be unable to clear established RV infections in adult rats [10, 90]. It should also be stated that parvoviruses have been reported to be both poor [84, 91] and highly susceptible [92] targets to the antiviral effects of interferons.

Parvoviral Interference with the Host Immune System

Besides being targets for host antiviral responses, a number of parvoviruses can also interfere with the host immune system. In particular, some rat (RV, RPV-1) and mouse (MPV-1, MVMi) parvoviruses were found to replicate in lymphoid tissues and to have lymphocytotropic properties [10, 11, 21–23, 74, 75, 81, 83]. T cells proved to be targets for these viruses, although B cells, NK cells and macrophages can also be infected [10, 84, 85, 93, and references therein]. Furthermore, several rodent parvoviruses, including H-1 virus, are able to grow in human T-cell lines [69, 71]. By infecting lymphocytes, rodent parvoviruses can potentially perturb immune responses to both the infecting virus (accounting for prolonged infection and delayed virus clearance) and other antigens (resulting in an interference with transplantation and oncology studies). Indeed, lymphocytotropic rodent parvoviruses were found to inhibit distinct T-cell effector functions in vivo and/or in vitro, including mitogen and antigen-induced lymphocyte proliferation, generation of cytotoxic T cells in mixed leukocyte cultures and activation of T-cell-dependent B-lymphocyte responses [10, 85]. Paradoxically, despite its immunosuppressive properties, MPV-1 was able to potentiate the rejection of tumor allografts in a T-cell-mediated fashion [11]. This enhanced rejection took place while the alloantigen-reactive lymphocytes were depressed and MPV-1 was most unlikely to directly kill or xenogenize tumor cells, suggesting that the virus may induce a bystander effect. By infecting lymphocytes, MPV-1 may conceivably induce the release of

cytokines which could in turn be responsible for the graft failure due to their direct action on the tumor or the induction of either a local inflammation or an imbalanced immune response. The possibility that some rodent parvoviruses may disturb the cytokine network of infected hosts is in keeping with another intriguing observation, i.e. MPV-1 also induces syngeneic graft rejection by stimulating autoreactive T cells [94]. Furthermore, it has been reported that RV causes autoimmune diabetes in certain rats by activating silent autoreactive T cells [86]. Although a stimulating role of cross-reacting viral antigens cannot be ruled out, the activation of quiescent autoreactive lymphocytes is assumed to be due to the spreading of presented epitopes, as a result of cytokine-induced alterations in either antigen processing, recruitment of antigen-presenting cells or expression of cell surface molecules involved in T-cell activation. Therefore, some of the immunological disorders observed in parvovirus-infected animals may be mediated by changes in cytokine profiles. In agreement with this hypothesis, RV was found to upregulate the production in splenic lymph nodes of tumor necrosis factor-α, interferon-γ and the interleukin (IL)-2 and IL-12 [93]. These cytokines may favor tumor rejection through their toxicity for neoplastic or vascular cells, and their ability to cause local inflammation or activate specific T-cell subsets. These immune responses may constitute a component of parvovirus oncosuppression, which is independent of a direct virus–tumor cell interaction and thought to be responsible for the continued resistance of infected animals to oncogenesis long after virus inoculation [9, 95].

Genetic Engineering of Parvoviruses

The oncotropic and oncolytic properties of several rodent parvoviruses (see above), together with their stability and high-titer production, make them attractive agents for the gene therapy of cancer [6, 21, 22, 27]. However, parvovirus infections are not always potent enough to prevent cancer or to induce the rejection of established tumors. The mere fact that a number of parvoviruses have been isolated from growing neoplastic lesions indicates that the natural viruses are often incapable of eradicating the tumor they infect. Furthermore, in successful cases, large amounts of virus are usually required to facilitate the regression of preexisting tumors. Indeed, high-titer inocula must be used to induce the rejection of human tumor allografts in immunodeficient animals, even though input virus neutralization is minimized under these conditions [7, 8]. There is thus a requirement for the genetic engineering of parvoviruses in order to enhance their anticancer potency or improve their targeting. Two types of genetically modified rodent parvoviruses have been produced to this end, which

are both competent for DNA replication but differ in their infectivity. DNA replication-deficient parvoviral vectors (NS^-VP^-) have also been generated [96–98], but will, however, not be considered here given the focus of the present book on replication-competent viruses.

Defective Recombinant Viruses Competent for DNA Replication

Rationale

Recombinant parvovirus-based vectors were developed which retain the NS transcription unit (left-hand ORF), but in which the gene encoding the capsid proteins (right-hand ORF) were removed or truncated and replaced by a therapeutic or reporter gene [99–102]. As depicted in figure 1, these NS^+VP^- vectors retain the viral genomic telomeres (containing the origins of DNA replication and other replication signals) and the P4 promoter-directed NS transcription unit (encoding the NS proteins required for viral DNA replication, expression, and cytotoxicity). It follows that these vectors possess all the above-mentioned elements that have been identified as participating in the oncotropism (DNA replication origins, P4 promoter, NS proteins) and oncolytic activity (NS proteins) of the parental parvoviruses. Upon transfer into target cells, the genome of NS^+VP^- vectors can thus be expected to be amplified, expressed, and to exert a NS-mediated cytopathic effect in an oncogene-dependent fashion. In addition, these recombinants harbor transgenes that are placed under the control of the genuine viral promoter P38, and which should be overexpressed in permissive neoplastic cells due to vector DNA replication and NS1-induced P38 transactivation (fig. 1). Such an efficient and preferential expression of parvovirus-transduced reporter and effector genes in transformed (including tumor-derived) versus nontransformed cells was indeed confirmed in cell systems [99, 100]. This allows us to reinforce the intrinsic oncosuppressive activity of natural parvoviruses by substituting anticancer transgenes for part of the viral VP gene. It should be stated, however, that parvovirus-based vectors of the NS^+VP^- type, though competent for DNA replication in target cells, are still defective since they are unable to produce capsid proteins and progeny virions. These vectors therefore achieve one-hit infections [99, 100] and cannot further spread in the tumor or test animal in the absence of wild-type helper virus, which may be a limitation to tumor destruction but an advantage from the safety point of view. As a probable result of the cytotoxicity of NS proteins, transgene expression achieved by typical NS^+VP^- parvoviral vectors is transient [99, 100] arguing against their use for gene replacement strategies but making them suitable for anticancer applications that involve toxic or immunomodulating transgenes whose long-term expression is not desirable.

Production of Recombinant Viruses

The starting materials for the production of recombinant viruses are infectious molecular clones – full-length viral genomes cloned into bacterial plasmids – which are capable of producing infectious virus upon transfection of permissive cells. Infectious DNA clones are available for a number of autonomous parvoviruses, with the notable exception of the human parvovirus B19, probably due to the deletion of palindromic sequences from the genome ends during DNA amplification in bacteria [4]. The NS1 polypeptides expressed upon cell transfection with an infectious DNA clone were shown to be required for excision of the parvoviral genome from the plasmid backbone [103, 104]. Transgene substitutions for (part of) the VP-coding region generate recombinant molecular clones which can be packaged into recombinant parvovirus particles in the presence of a helper construct that complements the VP gene defect by providing capsid proteins in trans. This helper DNA can be supplied concurrently with the recombinant parvoviral genome via cotransfection [99–102]. Alternatively, a packaging cell line harboring the helper construct could be used, although so far only low recombinant parvovirus yields have been obtained using this method [105]. Interestingly, it is possible to pseudotype, i.e. to package the genome of a given parvovirus into capsids of related viruses [97, 98, and C. Wrzesinski, unpublished data], which may be used to modify the virus host range or circumvent the antiviral immunity which develops during a repeated injection protocol. Yields of recombinant parvovirus have been significantly increased over the past few years. These improvements have been made through the use of shuttle helper plasmids, new producer cell lines and, above all, modified parvoviral DNA vectors [102]. Indeed, one of the reasons why the titers of first-generation recombinant parvovirus stocks, including the DNA replication-deficient vectors, were so low [96–102] could be traced back to the fact that the original infectious molecular clones terminated on the left-hand side in an incomplete replication origin, i.e., in a sequence which was suboptimal for resolution through the nicking activity of the NS1 protein [34, 103, 104]. At present, second-generation parvoviral DNA vectors yield recombinant MVM and H-1 virus titers that are up to one thousandfold higher than initial titers, reaching 5×10^7 replication units/ml of crude cell extract [102, and our unpublished data].

Given their competence for DNA replication, NS^+VP^- recombinant viruses can be routinely titrated using an infected cell hybridization assay that reveals the amplification of incoming vector DNA at the single-cell level [99–102]. A problem inherent in the helper-assisted production of defective viruses lies in the generation of wild-type particles (usually referred to as RCVs, for replication-competent viruses) through homologous recombination between vector and helper DNA constructs. Parvoviral vectors are no exceptions

and were initially found to be highly (up to 80%) contaminated with RCVs [99, 100]. This generation of nondefective parvoviruses has now been decreased to virtually undetectable levels by limiting the helper plasmid copy number through the use of a packaging cell line [105], by reducing DNA overlaps between the helper construct and the vector to be packaged [102, by pseudotyping corzesinsky, unpublished data], by minimizing the homology of residual overlapping sequences through mutagenesis [106] or production of chimeras between related parvoviruses [107]. A definite limitation of recombinant parvoviruses concerns the size of the transgenes which they can accommodate. This restriction does not affect vector DNA replication and is actually due to the low tolerances existing for encapsidation with respect to both increases in the total genome length over 5–10% of wild-type DNA (i.e., about 500 bp or less) and extensive deletions of viral DNA sequences [101, 102]. It has been hypothesized that these deletions impair encapsidation by removing NS1-binding sites which are scattered at many positions along the parvoviral genome and may contribute to the NS1-facilitated incorporation of viral DNA into nascent virions [102, 108]. The size and sequence requirements for encapsidation do not raise problems as far as small transgenes are concerned. Thus, foreign DNAs of up to 800 bp in length can be substituted for VP sequences without significant losses in recombinant virus yields compared with wild-type virus derived from the infectious molecular clone [102]. It is still possible to produce NS^+VP^- viruses that harbor larger transgenes of up to 1,600 bp, however, these have a lower virus titer [102].

The DNA replication-competent NS^+VP^- vectors developed so far for human cell transduction are based on the rodent parvoviruses H-1 and MVM [99–102, 107, 109]. Besides these agents, other long-known rodent parvoviruses, in particular RV and LuIII, have been shown to grow in various transformed human cells in vitro [21, 69] and are thus candidates for anticancer vector production. In addition, new rodent parvovirus isolates (MPV-1, RPV-1) have been described which deserve special consideration for vector development, owing to their intrinsic oncosuppressive properties and lack of pathogenic activity in natural hosts [11, 22, 23, 85]. As stated above, several rodent parvoviruses have lymphocyto- and endotheliotropic features which may contribute to their ability to suppress tumor growth, regardless of their direct interaction with neoplastic cells. Advantage may be taken of this tropism to devise recombinant vectors that specifically modulate tumor angiogenesis and immune constraint (see below). It is also worth mentioning that other mammalian parvoviruses were found to display antineoplastic properties, as exemplified by the capacity of canine parvovirus for suppressing venereal sarcomas in dogs [110]. Further investigations are required to determine whether parvoviruses of origins other than rodent could be of use in designing vectors for human applications. Though pathogenic and

not available in the form of an infectious molecular clone, the genuine human parvovirus B19 could still be used to supply other vectors with erythrotropic elements [67], allowing these vectors to target cells from the erythroid lineage.

Choice of Anticancer Transgenes

A promising approach to the gene therapy of cancer lies in the vector-mediated transduction of tumor cells with genes encoding proteins that lead directly or indirectly to the destruction of these cells. The most promising strategies involve the use of immunostimulatory and suicide transgenes, whose mechanisms of action involve bystander effects. These allow tumor cells that are not themselves hit by the vector to be targets for the cytotoxic response elicited from transduced neighboring cells. The suicide thymidine kinase gene of herpes simplex virus was introduced into a parvovirus MVMp-based vector, allowing the recombinant virus to kill cultures of target tumor cells in an acyclovir-dependent fashion [111]. Alternatively, attempts are being made to use recombinant parvoviruses to express immunostimulatory factors in neoplastic lesions, with the aim of achieving tumor rejection and establishment of a long-term immunity to prevent tumor regrowth. This modulation of host immune responses is controlled by various secreted peptides of the cytokine family. Some of these, in particular IL-2, IL-12 and granulocyte-macrophage-colony stimulating factor, have been shown to be especially potent stimulators of anti-tumor immunity [112, 113]. Besides their immunomodulating activity, certain cytokines may also inhibit neoangiogenesis and thus inhibit tumor progression [114]. Because cytokines are relatively small (approximately 100 amino acids), their corresponding cDNAs can be easily incorporated into NS^+VP^- parvoviral vectors. Recombinant MVMp and H-1 viruses have been produced which harbor the cDNAs of various cytokines: IL-2, IL-4 [99], monocyte chemotactic protein (MCP)-1 [102, 115], MCP-3 [116] and interferon-γ-inducible protein 10 (IP-10) [117]. Due to difficulties in obtaining recombinant virus stocks of adequately high titer, the assessment of these vectors in tumor-bearing animals could only recently be initiated [115–117]. Preliminary results from both ex vivo and in vivo experiments indicate that NS^+VP^- parvoviral vectors supplemented with distinct cytokine genes exhibit a significantly enhanced antineoplastic activity in at least some tumor models, compared with the corresponding wild-type viruses (see below). Besides cytokines, cell surface molecules (e.g. the costimulatory ligands B7-1 and B7-2) involved in T-cell activation are also good candidates for parvovirus-mediated transduction and stimulation of anti-tumoral cellular immunity [118]. Furthermore, parvoviruses may be suitable for expressing tumor antigens over an extended period and eliciting in this way a sustained antitumoral immune response [119], given that these viruses often persist in infected organisms (albeit in a presently unclear form).

Modified Nondefective Viruses

For certain applications, it may be necessary to hit the majority of cells constituting an established tumor with parvoviral vectors. This cannot, however, realistically be expected following a single inoculation, even if very high-titer virus stocks were to be available. The fraction of tumor cells becoming infected can be increased through repeated injections or, better still, through the use of nondefective viruses that are able to multiply in target tumors and spread from primarily infected neoplastic cells to neighboring cells. As stated above, some natural parvoviruses can be used to this end owing to their capacity for preferential intratumoral growth (oncotropism) and toxicity (oncolysis). Yet, in many instances, the parvovirus does not appear to be the winner in the race between its propagation and the multiplication of tumor cells, resulting in the continuing growth of infected tumors. Thus there is a requirement for the modification of parvoviruses to increase their anticancer potential whilst retaining their infectious potential. Parvoviral DNA could theoretically be engineered in several ways to achieve this goal. Although parvoviruses have a very compact genetic organization, a few regions of the viral genome may be nonessential and thus used to insert therapeutic transgenes without impairing infectivity [1, 27]. Despite the limited size of potential target regions of parvoviral DNA and the above-mentioned constraints on the overall genome length, this approach should be applicable for small transgenes, such as antisense RNAs/ribozymes. Feasibility studies showing that such nondefective recombinant parvoviruses can indeed be obtained are, however, lacking at present.

Besides transgene insertion, parvovirus engineering could also involve the modification of existing parvoviral genes and/or regulatory elements. This strategy would allow the production of genetically modified viruses that have acquired novel biological features without becoming defective, regardless of whether or not a foreign gene is inserted. As far as cancer therapy is concerned, specific changes could be made to the elements controlling the oncotropic and oncolytic properties of parvoviruses in cis (e.g. P4 promoter) or trans (e.g. NS-coding sequences), in order to increase the extent and/or specificity of the antineoplastic activity of these viruses. Parvoviruses engineered in this way have not yet been described, yet current improvements in our understanding of the regulation of parvoviral protein expression and functioning (see above) make the generation of such modified viruses a most interesting possibility. The feasibility of this approach is supported by recent work showing that substitution of the human immunodeficiency virus (HIV) TAT protein-response (tar) element for the proximal region of the P4 promoter of parvovirus MVMi allows modified viruses to be generated, which still possess a full productive life cycle, yet in a TAT-dependent fashion [109]. This demonstrates that parvoviruses can be engineered so as to change the host range without them becoming defective.

Along the same line, the NS region of parvovirus MVMp was modified by site-directed mutagenesis to generate viruses that encode functional NS1 but nonfunctional NS2 proteins and, as a consequence, are impaired for growth in murine cells whilst remaining fully infectious for transformed human cells [30, 120]. In conclusion, genetic engineering allows nondefective parvoviruses to better target and eliminate specific cells. While the above-mentioned tar-substituted MVMi virus is aimed at killing HIV-infected cells [109], parvoviruses modified in other ways may be used to destroy neoplastic tissues more efficiently and/or selectively than the natural viruses. Autonomous parvoviruses that can grow in human cells are candidates for this approach and include the above-mentioned rodent viruses and, should its pathogenic risk [4] be overcome, the human B19 virus.

Antitumor Activity

Parvoviruses are unique among DNA viruses in that they do not have any tumorigenic members [1]. Furthermore, the autonomous parvoviruses are not known to integrate into the host cell genome [35], and are therefore unlikely to pose a significant risk of insertional mutagenesis. On the contrary, autonomous parvoviruses have been shown to be oncosuppressive in a number of tumor models [6, 9, 11, 95, 110], and in human tumor transplants in recipient animals [7, 8, 115, 116], creating a heightened interest in the mechanisms involved in this oncosuppression and their possible application in human cancer therapy. As discussed above, as well as the genuine human parvovirus B19, several autonomously growing parvoviruses of animal origin (in particular rodent) are able to replicate in some human cells, and may be used to this end.

Direct Parvovirus Toxicity in Tumor Cells

As reviewed in previous sections, the oncotropic and oncolytic properties of parvoviruses have been well documented in vitro, comparing transformed or tumor-derived cell lines with their nontransformed counterparts [12–19, 24–26]. The physiological relevance of these observations is, however, questionable given the drift known to occur in vitro during the establishment of cell lines. A first hint as to the capacity of parvoviruses for interfering with the growth of at least some original tumor cells was given by studies using non-established short-term cultures, showing that the H-1 virus exerts cytostatic and cytopathic effects on freshly isolated human breast and liver carcinoma cells [20, 121]. There are also indirect indications that some tumor cells can be targets for the cytotoxic activity of parvoviruses under in vivo conditions, i.e. oncolysis may contribute to parvovirus oncosuppression. As stated above,

several rodent parvoviruses were isolated from human transplantable neoplasms under conditions in which they could not be detected in the normal tissues of recipient animals [21–23], arguing for the ability of these tumors to sustain a productive parvovirus infection that can be assumed to lead to the death of at least a fraction of the neoplastic cells. Additional support for the contention that parvoviruses can have an oncolytic activity in vivo is given by the capacity of H-1 virus for suppressing human tumor transplants in immunodeficient mice [7, 8]. Recipient mouse cells cannot be infected with this virus, while signs of virus replication were found in implanted human neoplastic cells. In particular expression of the cytotoxic viral protein NS1 was demonstrated in the vicinity of tumor necrotic areas [8].

Additional data are clearly needed to assess the oncolytic capacity of parvoviruses in vivo. The extent of oncolysis can be expected to depend on the target tumor type. Indeed, transformed cultures were found to vary in their sensitization to a given parvovirus under in vitro conditions, depending on the cell type and transforming agent used [17–19]. Furthermore, the outcome of infection will also vary between parvoviruses, since different viral strains have distinct host ranges [3, 22, 77–79], and cell differentiation-related restrictions to parvovirus replication may not be overcome as a result of neoplastic transformation [3, 17, 27]. Thus, tumors that are most sensitive to a certain parvovirus would be predicted to be derived from tissues for which this virus has a preferential tropism and which serve as natural virus reservoirs (even though virus replication may be limited enough to remain asymptomatic under normal conditions). As stated above, a number of rodent parvoviruses (RV, MPV-1, RPV-1, H-1) exhibit a tropism for lymphoid tissues, leading one to speculate that hematopoietic tumors may be suitable targets for virus-induced destruction. This would be consistent with in vitro studies which showed that a number of human leukemia and lymphoma cell lines are especially sensitive to H-1 virus or RV-induced killing [57, 62, 69, 71, 72]. It is noteworthy in this respect that most rodent parvoviruses are also endotheliotropic [22, 32], pointing to vascular tumors and hyperplasias as candidate virus-sensitive targets. Yet, these viruses may also jeopardize the survival of other types of tumors, although possibly to a lesser extent, since H-1 virus proved able to lytically infect transformed human fibroblasts and epithelial cells in vitro [6, 12–14, 16] and to suppress the growth of human carcinoma transplants in vivo [7, 8, 115, 116].

Immune System-Mediated Antitumoral Effects of Parvoviruses

There is also indirect evidence to suggest that tumor suppression by natural parvoviruses involves an immune component. For instance, mice coinjected with ascites cells and parvovirus MVMp acquire a long-term immunity to reinjection of the tumor cells alone in the absence of a detectable chronic infection [9].

The mechanism through which parvoviruses may elicit a lasting anti-tumor immunity is unclear at present. Although parvoviruses are non-budding agents and are thus unlikely to xenogenize infected tumor cells, the possibility must not be dismissed that viral antigens released by dying infected cells are taken up by dendritic cells, triggering an antiviral cytotoxic T lymphocyte (CTL)-mediated response that may cross-react with tumor antigens [122]. Alternatively, parvoviruses may stimulate natural tumor-specific immunity by inducing the appearance of dying cancer cells that provide dendritic cells with tumor antigens to elicit an antitumoral CTL response [123]. On the other hand, parvoviruses may act by disturbing the cytokine network, thereby raising inflammatory, cytotoxic and/or immune reactions directed against tumors. In agreement with this possibility, human tumor transplants that were induced to regress in recipient mice upon infection with H-1 virus were found to become infiltrated with activated NK cells [115]. Furthermore, infection of rats with parvovirus RV was reported to cause a selective increase in the expression of specific cytokines with immunomodulating, antitumoral and/or antiangiogenic activities [93]. These changes in cytokine profile may be direct or indirect consequences of parvovirus interaction not only with tumor cells but also with immune or related cells. This complexity can be exemplified by the ability of parvovirus MPV-1 to induce mice to reject tumor allografts, although these are resistant to virus infection [11]. This effect proved to be T-cell-mediated and was proposed to result from a virus-induced, cytokine-mediated reprogramming of the immune response at the expense of tumors, resulting in the development of an autoimmune reaction [11, 94]. Incidentally, an imbalanced cytokine environment was also hypothesized to be responsible for the apoptotic death of uninfected erythroid precursors in MVMi-infected mice [75]. Like its oncolytic counterpart, the immune component of parvoviral oncosuppression is expected to be of varying importance, depending on the virus and tumor under consideration. Parvoviruses exhibiting a tropism for lymphoid tissues may be particularly efficient in altering the immune status of infected hosts.

Recombinant Parvoviruses

As outlined above, parvoviral genomes harboring deletions within the VP gene are competent for DNA replication and can be packaged in homologous capsids, generating vectors that appear to retain the host range (in particular the oncotropism) of wild-type viruses, at least under in vitro conditions [99, 100, 111]. This gives an impetus to the production of $NS^{+}VP^{-}$ recombinants that carry transgenes capable of reinforcing the intrinsic antineoplastic activity of the natural viruses. Although the in vivo assessment of this strategy is still in its infancy, there is some evidence from in vitro and ex vivo experiments to suggest that the cytotoxic and immunomodulating properties of parvoviruses can both

be enhanced through supplementing these viruses with appropriate transgenes. Thus, MVMp VP gene replacement with a suicide gene led to the production of recombinant viruses whose cytocidal capacity was enhanced in a conditional manner upon infection of transformed or tumor-derived cell cultures [111]. On the other hand, mouse melanoma implants in immunocompetent mice were prevented from forming tumors as a result of their preimplantation infection with MVMp-based vectors expressing the chemokine MCP-3 [K. Wetzel, unpublished data], while H-1/IL-2 recombinants also suppressed human cervical carcinoma cell implants in immunodeficient mice [115]. The cytokine gene-transducing parvoviruses achieved this protection at multiplicities of infection that were insufficient for the wild-type virus and empty vector to show their intrinsic antineoplastic activity. Thus, under these admittedly artificial conditions, the oncosuppressive capacity of parvoviruses could be significantly enhanced by adding a cytokine transgene to the viral replicon. It remains to be determined whether this potentiation will also occur following the vector's administration in vivo. It should also be stated that other targets, besides immune cells, may be responsive to parvovirus-transducing cytokines and participate in the suppression of tumor progression. These targets include endothelial cells involved in tumor neo-angiogenesis, as supported by the recent finding that MVMp-mediated expression of the antiangiogenic chemokine IP-10 strongly enhances the oncosuppressive effect of the parvovirus in a mouse model of Kaposi's sarcoma [117]. Indeed, immunocompetent mice could be efficiently protected against fatal tumor recurrence and metastases formation from engrafted hemangioma cells, as a result of the in vivo infection of primary tumor-bearing animals with MVMp/IP-10 viruses [117]. This protection was achieved using the repeated vector injection procedure that had no detectable adverse side effects and was not effective when wild-type MVMp was used instead of the IP10-transducing vector. These data provide the first indications that cytokine transgene substitutes for VP genes may increase the antineoplastic potential of parvoviral vectors, compared to parental viruses, in spite of the fact that the recombinants become deficient in progeny virus production. It is quite possible that this additional feature of recombinant vectors will not apply to all tumors, in particular those which are highly permissive for wild-type virus propagation in vivo [9, 115]. Yet, in the systems studied, the acquisition of transgene expression, combined with the maintenance of NS-dependent cytotoxicity and competence for DNA amplification, appears to prevail over defectiveness, resulting in an enhanced oncosuppressive capacity of recombinant vectors compared to the natural viruses.

Parvovirus Administration Regimen

Some of the above-mentioned animal studies have shown that parvoviruses can find their way to tumors and exert an oncosuppressive effect after systemic

administration through intravenous injection [7]. Yet, this route of parvovirus delivery is likely to lead to the loss of many input particles, since most cells from infected organisms are able to take up these viruses even though they are not permissive for their full replication [13, 16, 17, and N. Giese, unpublished data]. To avoid parvoviruses getting trapped by normal tissues before they can reach the tumors, a local delivery of the virus inoculum within or in the vicinity of neoplastic lesions seems therefore advisable.

The optimal method for delivering (recombinant) parvoviral genomes is a priori in the form of full viral particles, since viruses are made to efficiently infect target cells. This has been achieved up until now by packaging DNA into parvoviral capsids. However, alternative strategies must not be dismissed. Parvoviral DNA constituents could be transferred via heterologous delivery systems allowing their transduction into target cells through non-parvoviral particles [124, 125]. Furthermore, parvoviral vectors may conceivably be administered in the form of naked DNA, as reported for in vivo gene transfer from other DNA viruses [126]. Since the aforementioned limitation to the overall size of the parvoviral genome concerns packaging in parvoviral capsids but not DNA replication [101, 102], these alternative approaches would have the advantage of allowing larger transgenes to be accommodated.

In many instances, it may be necessary to perform multiple virus injections in order to increase the fraction of target cells that become infected with natural or recombinant parvoviruses. A humoral response develops within 2 weeks of infection, and has a neutralizing effect on most parvoviruses (with the notable exception of ADV) [21, 22, 88]. Although the cellular component of antiviral immunity may be required for full eradication of the virus which can persist at least to some extent in the presence of neutralizing antibodies [22], this seroconversion is likely to limit the efficiency of repeated parvovirus injections. As mentioned above, pseudotypes can be generated by packaging the genome of a given parvovirus into capsids from related parvoviruses [97, 98, and our unpublished results]. By using such pseudotypes for reinfection, it may be possible to circumvent the neutralizing immune response directed against previously injected virions. Another major impediment to the repeated inoculation of certain viruses (e.g. adenoviruses) lies in the occurrence of life-threatening inflammatory reactions [127]. This problem was, however, not encountered in our recent attempts to suppress tumor graft growth in immunocompetent mice by means of the fibrotropic strain (p) of MVM or recombinant derivatives. Up to 15 virus injections could be made over a prolonged period without inducing any detectable inflammatory reaction [117]. This probably reflects, at least in part, the narrow spectrum of cells that can be targets for MVMp cytotoxicity and function as antigen presenters. It remains to be determined whether a similar tolerance to multiple infections also applies to animals treated with

lymphocytotropic parvoviruses and/or bearing tumors of varying permissiveness for parvoviruses.

Local delivery may not be enough to allow parvoviruses or their recombinant derivatives to stabilize or revert tumor growth, due to the limiting amounts of virions and/or poor accessibility of some neoplastic cells. Therefore, the combination of parvovirus therapy with other treatments deserves consideration. Parvoviruses may be administered after reducing tumor burden by conventional mechanical or chemo/radiotherapeutic treatments, in the hope of preventing the recurrence of neoplastic growth from residual tumor cells. The dose of conventional anticancer agents may also be decreased when given in combination with parvoviruses, thereby reducing deleterious side effects. Yet, it is worth stressing that cell proliferation is a prerequisite for parvovirus replication and therefore these viruses cannot be used to kill resting tumor cells or cells whose quiescence has been induced by a cytostatic treatment. In this respect, a most appealing treatment would combine toxins specific for cycling cells with recombinant parvoviruses expressing a cocktail of immunostimulatory factors, in the hope that any tumor cells escaping the former agent may be sensitive to the bystander effect of the viral vector's gene product.

Safety Issues and Clinical Trials

As previously mentioned, antineoplastic properties can be assigned to parvoviruses which were originally isolated from human tumor transplants in rodents. Despite initial claims that they are of human origin [21], these agents are presently viewed as being rodent parvoviruses that opportunistically infect human neoplastic cells engrafted in virus-carrying animals. Indeed, these viruses are prevalent in rodent populations [21, 22], and the initial question as to whether they could also be isolated from human material has been discussed [21]. Serological data confirm that natural infections with rodent parvoviruses in the human population are, at the most, rare events even in laboratory personnel working with these agents [21]. This low natural infection frequency can be seen as an advantage, not only for the scientific and technical staff working with these viruses but also for prospective patients receiving a parvovirus-based therapy, since less than a few percent of the patients will have neutralizing antibodies at the time of therapeutic virus injection [21]. Except for some neonatal infections, rodent parvovirus infections are usually clinically inapparent in their respective animal hosts (see above). If applicable to humans, this relative innocuousness would argue for the safety of rodent parvovirus administration to cancer patients. Yet, the biological effects of these viruses cannot be readily extrapolated from one animal species to the other, as exemplified by the

capacity of MVMp, which is nonpathogenic for mice, to cause severe illness in newborn multimammate rats [82]. Therefore, safety is an important issue when considering rodent parvoviruses for human treatments. This question was initially addressed for suspected cases of natural infection of humans with H-1 and related viruses. No consistent correlation was found between any human disease and serological evidence of a previous infection with these viruses [21]. Since some rodent parvoviruses are particularly pathogenic in fetuses and neonates, histories of repeated abortion and stillbirth were investigated for their possible relationship with H-1 virus infections. Although the virus was claimed by some authors to be prevalent in women with such histories [128], this finding could not be confirmed in other studies [21]. Altogether, these data tip the balance between potential risks and benefits from H-1 and related viruses in favor of the latter in cancer patients, warranting the further assessment of these agents in humans.

Although infection through natural routes is rare, H-1 can be experimentally inoculated in patients and seen to proliferate. A pioneer study was conducted as far back as 1965 by Toolan et al. [5] who used H-1 virus to treat 2 patients with advanced disseminated osteosarcomas that had proved to be resistant to other therapies. The result was a transient viremia followed by seroconversion. In a more recent phase-I clinical trial involving 12 cancer patients, live H-1 virus was directly injected into (sub)cutaneous metastases from different kinds of solid tumors which had proved to be resistant to conventional therapies [84]. These studies indicate that H-1 virus infection usually remains fully asymptomatic, while causing only transient and mild clinical signs (e.g., low fever) in a minority of infected patients. A maximum tolerated dose could thus not be determined, even though high amounts of virus were inoculated (up to 3×10^{10} infectious units/patient). Although the H-1 virus inocula used in these studies were not able to induce tumor regression, some treated neoplastic lesions showed evidence of growth stabilization and in situ virus replication. These effects, together with the good tolerance of H-1 virus, are provocative and encourage the consideration of this and related viruses, in natural or recombinant forms, for anticancer applications in humans.

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References

1 Berns KI: *Parvoviridae:* The viruses and their replication; in Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, Roizman B, Straus SE (eds): Virology. Philadelphia, Lippincott-Raven, 1996, pp 2173–2197.
2 Deleu L, Pujol A, Faisst S, Rommelaere J: Activation of promoter P4 of the autonomous parvovirus minute virus of mice (MVM) at the early S phase is required for productive infection. J Virol 1999;73:3877–3885.
3 Spalholz BA, Tattersall P: Interaction of minute virus of mice with differentiated cells: Strain-dependent target cell specificity is mediated by intracellular factors. J Virol 1983;46:937–943.
4 Young NS: Parvoviruses; in Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, Roizman B, Straus SE (eds): Virology. Philadelphia, Lippincott-Raven, 1996, pp 2199–2220.
5 Toolan HW, Saunders EL, Southam CM, Moore AE, Levin AG: H-1 virus viremia in the human. Proc Soc Exp Biol Med 1965;119:711–715.
6 Rommelaere J, Cornelis JJ: Antineoplastic activity of parvoviruses. J Virol Meth 1991;33:233–250.
7 Dupressoir T, Vanacker J-M, Cornelis JJ, Duponchel N, Rommelaere J: Inhibition by parvovirus H-1 of the formation of tumors in nude mice and colonies in vitro by transformed human mammary epithelial cells. Cancer Res 1989;49:3203–3208.
8 Faisst S, Guittard D, Benner A, Cesbron JY, Schlehofer JR, Rommelaere J, Dupressoir T: 1998. Dose-dependent regression of HeLa cell-derived tumours in SCID mice after parvovirus H-1 infection. Int J Cancer 1998;75:584–589.
9 Guetta E, Graziani Y, Tal J: Suppression of Ehrlich ascites tumors in mice by minute virus of mice. J Natl Cancer Inst 1986;76:1177–1180.
10 McKisic MD, Paturzo FX, Gaertner DJ, Jacoby RO, Smith AL: A nonlethal rat parvovirus infection suppresses rat T lymphocyte effector functions. J Immunol 1995;155:3979–3986.
11 McKisic MD, Paturzo FX, Smith AL: Mouse parvovirus infection potentiates rejection of tumor allografts and modulates T cell effector functions. Transplantation 1996;61:292–299.
12 Chen YQ, De Foresta F, Hertoghs J, Avalosse BL, Cornelis JJ, Rommelaere J: Selective killing of simian virus 40-transformed human fibroblasts by parvovirus H-1. Cancer Res 1986;46: 3574–3579.
13 Cornelis JJ, Becquart P, Duponchel N, Salomé N, Avalosse B, Namba M, Rommelaere J: Transformation of human fibroblasts by ionizing radiation, a chemical carcinogen or SV40 correlates with an increase in their susceptibility to the autonomous parvoviruses H-1 and minute-virus-of-mice. J Virol 1988;62:1679–1686.
14 Cornelis JJ, Chen YQ, Spruyt N, Duponchel N, Cotmore SF, Tattersall P, Rommelaere J: Susceptibility of human cells to killing by the parvoviruses H-1 and minute virus of mice correlates with viral transcription. J Virol 1990;64:2537–2544.
15 Cornelis JJ, Spruyt N, Spegelaere P, Guetta E, Darawshi T, Cotmore SF, Tal J, Rommelaere J: Sensitization of transformed rat fibroblasts to killing by parvovirus minute virus of mice correlates with an increase in viral gene expression. J Virol 1988;62:3438–3444.
16 Chen YQ, Tuynder MC, Cornelis JJ, Boukamp P, Fusenig E, Rommelaere J: Sensitization of human keratinocytes to killing by parvovirus H-1 takes place during their malignant transformation but does not require them to be tumorigenic. Carcinogenesis 1989;10:163–167.
17 van Hille B, Duponchel N, Salomé N, Spruyt N, Cotmore SF, Tattersall P, Cornelis JJ, Rommelaere J: Limitations to the expression of parvoviral nonstructural proteins may determine the extent of sensitization of EJ-ras-transformed rat cells to minute virus of mice. Virology 1989;171:89–97.
18 Guetta E, Mincberg M, Mousset S, Bertinchamps C, Rommelaere J, Tal J: Selective killing of transformed rat cells by minute virus of mice does not require infectious virus production. J Virol 1990;64:458–462.
19 Salomé N, van Hille B, Duponchel N, Meneguzzi G, Cuzin F, Rommelaere J, Cornelis JJ: Sensitization of transformed rat cells to parvovirus MVMp is restricted to specific oncogenes. Oncogene 1990;5:123–130.

20 Van Pachterbeke C, Tuynder M, Cosyn JP, Lespagnard L, Larsimont D, Rommelaere J: Parvovirus H-1 inhibits growth of short-term tumor-derived but not normal mammary tissue cultures. Int J Cancer 1993;55:672–677.
21 Siegl G: Biology and pathogenicity of auonomous parvoviruses; in Berns KI (ed): The Parvoviruses. New York, Plenum Press, 1984, pp 297–362.
22 Jacoby RO, Ball-Goodrich LJ, Besselsen DG, McKisic MD, Riley LK, Smith AL: Rodent parvovirus infections. Lab Anim Sci 1996;46:370–380.
23 Ball-Goodrich LJ, Leland SE, Johnson EA, Paturzo FX, and Jacoby RO: Rat parvovirus type 1: The prototype for a new rodent parvovirus serogroup. J Virol 1998;72:3289–3299.
24 Spegelaere P, van Hille B, Spruyt N, Faisst S, Cornelis JJ, Rommelaere J: Initiation of transcription from the minute virus of mice P4 promoter is stimulated in rat cells expressing a c-Ha-ras oncogene. J Virol 1991;65:4919–4928.
25 Mousset S, Youssef Q, Caillet-Fauquet P, Rommelaere J: The cytotoxicity of the autonomous parvovirus minute virus of mice nonstructural proteins in FR3T3 cells depends on oncogene expression. J Virol 1994;68:6446–6453.
26 Anouja F, Wattiez R, Mousset S, Caillet-Fauquet P: The cytotoxicity of the parvovirus minute virus of mice nonstructural protein NS1 is related to changes in the synthesis and phosphorylation of cell proteins. J Virol 1997;71:3671–4678.
27 Cotmore SF, Tattersall P: The autonomously replicating parvoviruses of vertebrates. Adv Virus Res 1987;33:91–174.
28 Rhode SL: *Trans*-activation of parvovirus P38 promoter by the 76K noncapsid protein. J Virol 1985;55:886–889.
29 Lorson C, Pearson J, Burger L, Pintel DJ: An SP1-binding site and TATA element are sufficient to support full transactivation by proximally bound NS1 protein of minute virus of mice. Virology 1989;240:326–337.
30 Naeger LK, Cater J, Pintel DJ: The small nonstructural protein (NS2) of the parvovirus minute virus of mice is required for efficient DNA replication and infectious virus production in a cell-type-specific manner. J Virol 1990;64:6166–6175.
31 Cotmore SF, D'Abramo Jr. AM, Carbonell LF, Bratton J, Tattersall P: The NS2 polypeptide of parvovirus MVM is required for capsid assembly in murine cells. Virology 1997;231:267–280.
32 Brownstein DG, Smith AL, Johnson EA, Pintel DJ, Naeger LK, Tattersall P: The pathogenesis of infections with minute virus of mice depends on expression of the small nonstructural protein NS2 and on the genotype of the allotropc determinants VP1 and VP2. J Virol 1992;66: 3118–3124.
33 Agbandje-McKenna M, Llamas-Saiz AL, Wang F, Tattersall P, Rossmann MG: Functional implications of the structure of the murine parvovirus, minute virus of mice. Structure 1998;6: 1369–1381.
34 Cotmore SF, Tattersall P: DNA replication in the autonomous parvoviruses. Semin Virol 1995; 6:271–281.
35 Richards RG, Armentrout RW: Early events in parvovirus replication: lack of integration by minute virus of mice into host cell DNA. J Virol 1979;30:397–399.
36 Bashir T, Hörlein R, Rommelaere J, Willwand K: Cyclin A activates the DNA polymerase δ-dependent elongation machinery in vitro: A parvovirus DNA replication model. Proc Natl Acad Sci USA 2000;97:5522–5527.
37 Christensen J, Cotmore SF, Tattersall P: A novel cellular site-specific DNA binding protein cooperates with the viral NS1 polypeptide to initiate parvovirus DNA replication. J Virol 1997; 71:1405–1416.
38 Cotmore SF, Tattersall P: High-mobility group 1/2 proteins are essential for initiating rolling-circle-type DNA replication at a parvovirus hairpin origin. J Virol 1998;72:8477–8484.
39 Richards RG, Linser P, Armentrout RW: Kinetics of assembly of a parvovirus, minute virus of mice, in synchronized rat brain cells. J Virol 1977;22:778–793.
40 Tullis GE, Burger LR, Pintel DJ: The minor capsid protein VP1 of the autonomous parvovirus minute virus of mice is dispensable for encapsidation of progeny single-stranded DNA but is required for infectivity. J Virol 1993;67:131–141.

41 Kuntz-Simon G, Bashir T, Rommelaere J, Willwand K: Neoplastic transformation-associated stimulation of the in vitro resolution of concatemer junction fragments from minute virus of mice DNA. J Virol 1999;73:2552–2558.
42 Deleu L, Fuks F, Spitkovsky D, Hörlein R, Faisst S, Rommelaere J: Opposite transcriptional effects of cyclic AMP-responsive elements in confluent or $p27^{KIP}$-overexpressing cells versus serum-starved or growing cells. Mol Cell Biol 1998;18:409–419.
43 Perros M, Deleu L, Vanacker J-M, Kherrouche Z, Spruyt N, Faisst S, Rommelaere J: Upstream CREs participate in the basal activity of minute virus of mice promoter P4 and in its stimulation in *ras*-transformed cells. J Virol 1995;69:5506–5515.
44 Fuks F, Deleu L, Dinsart C, Rommelaere J, Faisst S: *Ras* oncogene-dependent activation of the P4 promoter of minute virus of mice through a proximal P4 element interacting with the Ets family of transcription factors. J Virol 1996;70:1331–1339.
45 Gu Z, Plaza S, Perros M, Cziepluch C, Rommelaere J, Cornelis JJ: NF-Y controls transcription of the minute virus of mice P4 promoter through interaction with an unusual binding site. J Virol 1995;69:239–246.
46 Gu Z, Kuntz-Simon G, Rommelaere J, Cornelis J: Oncogenic transformation-dependent expression of a transcription factor NF-Y subunit. Mol Carcinog 1999;24:294–299.
47 Wolter S, Richards R, Armentrout RW: Cell cycle-dependent replication of the DNA of minute virus of mice, a parvovirus. Biochim Biophys Acta 1980;607:420–431.
48 Rhode SL: Construction of a genetic switch for an inducible *trans*-activation of gene expression in eukaryotic cells. J Virol 61;1990:1448–1456.
49 Li X, Rhode SL: Mutation of lysine 405 to serine in the parvovirus H-1 NS1 abolishes its function for viral DNA replication, late promoter *trans* activation and cytotoxicity. J Virol 1990;64: 4654–4660.
50 Brandenburger A, Legendre D, Avalosse B, Rommelaere J: NS-1 and NS-2 proteins may act synergistically in the cytopathogenicity of parvovirus MVMp. Virology 1990;174:576–584.
51 Caillet-Fauquet P, Perros M, Brandenburger A, Spegelaere P, Rommelaere J: Programmed killing of human cells by means of an inducible clone of parvoviral genes encoding non-structural proteins. EMBO J 1990;9:2989–2995.
52 Legendre D, Rommelaere J: Terminal regions of the NS-1 protein of the parvovirus minute virus of mice are involved in cytotoxicity and promoter *trans* inhibition. J Virol 1992;66:5705–5713.
53 Moffat S, Yaegashi N, Tada K, Tanaka N, Sugamura K: Human parvovirus B19 nonstructural (NS1) protein induces apoptosis in erythroid lineage cells. J Virol 1998;72:3018–3028.
54 Momoeda M, Wong S, Kawase M, Young NS, Kajigaya S: A putative nucleoside triphosphate-binding domain in the nonstructural protein of B19 parvovirus is required for cytotoxicity. J Virol 1994;68:8443–8446.
55 Ohshima T, Iwama M, Ueno Y, Sugiyama F, Nakajima T, Fukamizu A, Yagami J-I: Induction of apoptosis in vitro and in vivo by H-1 parvovirus infection. J Gen Virol 1998;79:3067–3071.
56 Morey AL, Ferguson DJ, Fleming KA: Ultrastructural features of fetal erythroid precursors infected with parvovirus B19 in vitro: Evidence of cell death by apoptosis. J Pathol 1993;169:213–220.
57 Rayet B, Lopez-Guerrero J-A, Rommelaere J, Dinsart C: Induction of programmed cell death by parvovirus H-1 in U937 cells: Connection with the tumor necrosis factor alpha signalling pathway. J Virol 1998;72:8893–8903.
58 Op de Beeck A, Anouja F, Mousset S, Rommelaere J, Caillet-Fauquet P: The nonstructural proteins of the autonomous parvovirus minute virus of mice interfere with the cell cycle, inducing accumulation in G2. Cell Growth Differ 1995;6:781–787.
59 Op de Beeck A, Caillet-Fauquet P: The NS1 protein of the autonomous parvovirus minute virus of mice blocks cellular DNA replication: A consequence of lesions to the chromatin? J Virol 1997; 71:5323–5329.
60 Ran Z-H, Rayet B, Rommelaere J, Faisst S: Parvovirus H-1 induced cell death: Influence of intracellular NAD consumption on the regulation of necrosis and apoptosis. Virus Res 1999;65:161–174.
61 Vanacker J-M, Laudet V, Adelmant G, Stéhelin D, Rommelaere J: Interconnection between thyroid hormone receptor signalling pathways and parvovirus cytotoxic functions. J Virol 1993;67: 7668–7672.

62 Telerman A, Tuynder M, Dupressoir T, Robaye B, Sigaux F, Shaulian E, Oren M, Rommelaere J, Amson R: A model of tumor suppression using H-1 parvovirus. Proc Natl Acad Sci USA 1993; 90:8702–8706.
63 Nüesch JPF, Dettwiler S, Corbeau R, Rommelaere J: Replicative functions of minute virus of mice NS1 protein are regulated in vitro by phosphorylation through protein kinase C. J Virol 1998; 72:9966–9977.
64 Corbau R, Duverger V, Rommelaere J, Nüesch JPF: Regulation of MVM NS1 by protein kinase C: Impact of mutagenesis at consensus phosphorylation sites on replicative and cytopathic effects. Virology 2000;278:151–167.
65 Dettwiler S, Rommelaere J, Nüesch JPF: DNA unwinding functions of minute virus of mice NS1 protein are modulated specifically by the lambda isoform of protein kinase C. J Virol 1999;73: 7410–7420.
66 Brown KE, Anderson SM, Young NS: Erythrocyte P antigen: Cellular receptor for B19 parvovirus. Science 1993;262:114–117.
67 Wang X-S, Yoder MC, Zhou SZ, Srivastava A: Parvovirus B19 promoter at map unit 6 confers autonomous replication competence and erythroid specificity to adeno-associated virus 2 in primary human hematopoietic progenitor cells. Proc Natl Acad Sci USA 1995;92:12416–12420.
68 Pallier C, Greco A, Le Junter J, Saib A, Vassias I, Morinet F: The 3′ untranslated region of the B19 parvovirus capsid protein mRNAs inhibits its own mRNA translation in nonprmissive cells. J Virol 1997;71:9482–9489.
69 Bass LR, Hetric FM: Human lymphoblastoid cells as hosts for parvoviruses H-1 and rat virus. J Virol 1978;25:486–490.
70 Su Z, Luo Z, Guo L, Li J, Liu Y: Inhibitory effect of parvovirus H-1 in cultured human tumour cells or transformed cells. Sci Sin [B] 1988;31:69–80.
71 Faisst S, Schlehofer JR, Zur Hausen H: Transformation of human cells by oncogenic viruses supports permissiveness for parvovirus H-1 propagation. J Virol 1989;63:2152–2158.
72 Lopez-Guerrero J-A, Rayet B, Rommelaere J, Dinsart C: Constitutive activation of U937 promonocytic cell clones selected for their resistance to parvovirus H-1 infection. Blood 1997;89:1642–1653.
73 Takahashi T, Ozawa K, Takahashi K, Asano S, Takabu F: Susceptibility of human erythopoietic cells to B19 parvovirus in vitro increases with differentiation. Blood 1990;75:603–610.
74 Segovia JC, Bueren JA, Almendral JM: Myeloid depression follows infection of susceptible newborn mice with the parvovirus minute virus of mice (strain i). J Virol 1995;69:3229–3232.
75 Segovia JC, Gallego JM, Bueren JA, Almendral JM: Severe leukopenia and dysregulated erythropoiesis in SCID mice persistently infected with the parvovirus minute virus of mice. J Virol 1999;73:1774–1784.
76 Ball-Goodrich LJ, Tattersall P: Two aminoacid substitutions within the capsid are coordinately required for acquisition of fibrotropism by the lymphotropic strain of minute virus of mice. J Virol 1992;66:3415–3423.
77 Truyen U, Parrish CR: The evolution and control of parvovirus host ranges. Semin Virol 1995; 6:311–317.
78 Colomar MC, Hirt B, Beard P: Two segments in the genome of the immunosuppressive minute virus of mice determine the host-cell specificity, control viral DNA replication and affect viral RNA metabolism. J Gen Virol 1998;79:581–586.
79 Previsiani N, Fontana S, Hirt B, Beard P: Growth of the parvovirus minute virus of mice MVMp3 in EL4 lymphocytes is restricted after cell entry and before viral DNA amplification: cell-specific differences in virus uncoating in vitro. J Virol 1997;71:7769–7780.
80 Vihinen-Ranta M, Kalela A, Mäkinen P, Kakkola L, Marjomäki V, Vuento M: Intracellular route of canine parvovirus entry. J Virol 1996;72:802–806.
81 Brownstein DG, Smith AL, Jacoby RO, Johnson EA, Hansen G, Tattersall P: Pathogenesis of infection with a virulent allotropic variant of minute virus of mice and regulation by host genome. Lab Invest 1991;65:357–364.
82 Haag A, Wayss K, Rommelaere J, Cornelis JJ: Experimentally induced infection with autonomous parvoviruses, minute virus of mice and H-1, in the african multimammate mouse (Mastomys coucha). Comp Med 2000;50:613–621.

83 Jacoby RO, Johnson EA, Ball-Goodrich LJ, Smith AL and McKisic MD: Characterization of mouse parvovirus infection by in situ hybridization. J Virol 1995;69:3915–3919.
84 Engers HD, Louis JA, Zubler RH, Hirt B: Inhibition of T cell-mediated functions by MVMi, a parvovirus closely related to the minute virus of mice. J Immunol 1981;127:2280–2285.
85 McKisic MD, Lancki DW, Otto G, Padrid P, Snook S, Cronin DC, Lohmar PD, Wong T, Fitch FW: Identification and propagation of a putative immunosuppressive orphan parvovirus in cloned T cells. J Immunol 1993;150:419–428.
86 Ellerman KE, Richards CA, Guberski DL, Shek WR, Like AA: Kilham rat virus triggers T cell-dependent autoimmune diabetes in multiple strains of rat. Diabetes 1996;45:557–562.
87 Le Cesne A, Dupressoir T, Janin N, Spielmann M, Le Chevalier T, Sancho-Garnier H, Paoletti C, Rommelaere J, Stehelin D, Tursz T: Intra-lesional administration of a live virus, parvovirus H-1 (PVH-1) in cancer patients: A feasibility study. Proc Annu Meet Am Soc Clin Oncol 1993, vol 12, p 297.
88 Porter DD, Larsen AE: Mink parvovirus infections; in Tijssen P (ed): Handbook of Parvoviruses. Boca Raton, CRC Press, 1990, vol 2, pp 87–101.
89 Marjolis G, Kilham L, Ruffolo PR: Rat virus disease, an experimental model of neonatal hepatitis. Exp Mol Pathol 1968;8:1–20.
90 Gaertner DJ, Jacoby RO, Smith AL, Ardito RB, Paturzo FX: Persistence of rat parvovirus in athymic rats. Arch Virol 1989;105:259–268.
91 Wiedbrauk DL, Bloom ME, Lodmell DL: Mink parvoviruses and interferons: In vitro studies. J Virol 1986;60:1179–1182.
92 Harris RE, Coleman PH, Morahan PS: Erythrocyte association and interferon production by minute virus of mice. Proc Soc Exp Biol Med 1974;145:1288–1292.
93 Chung Y-H, Jun H-S, Kang Y, Hirasawa K, Lee B-R, Van Rooijen N, Yoon J-W: Role of macrophages and macrophage-derived cytokines in the pathogenesis of kilham rat virus-induced autoimmune diabetes in diabetes-resistent biobreeding rats. J Immunol 1997;159:466–471.
94 McKisic MD, Macy JD, Delano ML, Jacoby RO, Paturzo PX, Smith AL: Mouse parvovirus infection potentiates allogeneic skin graft rejection and induces syngeneic graft rejection. Transplantation 1998;65:1436–1446.
95 Toolan HW, Rhode SL, Gierthy JF: Inhibition of 7,12-dimethylbenz(a)anthracene-induced tumors in syrian hamsters by prior infection with H-1 virus. Cancer Res 1982;42:2552–2555.
96 Maxwell IH, Maxwell F, Rhode SL III, Corsini J, Carlson JO: Recombinant LuIII autonomous parvovirus as a transient transducing vector for human cells. Hum Gene Ther 1993;4:441–450.
97 Maxwell IH, Long CJ, Carlson JO, Rhode SL III, Maxwell F: 1993. Encapsidation of a recombinant LuIII parvovirus genome by H1 virus and the fibrotropic or lymphotropic strains of minute virus of mice. J Gen Virol 1993;74:1175–1179.
98 Spitzer AL, Maxwell F, Corsini J, Maxwell IH: Species specificity for transduction of cultured cells by recombinant LuIII rodent parvovirus genome encapsidated by canine parvovirus or feline panleukopenia virus. J Gen Virol 1996;77:1787–1792.
99 Russell SJ, Brandenburger A, Flemming CL, Collins MKL, Rommelaere J: Transformation-dependent expression of interleukin genes delivered by a recombinant parvovirus. J Virol 1992; 66:2821–2828.
100 Dupont F, Tenenbaum L, Guo LP, Spegelaere P, Zeicher M, Rommelaere J: Use of an autonomous parvovirus vector for selective transfer of a foreign gene into transformed human cells of different tissue origins and its expression therein. J Virol 1994;68:1397–1407.
101 Brandenburger A, Coessens E, El Bakkouri K, Velu T: Influence of sequence and size of DNA on packaging efficiency of parvovirus MVM-based vectors. Hum Gene Ther 1999;10: 1229–1238.
102 Kestler J, Neeb B, Struyf S, van Damme J, Cotmore SF, D'Abramo A, Tattersall P, Rommelaere J, Dinsart C, Cornelis JJ: *Cis*-requirements for the efficient production of recombinant DNA vectors based on autonomous parvoviruses. Hum Gene Ther 1999;10:1619–1632.
103 Merchlinsky MJ, Tattersall PJ, Leary JJ, Cotmore SF, Gardiner EM, Ward DC: Construction of an infectious molecular clone of the autonomous parvovirus minute virus of mice. J Virol 1983; 47:227–232.

104 Rhode SL: Both excision and replication of cloned autonomous parvovirus DNA require the NS1 (rep) protein. J Virol 1989;63:4249–4256.
105 Brandenburger A, Russell SJ: A novel packaging system for the generation of helper-free oncolytic MVM vector stocks. Gene Ther 1996;3:927–931.
106 Karim A, Avalosse B, Maron A, Mine N, Burny A, Dupont F: Development of an advanced MVMp-based vector system designed specifically to prevent the generation of replication-competent helper virus via homologous recombination (abstract 5.3). 7th Parvovirus Workshop, Heidelberg 1997.
107 Palmer GA, Tattersall P: MVM-based gene therapy vectors that transduce genes into human and murine T cells (abstract W34-6). 17th Annu Meet Am Soc Virol, Vancouver, BC, 1998, p 119.
108 Cotmore SF, Christensen J, Nüesch JPF, Tattersall P: The NS1 polypeptide of the murine parvovirus minute virus of mice binds to DNA sequences containing the motif [ACCA] 2-3. J Virol 1995;69:1652–1660.
109 Tattersall P, D'Abramo A Jr, Cotmore SF: HIV TAT transactivator dependent, replication-competent chimeric MVM vectors. 17th Annu Meet Am Soc Virol, Vancouver, BC, 1998, p 120.
110 Yang TJ: Parvovirus-induced repression of canine transmissible venereal sarcoma. Am J Vet Res 1987;48:799–800.
111 Dupont F, Avalosse B, Karim A, Mine N, Bosseler M, Maron A, Van den Broeke A, Ghanem GE, Burny A, Zeicher M: Tumor-selective gene transduction and cell killing with an oncotropic autonomous parvovirus-based vector. Gene Ther 2000;7:790–796.
112 Parmiani G, Colombo MP, Melani C, Arienti F: Cytokine gene transduction in the immunotherapy of cancer. Adv Pharmacol 1997;40:259–307.
113 Pusieux I, Odin L, Poujol D, Moingeon P, Tartaglia J, Cox W, Favrot M: Canarypox virus-mediated interleukin 12 gene transfer into murine mammary adenocarcinoma induces tumor suppression and long-term antitumoral immunity. Hum Gene Ther 1998;9:2481–2492.
114 Sato TN: A new approach to fighting cancer? Proc Natl Acad Sci USA 1998;95:5843–5844.
115 Haag A, Menten P, Van Damme J, Dinsart C, Rommelaere J, Cornelis JJ: Highly efficient transduction and expression of cytokine genes in human tumor cells by means of autonomous parvovirus vectors: Generation of antitumor responses in recipient mice. Hum Gene Ther 2000;11: 597–609.
116 Wetzel K, Menten P, Opdenakker G, Van Damme J, Gröne HJ, Giese N, Vecchi A, Sozzani S, Cornelis JJ, Dinsart C: Transduction of human MCP-3 by a parvoviral vector induces leukocyte infiltration and reduces growth of human cervical carcinoma cell xenografts. J Gene Med 2001;3: in press.
117 Gregoriades G and McCormack B (eds): Proc NATO ASI on Targeting of Drugs: Strategies for Gene Constructs and Delivery. Amsterdam, IOS Press, 2000, pp 34–52.
118 Thomson CB: Distinct roles for the costimulatory ligands B7-1 and B7-2 in T helper cell differentiation. Cell 1995;81:979–982.
119 Rosenberg SA, Zhai Y, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Seipp CA, Einhorn JH, Roberts B, White DE: Immunizing melanoma using recombinant adenoviruses encoding MART-1 or gp100 melanoma antigens. J Natl Cancer Inst 1998;90: 1894–1900.
120 Cater JE, Pintel DJ: The small non-structural protein NS2 of the autonomous parvovirus minute virus of mice is required for virus growth in murine cells. J Gen Virol 1992;73: 1839–1843.
121 Lin W, Cui Y, Yu B, Luo Z, Lin Z: Preliminary comparison of sensitivity toward parvovirus H-1 of human hepatoma cells and parahepatoma tissue. Chin J Cancer Res 1989;1:15–20.
122 Sigal LJ, Crotty S, Andino R, Rock KL: Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. Nature 1999;398:77–80.
123 Soruri A, Fayyazi A, Gieseler R, Schlott T, Rünger TM, Neumann C, Hinrich Peters J: 1998. Specific autologous anti-melanoma T cell response in vitro using monocyte-derived dendritic cells. Immunobiology 1998;198:527–538.
124 Jacoby DR, Fraefel C, Breakefield XO: Hybrid vectors: A new generation of virus-based vectors designed to control the cellular fate of delivered genes. Gene Ther 1997;4:1281–1283.

125 Recchia A, Parks RJ, Lamartina S, Toniatti C, Pieroni L, Palombo F, Ciliberto G, Graham FL, Cortese R, La Monica N, Colloca S: Site-specific integration mediated by a hybrid adenovirus/adeno-associated virus vector. Proc Natl Acad Sci USA 1999;96:2615–2620.
126 Cohen H, Benvenisty N, Reshef L: Fate of polyoma origin of replication after its direct introduction into mice. FEBS Lett 1987;223:347–351.
127 Yang Y, Li Q, Ertl HCJ, Wilson JM: Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J Virol 1995;69:2004–2015.
128 Guglielmino S, Tempera G, Papalardo G, Castro A. H-1 and X14 parvovirus antibodies in woman with abortions or still-births. Acta Virol 1978;22:426–428.

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Monogr Virol. Basel, Karger, 2001, vol 22, pp 130–159

Vaccinia Virus

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Vaccinia virus has a distinctive history as a vaccine used for the eradication of smallpox, and the unique biology of this virus has been extensively investigated [1]. Because of its success as a vaccine and its safety profile in human vaccination, vaccinia has been explored as an expression vector to induce immune responses to a variety of antigens in the prevention or treatment of other viral illnesses such as rabies and HIV as well as for the expression of tumor-associated antigens in the treatment of cancer [2, 3]. In general, these approaches do not rely on targeting of any specific tissues and may not require viral replication. Rather they are designed to take advantage of the immune stimulatory effects of the complex viral particle and the efficient transcriptional machinery of the virus. Recently, it has been recognized that vaccinia has properties that make it worthy of exploration as a replicating vector for tumor directed gene therapy [4].

Using a replicating virus to target tumor cells and spread throughout a tumor prior to elimination by the immune system requires an extremely efficient virus or one that evades the immune system. Efficient viruses or viruses that evade the immune system are more pathogenic and pose a greater risk to the patient and population. Variola, the pox virus responsible for smallpox, was an example of a very efficient, yet virulent virus. The challenge is to make the virulent virus specific for tumor cells without decreasing its efficiency. Pox viruses are unique in that their entire life cycle occurs in the cytoplasm, including transcription and DNA replication, thus avoiding the potentially inefficient process of nuclear translocation [1]. Pox viruses produce their own transcriptional and DNA replication machinery which makes them resistant to the cell's attempt to shut down these processes. The virus produces a pronounced cytopathic effect on cells within hours of infection and synthesizes up to 10,000 copies of its genome within 12 h. The virus efficiently transfers from cell to cell such that a single

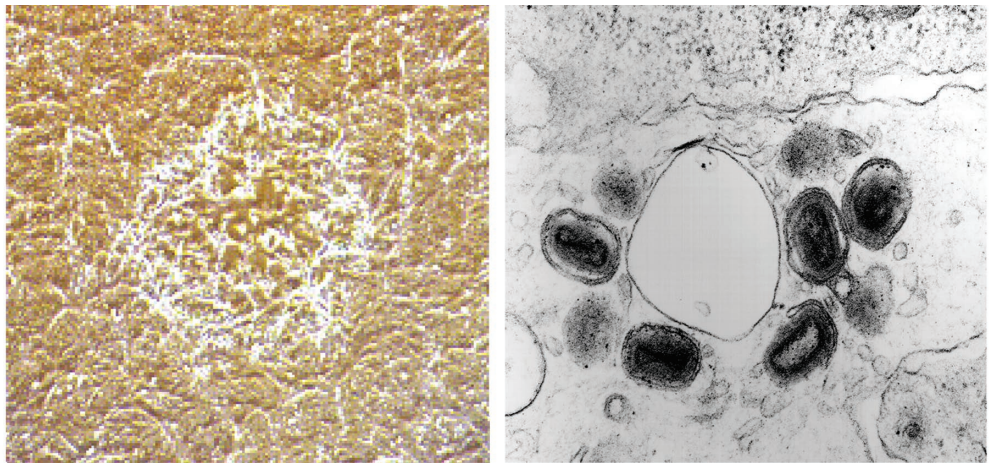


Fig. 1. ***a*** A typical vaccinia plaque on CV-1 cells is demonstrated revealing a marked cytopathic effect in cells within the infected region. Vaccinia progeny are released by membrane fusion and then remained cell-associated until infecting a neighboring cell. This results in a progressively enlarging plaque on confluent cells. ***b*** Vaccinia virus under electron microscope (courtesy of Maria Tsokos, MD, and Mones Abu-Asab, PhD, Laboratory of Pathology, NCI) revealing the characteristic enveloped oval virion with a biconcave central core.

virus particle can result in visible plaques on a cell monolayer within 36 h (fig. 1). In addition to its aggressive replicative cycle the virus has a broad host range infecting almost all cell types and multiple species ranging from rodents to humans.

Despite the observation that vaccinia virus replicates very efficiently in human cell lines, unlike other replicating viruses considered for gene therapy it does not routinely cause human infection, and it is not an endemic virus to any population. While the majority of people over the age of 21 have been immunized against vaccinia for the eradication of smallpox, patients under the age of 21 and future populations will not have preformed immunity to vaccinia as there will be no native exposure of the population to this virus. Other characteristics that make vaccinia an ideal vector for tumor-directed gene therapy include the ability to grow vaccinia in high titers and the ability to insert up to 25 kilobases of recombinant DNA without deletions in the virus. Strong synthetic pox promoters exist which allow for exceptionally high levels of gene expression. In this chapter we will review the unique biology of vaccinia virus and methods by which this virus can be targeted to human cancers.

Biology of Vaccinia Virus

Pox viruses are classified into two subfamilies, Chordopoxviridae (vertebrate poxviruses) and Entomopoxviridae (insect pox viruses), and at least 46 species [5]. The classification scheme is based on host range, sequence homology, and antigenicity. Vaccinia virus is a member of the orthopox virus genus, and its host of origin is unclear. Vaccinia is the virus that was used as a vaccine for smallpox, probably back as far as the 1850s. While Jenner originally used a cowpox virus isolated from a milkmaid for vaccination, in the 1930s it became clear that the virus in use for smallpox vaccination was genetically distinct from both cowpox virus and variola virus [6]. No known natural host exists for vaccinia virus, making it possible that mutations of cowpox or variola has led to this new species. It is perhaps more likely that vaccinia represents a unique species which is either extinct in its natural host or is so rare that it is difficult to identify [7].

All members of the orthopox genus have immune cross-reactivity and are relatively stable, allowing for the eradication of variola virus. Nevertheless, multiple strains of vaccinia viruses exist. As vaccination became widespread throughout the world, numerous centers produced and maintained the vaccine in different ways, resulting in numerous strains which differ in characteristics and pathogenicity. The original New York City Board of Health strain was obtained from England in 1856 and was originally used for smallpox vaccination in the United States [7]. The WR strain is a laboratory derivative of this strain and appears to be one of the more virulent strains in laboratory animals. It has not been utilized in patients to date.

The genome of the Copenhagen strain of vaccinia virus was completely sequenced and reported in 1990 [8] (other strains have been sequenced as well) [9]. The genome consists of double-stranded DNA with inverted terminal repeats and a terminal hairpin loop which mimics a large circular single-stranded DNA. The genome consists of 191,636 base pairs encoding approximately 2,063 proteins of 65 or more amino acids in length. As with all pox viruses the vaccinia virus is a double-stranded DNA virus whose entire life cycle exists within the cytoplasm of eukaryotic cells (fig. 2). The virus contains an outer envelope as well as an internal membrane and it carries the enzymes required for initiation of transcription. The extracellular enveloped form of the virus (EEV) is responsible for cell-to-cell spread, and has different properties compared to the intracellular mature virus (IMV) which is artificially released from the cell upon lysis [10]. The laboratory purified vaccinia is the IMV form, as it is collected from the lysed cellular fraction, and the extracellular envelope is too fragile to withstand the purification process.

Laboratory purified vaccinia enters the cell by membrane fusion, and recent data suggest that this is mediated by the A27L and D8L IMV membrane

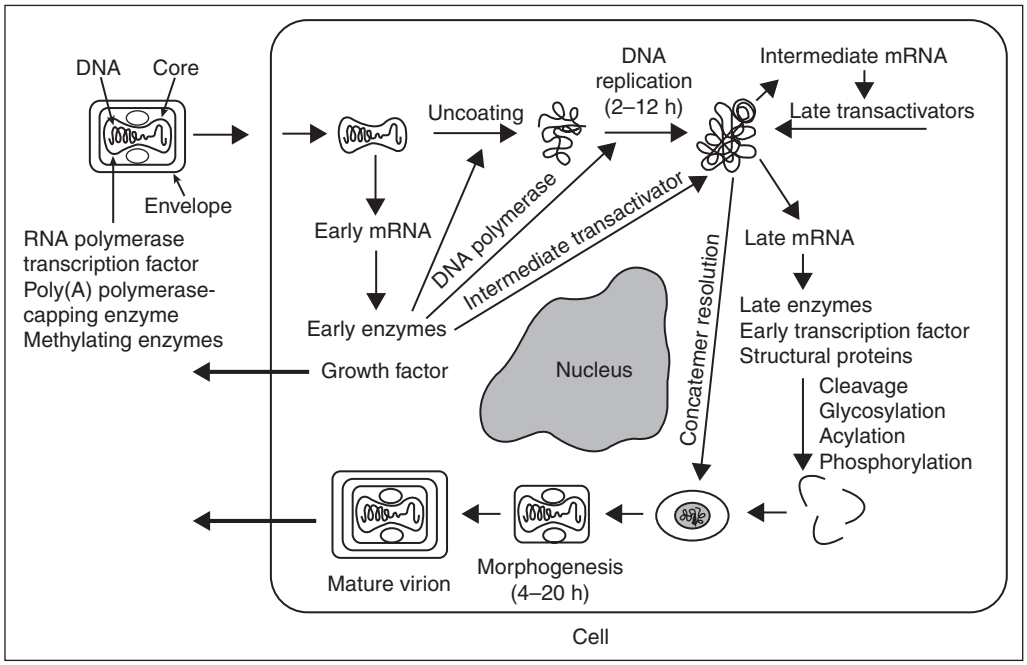


Fig. 2. Vaccinia life cycle. Reprinted from Science [2].

proteins which bind to heparin sulfate and chondroitin sulfate on the surface of the cell [11, 12]. This binding is followed by membrane fusion and entry of the virus core into the cytoplasm. As the virus enters the cell, transcriptional enzymes are released and immediately begin transcribing early messenger RNA [13]. Translation of this RNA produces early proteins which are involved in uncoating of the viral DNA, DNA replication, and intermediate transactivation for transcription of intermediate messenger RNA. Intermediate messenger RNA is then expressed which encodes for late transactivators leading to late messenger RNA synthesis. Late proteins include structural proteins for membrane formation and early transcription factors to be incorporated into the new virus particle. DNA replication occurs forming multiple concatamers of the genome. These concatamers are then resolved into individual genomes which are encapsulated along with the early transcription factors into Golgi-derived membranes. The virus has a total of three membrane layers immediately prior to release. The outermost viral membrane fuses with the cell membrane allowing release of the two-layered virus. This extracellular, enveloped virus particle, however, remains attached to the cell membrane, probably via the A34R gene product, theoretically allowing cell-to-cell spread of the virus without significant

shedding into the circulation [14]. The A34R gene may also be responsible for circulating EEV attaching to cells for viral uptake [15].

The entire replication cycle occurs in approximately 12 h. Early messenger RNA can be detected within 20 min of infection and DNA replication as early as 1–2 h after infection. A profound cytopathic effect occurs soon after viral entry as early enzymes act to shut down host cell function including complete inhibition of host protein synthesis by 4–6 h from the time of infection. This allows very efficient expression of viral genes and within 12 h after infection the majority of messenger RNA within the cytoplasm is from vaccinia-encoded genes. Approximately 10,000 copies of the viral genome are made within 12 h and approximately half of these are encapsulated into mature virions and released from the cell. Overall there is relatively little known direct interaction of cellular proteins with the viral life cycle. Recently, the cellular transcription factor YY1 has been demonstrated to bind to late vaccinia promoters to enhance late viral gene expression [16]. Another cell-derived intermediate transcription factor has been identified but not well characterized [17]. On the other hand, the virus encodes for many proteins which interact with the host as a means of protection against an antiviral host response. Many of these proteins have been well characterized, and include inhibitors of the interferon-induced apoptosis pathway, complement inhibitors, TNF receptor analogs, and serine protease inhibitors (serpins) [18].

A variety of vaccinia genes have been defined as host range genes by deletion mutants which lose their ability to replicate in certain cell types, but replicate normally in others. Early reports suggested that these genes were involved in resisting cellular apoptosis which may act as a natural cellular defense against viral infection. More recent data, however, obscure the relationship between viral replication and apoptosis [19, 20]. Nevertheless, some vaccinia proteins interact with the host as a means of protecting itself against host resistance, and deletion of these genes may lead to the inability of the virus to replicate in certain cell lines. The exact mechanism of these interactions, however, is still being investigated. Other vaccinia proteins inhibit the host's immune response against the virus (table 1).

Because the virus replication and life cycle occurs within the cytoplasm there is minimal to no risk of viral DNA incorporation into the genome. Productive infection uniformly results in the rapid death of cells such that recombination into the genome could not transform the cell. The viral genome appears to be quite stable. It was possible to eradicate smallpox worldwide through vaccinations without selective mutations leading to resistant strains. While multiple mutations of vaccinia virus have developed in different strains through in vitro and in vivo passages, none altered the immune recognition of the virus. Vaccinia has been inoculated through scarification of the skin in humans, and viral

Table 1. Anticytokine strategies encoded by pox viruses

Cytokine	Mechanism	Virus factor, ORF	Virus
IL-1β	Inhibits the IL-1β-converting enzyme	crmA (cow), B13R (vac)	cowpox, vaccinia
IFN-α/β/γ	blocks PKR activation by dsRNA	E3L (vac), E3L (var)	vaccinia, variola
IFN-α/β/γ	prevents phosphorylation of eIF2α by PKR	K3L (vac), C3L (var)	vaccinia, variola
TNF	soluble receptor	T2 (myx), crmB (cow), G2R (var)	myxoma, Shope fibroma, cowpox, vaccinia, variola
TNF	soluble receptor	crmC (cow), A53R (vac)	cowpox, vaccinia
IL-1β	soluble receptor	B15R (vac)	vaccinia, cowpox
IFN-γ	soluble receptor	T7 (myx), B8R (vac), C6L (swi) BBR (var)	myxoma, vaccinia, cowpox, camelpox, ectromelia, swinepox, variola
IFN-α/β	soluble receptor	B1BR (vac), B17R (var)	vaccinia, cowpox, camelpox, ectromelia, variola
chemokines	membrane chemokine receptor-like protein	K2R (swi), Q2/3L (she)	swinepox, sheeppox
chemokines	chemokine homologue	MC148R (mc)	molluscum contagiosum
chemokines	soluble binding protein	B29R (vac), T1 (myx)	vaccinia, cowpox, camelpox, variola, myxoma, Shope fibroma, racoonpox
chemokines	soluble binding protein	T7 (myx)	myxoma
IL-2/IL-5/IFN-γ	soluble binding protein	Not identified	tanapox

dsRNA = Double-stranded RNA; eIF2α = eukaryotic initiation factor 2α; IFN = interferon; IL-1β = interleukin-1β; IL-2 = interleukin-2; IL-5 = interleukin-5; ORF = open reading frame; PKR = dsRNA-dependent protein kinase; TNF = tumor necrosis factor.

The activity has been demonstrated in all cases except for the chemokine receptor-like protein and the chemokine homolog, which are predicted from sequence similarity.

The virus is indicated in parentheses: cow = cowpox; mc = molluscum contagiosum; myx = myxoma; she = sheeppox; swi = swinepox; vac = vaccinia strain Copenhagen (except for B15R and B18R which are strain WR); var = variola strain Bangladesh-1975.

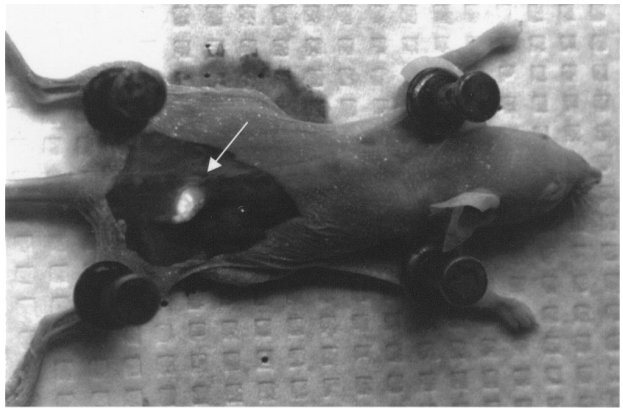
Reproduced from Smith et al. with permission [18].

replication in the skin leads to a characteristic vesicle formation known as a pox lesion. No known direct systemic inoculation of vaccinia virus has been reported in patients, however deaths from systemic viremia have occurred in vaccinated patients who have T-cell immune deficiencies. The immune response to vaccinia virus in humans is quite complex and includes an aggressive cellular response as well as the development of neutralizing antibodies. While preformed circulating antibodies may neutralize the intracellular mature form of the virus which is used for initial inoculation, they are not believed to be able to neutralize the EEV [21]. EEV is responsible for cell-to-cell spread, and antisera act to inhibit viral release from the cell surface without affecting plaque formation [22]. The viral immunity appears to be quite long lasting, as illustrated by the successful eradication of smallpox, however the current Center for Disease Control (CDC) recommendation is for repeat immunizations every 10 years for laboratory personnel actively working with vaccinia virus.

In vivo Biodistribution and Pathogenicity

When considering a virus as a vector for cancer gene therapy it is essential to know the biodistribution of the vector in humans in order to predict unacceptable toxicities and tumor transduction efficiency. It is well known that vaccinia virus forms characteristic pox lesions in the skin when administered by skin scarification. However, no data exist for whole body biodistribution. Given the virus' ubiquitous host range, it may be that animal models will provide some useful insight into biodistribution in humans. We and others have explored the biodistribution of vaccinia in murine models. Peplinski et al. [23] demonstrated that intravenous delivery of vaccinia virus (WR strain) in a subcutaneous murine tumor model led to 6-log higher recoverable virus plaque forming units (pfu) from the tumor compared to the liver and spleen. Our original intention was to explore the regional delivery of vaccinia virus in an isolated vascular perfusion system as a means of achieving high levels of gene expression within the tumors. When we compared levels of tumor marker gene activity in subcutaneous tumors after regional perfusion to levels after systemic injection, we consistently found that there was no advantage to regional perfusion because the systemically delivered virus efficiently targeted the tumor (unpublished data). We followed up with more formal biodistribution studies using systemically injected vaccinia virus (WR strain) [24].

We found that when injected systemically (intravenously or intraperitoneally) into mice bearing subcutaneous tumors, vaccinia infects and expresses genes in both the tumor and the ovary at much higher levels than in any other organs. Low levels of marker gene activity can also be picked up in the brain,



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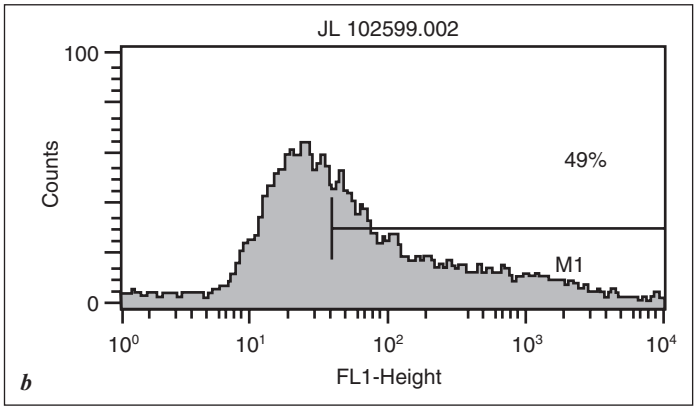

b

Fig. 3. A model of subcutaneous flank MC-38 tumors in nude mice treated with 10^8 pfu vaccinia expressing GFP injected into the peritoneal cavity (systemically). ***a*** Under UV light, 8 days after virus injection, the flank tumor (arrow) is fluorescent due to GFP expression as a result of vaccinia infection. Surrounding tissues show no evidence of fluorescence. ***b*** FACS analysis demonstrates that 49% of the cells within the tumor express GFP 8 days after injection. Slide is courtesy of Dr. John Lee, NCI.

bone marrow, liver, lung, and spleen, but on the order of 3 to 5 log-fold less than what is seen in the ovary and the tumor. FACS analysis of tumors after systemic injection of a vaccinia virus expressing the green fluorescent protein (GFP) demonstrates that up to 49% of the cells within the tumor express the gene 8 days after virus injection (fig. 3). High levels of tumor luciferase activity persists for greater than 21 days in athymic/nude mice, and for about 6 days in immunocompetent mice. The possible mechanisms for this apparent tropism and selective replication will be discussed in the next section. The observation

Table 2. Tissue luciferase activity (RLU/mg protein) after intravenous delivery of 10^6 vaccinia luciferase [4, 24]

Tumor model	Tumor	Ovary	Liver	Lung
Adenocarcinoma liver metastases in immunocompetent mice	46,000,000	1,450	–	600
Subcutaneous sarcoma in rat	4,337	0.74	0.023	0.056
VX-2 liver metastases in rabbit	2,103	132	9	7
Human melanoma in athymic mice	558,000	78,000	215	963

can be extended to many tumor models and hosts, suggesting that a similar phenomenon may occur in humans. We have studied the following tumors: MC38 (murine colon cancer) in immunocompetent mice; Pmel (human melanoma) in nude mice; MCA (sarcoma) in rat, and VX-1 (adenocarcinoma) in rabbits [4, 24]. All models demonstrated similar findings of selective tumor uptake and gene expression compared to other organs (table 2).

The biodistribution data alone do not clearly delineate the pathogenicity of this virus, and since it does not naturally cause human disease, it is difficult to define what organ systems are at risk from the virus. We often identify cystic/necrotic changes in the ovary after viral infection, but this is obviously not the cause of overall viral pathogenicity, and would be an acceptable side effect of systemic cancer therapy. We have found that 10^8 pfu of a WR strain, TK-deleted vaccinia injected into a tumor-bearing nude mouse will reliably result in the death of that mouse within 40 days. Animals become progressively cachectic and develop pox lesions on the skin. Laboratory tests do not reveal specific organ dysfunction, and postmortem histologic examination reveals no specific organ necrosis (except ovary) from vaccinia replications [25]. Presumably, neurologic complications, direct effects from secreted viral proteins, or detrimental effects of host proteins in response to the virus ultimately lead to the death of the host. The goal for enhancing tumor specificity and improving safety should be to address these possibilities. It should be noted that doses of up to 10^{10} pfu of a thymidine kinase-deleted WR strain of vaccinia virus injected intraperitoneally can be tolerated in an immunocompetent mouse.

Tumor Targeting

The mechanism for the ovary and tumor tropism that we and others have observed is not defined. The most obvious possibility is an increased density of cell surface receptors for the virus, but the virus efficiently infects all histologic

subtypes in vitro. As discussed above, the viral coat proteins appear to bind to heparin-sulfated glycosaminoglycans and chondroitin sulfate, leading to membrane fusion and viral uptake into cells. No direct evidence exists to suggest a higher density of heparin-sulfated glycosaminoglycans on tumor cells, but these glycoproteins are a component of the extracellular matrix within tumors [26], and digestion of the extracellular matrix during tumor cell invasion may lead to enhanced exposure of these molecules for vaccinia binding. This may trap the virus in the vicinity of the tumor, resulting in more tumor cell uptake of virus. It may be that exposure of the basement membrane in tumor neovasculature is somehow favorable to vaccinia binding and escaping the circulation. In addition, tumor neovasculature is leaky compared to vessels in other tissues. It has been clearly demonstrated that large proteins are more apt to escape the blood stream and concentrate in tumor tissue, simply because of this increased leakiness [27]. Vaccinia virus is a very large particle (350×270 nm), and a leaky vascular barrier would be a distinct advantage for viral escape from the circulation. Ovarian follicles have similar neovasculature and could explain why the tumor and ovary are similarly targeted [28].

Viruses like vaccinia, which replicate efficiently are likely to be more successful in rapidly dividing cells such as those seen within a tumor. This observation may help explain the tumor tropism. Dividing cells have an accessible pool of nucleotides, and are resistant to some pathways of apoptosis (which may or may not be involved in resistance to viral infection as discussed above). In fact, pox viruses encode for a secreted protein known as vaccinia growth factor (VGF) which stimulates surrounding cells to divide in order to prime them for vaccinia infection [29]. On the other hand, if vaccinia tropism is completely based on the presence of dividing cells, then it would be expected for vaccinia to preferentially replicate in gastrointestinal mucosal cells and bone marrow, such that toxicity would be similar to that seen with standard chemotherapy agents which target dividing cells. We have not been able to demonstrate vaccinia gene expression or viral recovery from gastrointestinal mucosa in repeated attempts. We can recover vaccinia from bone marrow, but the significance of this is unclear. Animals dying of vaccinia infection do not have neutropenia or thrombocytopenia, [25]. While we can take advantage of the receptiveness of dividing cells within a tumor to increase the specificity of the vector, it probably does not completely explain the native tropism.

By deleting genes which are required for vaccinia virus replication in nondividing cells, the virus may be more specific for tumor cells. We have focused on the thymidine kinase gene which is essential for the synthesis of deoxythymidine monophosphate (dTMP) and deoxyuridine monophosphate (dUMP) for DNA and RNA synthesis. In nondividing cells the host cell's stores of available nucleotides is limited, and therefore the viral thymidine kinase gene becomes

essential for the viral life cycle. After deleting the thymidine kinase gene the viral replication is markedly attenuated in nondividing cells, and therefore is less pathogenic in vivo [30]. Marker studies demonstrate that there is less viral recovery from normal host cells after thymidine kinase deletion [31]. A greater than 10-fold higher titer of thymidine kinase-deleted virus can be safely inoculated into the mouse brain compared to the wild-type virus, suggesting limited replication in brain cells in the absence of thymidine kinase [30]. In dividing cells such as tumor cells, however, the host cell nucleotide pool is ample and this seems to compensate for the loss of viral thymidine kinase. Dividing cells in culture allow for efficient viral replication in the absence of viral thymidine kinase and the deleted virus can grow to high titers. Thymidine kinase deletion alone, therefore, decreases the pathogenicity of the virus in vivo without affecting its ability to replicate in tumor cells, providing a selective therapy for tumor cells in vivo. We have demonstrated that thymidine kinase-deleted WR strain of vaccinia virus achieves a greater than 4 log-fold increase in tumor gene expression compared to the liver, spleen or brain [24]. Expressing an enzyme for conversion of a nontoxic prodrug with this marked therapeutic ratio between tumor cells and normal cells should allow for a reasonable tumor response.

There are many other genes involved in DNA synthesis such as ribonucleotide reductase, thymidine kinase, DNA ligase, and dUTPase that can similarly be deleted to allow selective advantage in dividing tumor cells. One concern is that, as more genes are deleted, the virus will become less efficient within tumor cells, negatively impacting the ability of the virus to treat cancer. We have studied a virus with combined deletions of the thymidine kinase gene and the vaccinia growth factor gene [32].

Vaccinia growth factor gene, as discussed above, is expressed by an early promoter and encodes a protein which is secreted by vaccinia-infected cells. It is thought to bind to surrounding cell membrane receptors such as the epidermal growth factor receptor and induce the cell to begin the process of dividing. This makes the surrounding cells more receptive to vaccinia virus. Deletions of the VGF gene reduces the virulence of the virus [29]. In the setting of a thymidine kinase-deleted virus, VGF may act to compensate for the loss of thymidine kinase by stimulating the surrounding cells to divide and synthesize nucleotides. Although not proven, it is possible that vaccinia growth factor actually increases the activity of cellular thymidine kinase in the surrounding cells which compensates for the loss of the viral thymidine kinase. It is our hypothesis that deletion of the vaccinia growth factor gene in parallel with deletion of the thymidine kinase gene will increase the attenuation of the virus in normal host cells but leave viral replication unabated in cancer cells. Preliminary data have demonstrated that the double-deleted virus is highly attenuated in vivo compared to either the thymidine kinase alone deleted virus or the

VGF-deleted virus [32]. On the other hand, the virus maintains the ability to replicate in tumor cells in vivo without a loss in the maximum marker gene activity within the tumor. In summary, similar to manipulations that have been performed with herpes virus, the vaccinia virus can be attenuated such that it will not divide well in normal host cells but can maintain its ability to replicate in dividing tumor cells.

Another targeting mechanism which can be utilized in many viral systems is transcriptional targeting. Nuclear DNA viruses which utilize eukaryotic RNA polymerase II for transcription of viral DNA can take advantage of cell-type specific expression using tissue-specific promoters and enhancers. Vaccinia virus, on the other hand, utilizes its own RNA polymerase which recognizes specific vaccinia promoter sequences and is not influenced by most host transcription factors. Tumor- or tissue-specific promoters and enhancers will not function in vaccinia virus. Even if vaccinia virus DNA could translocate into the nucleus and included a cellular promoter, it is unlikely that cell-type specific transcription would occur, because the virus somehow shuts down host cell transcription early in infection in order to maximize viral protein synthesis.

As discussed above, it has been demonstrated that cellular transcription factors are involved in the expression of intermediate and late vaccinia genes, and recently the YY1 host transcription factor has been shown to bind to vaccinia late promoter regions. This transcriptional activator is normally trafficked to the nucleus by a nuclear localizing sequence, but in the presence of vaccinia viral infection it is found primarily in the cytoplasm associated with viral DNA [16]. This finding potentially opens the door for other host cell transcription factors to be utilized artificially in a cell-type specific manner, but the interaction between host cell transcription factors and viral RNA polymerase needs to be better defined and perhaps manipulated.

Another targeting strategy involves the mutation of viral coat proteins responsible for virus binding to the cell surface in order to improve viral targeting, and the principle for this has been demonstrated in adenovirus and retrovirus. Such an approach is more difficult with vaccinia because of the complex nature of the viral coat, and because of the baseline ubiquitous cellular infectivity of vaccinia. As well, the different enveloped forms of the virus add to this complexity. As discussed above, the virus that is purified in the laboratory is mostly the IMV which has different properties compared to the EEV which is responsible for cell-to-cell spread of the virus after initial infection in vivo. Successful retargeting of the EEV form may serve no advantage to the systemic delivery of purified IMV. Retargeting the IMV may be futile when EEV is the primary circulating form during secondary viremia. Katz et al. [33] have demonstrated that an HIV glycoprotein can be preferentially targeted to be expressed on the surface of the EEV by fusing with the cytoplasmic and transmembrane domain

from the B5R EEV protein. The virus was in no way retargeted, but it did form syncytia with cells expressing the CD4 receptor for HIV. Galmiche et al. [34] have shown that a scFv can be expressed appropriately on the extracellular enveloped virus, and that it would function to bind an antigen, but this did not alter the infectivity of the virus. Other proteins can be expressed on the viral coat as a means of improving immunogenicity for vaccine approaches without altering viral uptake into cells.

Recently, two IMV coat proteins (A27L and D8L) have been described which mediate binding to cell surface heparin sulfate and chondroitin sulfate for IMV uptake into cells. Blocking antibodies to these proteins abrogate infection, as does soluble A27L and D8L. Studies of single and double mutants demonstrate that D8L is the most important of the two proteins for cell surface binding [35]. Many questions and contradictions remain, however, and it is possible that in the wild-type virus numerous IMV coat proteins exist for cell surface binding. Nevertheless, these findings open the possibility for redirection of infection based on mutation of this protein, but no attempts at this have been published to date. It is not clear whether retargeting the IMV is sufficient for enhanced specificity of the vector, or whether EEV would also require retargeting. After initial infection, subsequent spread of the virus is mediated by EEV. It must be kept in mind that significant alterations in the IMV or EEV envelope proteins may affect the formation of the extracellular envelope which would alter virion formation and release.

In general, vaccinia virus appears to have minimal dependence and interaction with host cellular factors (as demonstrated perhaps by its wide host range), which makes it difficult to imagine creating a cell-specific virus. It is of interest, therefore, when deletions of viral genes result in host range defects where the virus will still replicate in certain host cells but not in others. At least 5 host range genes have been described in pox viruses, including Chinese hamster ovary (CHO)hr, C7L, K1L, E3L, and SPI-1 [36, 37].

The products of these host range genes interact with the cell in some selective way to allow for cell-specific replication. It may be that a cellular protein present in some cells but not others compensates for the loss of the essential viral protein, or that some cells have better antiviral defense mechanisms in place which require a viral-blocking protein. Indeed, some host range genes are thought to function as inhibitors of apoptosis within some cells, but are not required in other cells. A general host cell defense against virus is the induction of apoptosis, shutting down host cell processes which may be essential for viral replication. Many viruses produce proteins which inhibit programmed cell death or apoptosis. In fact, viral-mediated cell transformation occurs when these viral proteins which resist apoptosis are inserted into the genome and become constitutively activated. Some pox virus host range genes are known to

inhibit apoptosis. The CrmA gene of cowpox virus is known to inhibit caspases, including the interleukin-1B-converting enzyme, a downstream mediator of apoptosis [38]. The vaccinia homologue, spi-2 is less well characterized, and while it can function to inhibit apoptosis it is not clear what significance this has for viral replication as an SPI-2 deleted mutant is not attenuated in vivo [39]. Perhaps intrinsic defects in apoptosis within tumor cells can compensate for the intentional deletion of viral anti-apoptotic genes as has been suggested in other viral systems, thus creating a tumor-specific virus. This avenue has not been explored in vaccinia virus.

However, the pathway for apoptosis resistance involved in tumor cell transformation may be different than the pathway involved in resistance to pox virus infection. The vaccinia E3L gene product (another host range gene) is known to bind to double-stranded RNA and prevent the induction of apoptosis via protein kinase (PKR) activation [40, 41], and the K3L gene is a competitive inhibitor of eukaryotic translation initiation factor (eIF-2a) [42]. Both of these proteins are mediators of interferon-induced apoptosis, and interferon is considered the primary defense mechanism against viral infection in mammalian cells [41]. Interferon, on the other hand, induces apoptosis in many tumor cells so efficiently that it has been utilized as antitumor therapy. While apoptosis resistance in the p53 pathway may be required for tumor cell transformation, resistance in the interferon pathway is not. No specific vaccinia protein interactions with the p53-mediated apoptotic pathway have been identified.

Recently the importance of apoptosis in vaccinia viral replication has been brought into question [19]. Because of the rapid life cycle of vaccinia virus and the nonreliance on host cell proteins in general, it may be apoptosis has very little relevance to vaccinia viral infection compared to its significance in other viruses such as adenovirus. Vaccinia itself shuts down host cell functions very early in infection which is similar to what would be expected in the initial stages of apoptosis. It is difficult to demonstrate apoptosis in vaccinia-infected cells, even in the setting of host range mutants in the absence of productive viral infection [19]. Intentional, artificial overexpression of a purely pro-apoptotic gene by vaccinia virus could potentially induce apoptosis in cells which were not transformed preventing viral replication. In tumor cells, where resistance to apoptosis is present as a means of its transformation, the virus should be able to replicate normally. However, even when apoptosis is intentionally induced upon vaccinia infection it does not alter vaccinia virus replication [43]. This avenue of obtaining specificity may not be feasible.

The vaccinia host range gene SPI-1 was originally demonstrated to function as an inhibitor of apoptosis during vaccinia infection, but new data place this observation in doubt [19]. Instead, the SPI-1 gene has been characterized as a serine protease inhibitor which binds to cathepsin G [44]. While the significance of

this is unknown, the squamous cell cancer antigen which is upregulated in most forms of squamous cell cancers has some homology to the SPI-1 protein of vaccinia virus and also acts to bind to cathepsin G [45]. The exact function of these proteins is not known, but upregulation of the cellular protein in transformed cells may compensate for deletion of the viral gene and provide a means for tumor specificity in squamous cell cancers. We are actively investigating this hypothesis.

In summary, the exploration of tumor-specific targeting of vaccinia virus is in its infancy. It may be possible to alter IMV or EEV envelope proteins in order to retarget the vaccinia virus, and it may be possible to alter host range genes to allow for selective replication in certain transformed tumor cells. Deletion of viral genes which are essential for viral replication in nondividing cells may also allow for tumor-specific replication. Despite the lack of careful investigation of these possibilities the WR strain of vaccinia virus naturally targets tumor cells remarkably well in vivo when delivered systemically. It may be that very little additional selectivity is necessary to achieve a safe virus that replicates and expresses genes well in tumor cells. It may also be possible to produce a gene product which has specificity for tumor cells. For example, in a suicide gene context, 5-fluorocytosine (5FC) can be converted to 5-fluorouracil (5FU) which has selective effects in dividing tumor cells and is already used as a systemically delivered chemotherapy agent. 5FU may have more of an effect when synthesized in tumor cells than in normal tissues. We are exploring the possibility of the vaccinia virus secreting a tumor-targeted protein consisting of an antibody/enzyme fusion. This may enhance both the specificity and the bystander effect of the virus.

Antitumor Effects

Much has been written on the ability of vaccinia virus to induce an immune response against tumor-associated antigens for the immunotherapy of cancer. The purpose of this review is, however, to focus more on the possibility of a replicating virus having an antitumor effect based on its ability to infect and kill cancer cells directly. Because vaccinia is such an efficient virus, as discussed above, it represents an ideal replicating virus for killing cancer cells. An intratumoral injection of a replication-competent vaccinia virus into a subcutaneous tumor can mediate a dramatic antitumor response [46]. In addition, we have found that a thymidine kinase-deleted WR strain of vaccinia has an antitumor response when injected systemically to treat a subcutaneous tumor in nude mice, without the addition of any toxic genes [25]. A characteristic pox vesicle can be seen specifically overlying a tumor which is infected with vaccinia after

a systemic injection of the virus, and virus can be recovered from the tumor during the response.

The ultimate goal with a vector like vaccinia is to express a gene which will induce the death of surrounding tumor cells which are not infected by the virus. We have added genes encoding enzymes for conversion of nontoxic prodrugs (viral-directed enzyme/prodrug therapy, VDEPT) in order to improve the antitumor response seen with vaccinia alone. We have investigated both the purine nucleoside phosphorylase gene which converts the nontoxic prodrug, 6-methylpurine deoxyriboside (6-MPDR) to the toxic 6-methyl-purine [47], and the cytosine deaminase (CD) gene which converts the nontoxic 5FC to the standard chemotherapy agent 5FU [48]. With both of these systems we have been able to demonstrate long-term cures in a murine model of hepatic metastases (fig. 4).

The interaction between the oncolytic effect of the replicating virus and the antitumor effect of the enzyme/prodrug system is quite complex. While the dividing virus itself can kill tumor cells, it will either be eliminated by the immune system or ultimately kill the host. On the other hand, the enzyme/prodrug system may lead to a significant bystander effect, but it may also inhibit viral replication and decrease the "oncolytic" activity of the virus [25]. Timing of the prodrug delivery is essential. The prodrug is not delivered until the virus has achieved its maximum effect alone such that the maximum percentage of cells within the tumor express the gene and the maximum tumor response has been achieved by the virus. At this stage the addition of the prodrug may serve to rescue the host from viral toxicity as well as enhance bystander killing of cells not infected with the virus. In vitro we have demonstrated that the addition of a prodrug enhances tumor cell killing by an efficient bystander effect where the converted prodrug is released into the cell supernatant and results in killing of distant cells. At a high viral multiplicity of infection (MOI) the virally induced cytopathic effect results in such efficient cell death that the prodrug addition is of no added benefit. At a very low MOI, however, the virus alone has very little effect over a finite period of time, yet the converted prodrug efficiently destroys many tumor cells [25]. It is likely that in vivo the converted prodrug will have the maximum effect as a low percentage of the tumor will be infected with virus. Of course other factors come into play in vivo including a potentially quick washout of diffusible toxins (converted prodrug) which may prevent a distant bystander effect within the tumor and lead to systemic toxicity. In fact, we have found that tumor conversion of the 6-MPDR into 6-MP leads to systemic toxicity from distant effects of the diffusible toxin [47].

We have attempted to demonstrate some of these complex interactions in vivo in a subcutaneous tumor in nude mice, using the 5FC/CD enzyme/prodrug

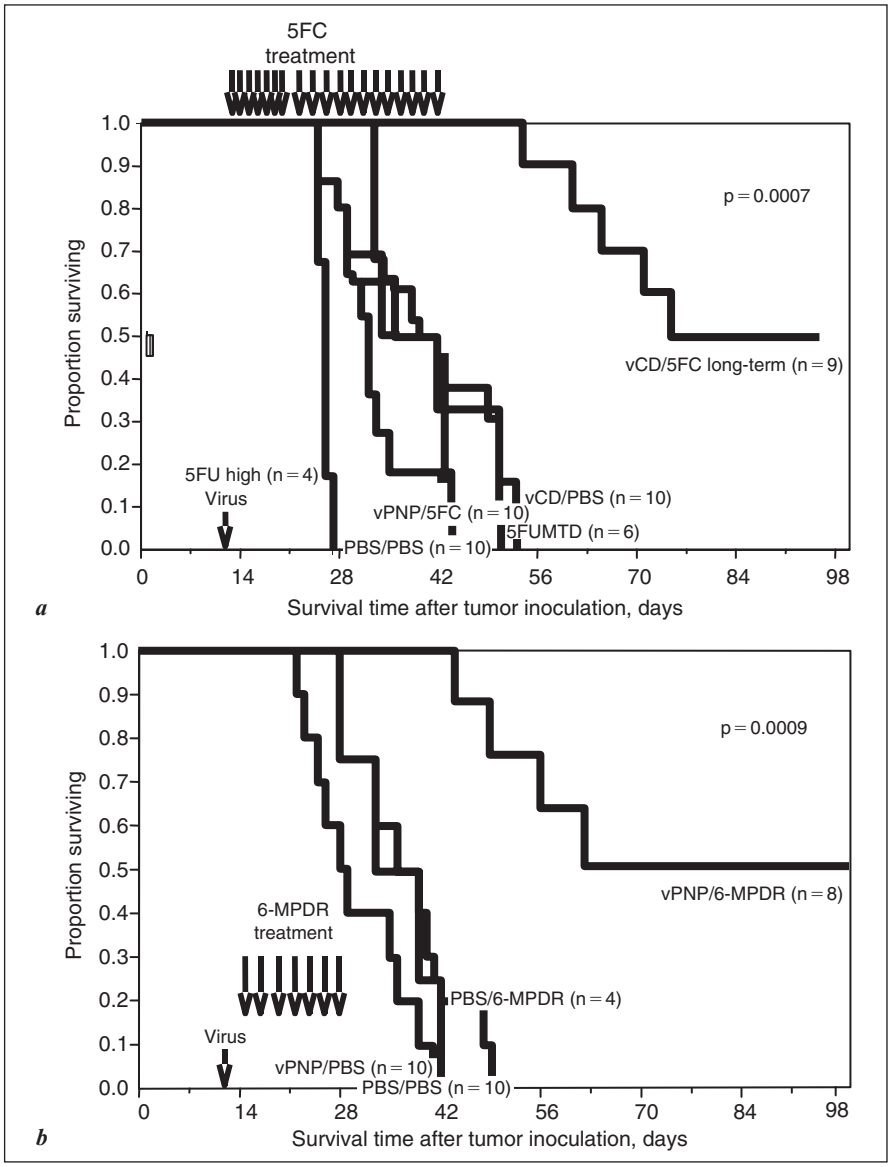

Fig. 4. Survival curves of athymic/nude mice with hepatic metastases using MC-38 cells, treated with 10^6 pfu intraperitoneal vaccinia: (***a***) vCD followed by 5FC or (***b***) vPNP followed by 6-MPDR. Treatment schedule is indicated by arrows on the graph. vCD = Vaccinia expressing cytosine deaminase; vPNP = vaccinia expressing purine nucleoside phosphorylase; PBS = phosphate-buffered saline; 5FC = 5-fluorocytosine (CD prodrug); 6-MPDR = 6-methylpurine deoxyriboside (PNP prodrug); 5FU = 5-fluorouracil chemotherapy control.

system. Intratumoral virus alone mediated a moderate antitumor response, but the addition of the 5FC prodrug resulted in some long-term cures. 5FC also prolonged survival in nude mice dying of virally mediated toxicity, suggesting that it may have inhibited viral replication. This was supported in vitro, as 5FC treatment resulted in decreased live virus recovery from vaccinia CD-infected cells [25]. We have also demonstrated that, in a hepatic metastases model in immunocompetent as well as nude mice, systemically delivered vaccinia virus expressing either the purine nucleoside phosphorylase gene or the CD gene can result in cures after systemic delivery of the prodrug [47–49]. The virus alone in this model, however, had no antitumor effect (a lower viral dose was used compared to the subcutaneous model). Palumbo et al. [46] reported treatment of subcutaneous tumors with a local injection of replicating vaccinia virus expressing the HSV-thymidine kinase gene for conversion of gancyclovir and bystander killing. They reported complete tumor responses, and improved immunologic protection following this approach.

The complex nature of these various effects requires further study. It is likely, however, that the combination of direct virally mediated oncolysis and a well-timed enzyme/prodrug system will combine for the most effective antitumor treatment. It should be noted that vaccinia virus is ideal for the expression of suicide genes and other toxic genes because of the efficient vaccinia RNA polymerase and very strong synthetic promoters [50]. It is believed that higher amounts of gene expression can lead to more efficient bystander killing with the enzyme/prodrug system.

Antiviral Immune Response

A major impediment to successful gene therapy using complex viral vectors is the immune response to the vector. This includes an intact immune system in a naive host eliminating the vector prior to it having a significant antitumor effect, as well as problems with preformed circulating antibodies and T-cell memory from prior exposure. Most viruses which infect human cells are also endemic in the population and therefore, the majority of patients will have circulating antibodies against the viruses and preformed cellular precursors. Vaccinia is unique in that as part of the smallpox eradication program all people over the age of approximately 21 have been immunized with vaccinia and, therefore, have circulating antibodies directed against orthopox viral proteins. As these circulating antibodies and memory T cells have led to the eradication of smallpox, it is quite likely this will also be an impediment to successful gene therapy with vaccinia virus. It may be that the remoteness of the immunization

influences the effectiveness of the circulating antibodies. Laboratory workers who undergo revaccination usually form pox vesicles, despite remote prior vaccination. This has also been demonstrated in tumor vaccine trials in patients previously immunized [51]. Some viruses can avoid circulating antibodies by mutating their coat proteins and changing serotype. This is not seen with vaccinia virus, as the complex viral coat proteins have proven stable over hundreds of years. It is also unlikely that intentional mutations of the coat proteins would be able to significantly alter the immune recognition without also changing the packaging and infectivity of the virus.

While the antibodies directed against vaccinia cross-react among all strains of vaccinia virus as well as other virus species within the orthopox genus, other pox viruses from different genera readily infect human cells and express genes without cross-reacting [52, 53]. Whether these viruses would show similar tumor tropism is unclear. It may be possible to construct a hybrid virus with a viral coat from one virus and the replication efficiency of vaccinia. This principle has been demonstrated by Scheiflinger et al. [54] who showed that a hybrid virus between fowlpox and vaccinia could be generated, though at low titers. Viruses from the Yatapox genus infect monkeys and secondarily have infected monkey caretakers [55]. These viruses do not cross-react with vaccinia, yet they cause human disease and replicate in human cells. The yaba-like disease (YLD) virus is under investigation as another replicating pox virus for tumor-directed gene therapy, as discussed in more detail below.

The T-cell response to vaccinia seems to be quite potent and is probably more important than antibodies in the host resistance to the virus. Table 3 summarizes the clinical response to vaccinia vaccination based on immunologic status. Progressive vaccinia correlates with a defect in cell-mediated immunity [56]. We have studied marker gene expression after systemic delivery of vaccinia in both immunocompetent and athymic/nude mice. In the absence of a functional T-cell population the virus is able to replicate and express genes within tumor cells at high levels for greater than 30 days [25] (fig. 5). On the other hand, in an immunocompetent host the window of gene expression only lasts for about 8 days with high levels of gene activity lasting approximately 4 days [24]. While this 4-day window may be enough for some very potent toxic genes or suicide gene systems to have an effect, it may be of some advantage to temporarily, reversibly inhibit the T-cell response to virus in order to allow for prolonged viral replication and spread through the tumor. Perhaps the recovering immune system would lead to bystander clearing of tumor cells as the intratumoral, antiviral, inflammatory response progresses. This is obviously a much safer procedure than manipulating the virus to be less recognizable by the immune system, but still requires a tumor-specific virus.

Table 3. Description of response to vaccinia vaccination based on immune status

Immunological condition	Cell-mediated immune status	Cell-mediated vac reactivity	Antibody immune status	Anti-vaccinia antibodies	Clinical response to vaccination	Vaccinia immune globulin helpful
Normal, vaccinated	+	+	+	+	Minimal reaction	N/A
Normal, unvaccinated	+	−	+	−	Primary vaccination	N/A
Thymic dysplasia	−	−	+	−	Progressive vaccinia	Not helpful
Bruton's syndrome	+	−	−	−	Primary vaccination, or if CMI overwhelmed, progressive vaccinia	May be helpful
Swiss syndrome	−	−	−	−	Progressive vaccinia	Not helpful
Acquired deficiencies	−	−	−	−	Progressive vaccinia (if CMI restored, then complete recovery)	May be helpful

Vac = Vaccinia, CMI = cell-mediated immunity.
Progressive vaccinia is a syndrome of progressive infection involving skin, but ultimately leading to death of the patient.
Adapted from Fenner et al. [56, fig. 4–6, p132].

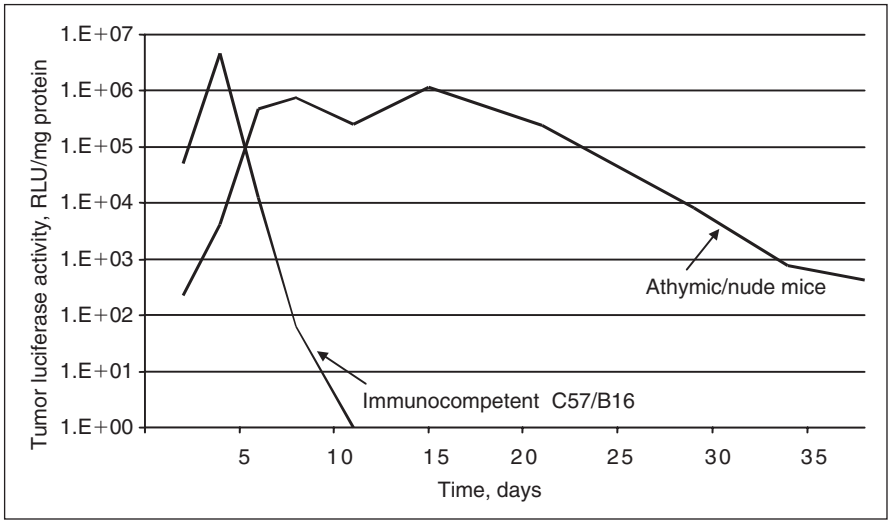

Fig. 5. Luciferase activity in hepatic metastases after intravenous delivery of vaccinia expressing luciferase. While the peak levels of activity are similar, the athymic mice have prolonged gene expression.

Safety Issues

Safety issues for cancer gene therapy vectors include direct toxicity of the vector, toxicity of the therapeutic gene product, genome insertion with transforming possibilities, germ line mutations and teratogenesis, and the ability to recombine with endemic virus to form a more virulent pathogen. Because vaccinia is a cytoplasmic virus, the viral DNA does not transport to the nucleus and therefore integration into the genome is very unlikely. In addition, there is no known latent infection with vaccinia virus and all cells infected by the virus will be killed by the virus. A theoretical concern exists with free viral DNA being released upon cell death that could be taken up into surrounding cells which are not infected with the virus and recombine into the genome [57]. The chances of this seem exceedingly low. In addition, since pox viruses are not endemic in the population, it is extremely unlikely for recombinations to occur in patients between attenuated strains and wild-type strains which would result in a more virulent virus with world health implications. The stability of the virus has already been proven during vaccination as part of the smallpox eradication program, so it is unlikely for spontaneous mutations to occur which would change the pathogenicity.

On the other hand, the properties that make it a useful virus for tumor-directed gene therapy also make it potentially more dangerous. It replicates

efficiently in human cells, and its pathogenicity as a systemically delivered virus is unknown. The scarification of the skin during vaccination for smallpox results in viral replication in the dermis, pox formation over 5–7 days, followed by an aggressive immune response against the virus which eliminates the virus and prevents systemic spread. A permanent scar in the skin results from the infection. It is not difficult to imagine that, if such an infection occurred in an organ such as the brain, this could result in a poor outcome. During vaccination for smallpox, patients with T-cell-deficient immune systems suffered progressive systemic infection and death from vaccinia [56]. In vaccine trials for HIV patients, deaths have been reported as a result of vaccinia viral replication, presumably secondary to systemic viremia in the setting of an immunocompromised host [58]. While intradermal delivery is quite safe for the vaccine strains, more virulent strains such as WR delivered systemically may be more pathogenic. These viruses need to be carefully studied in preclinical toxicology studies prior to human trials. Any mutations which result in improved tumor specificity and decreased systemic virulence should be considered (as discussed above).

Vaccinia and other pox viruses have been identified, designed, or treated such that they no longer replicate in human cells, but still efficiently express genes. These include the modified vaccinia ankara strain (attenuated by serial passage in chick embryo fibroblasts, until it no longer replicated in human cells), fowlpox virus, and entomopox viruses [52, 53, 59]. Vaccinia can also be reliably inactivated using UV light and psoralen such that early genes are still expressed but no cytopathic effect or replication occurs [60]. Also, viral mutants can be constructed with deletions in essential genes preventing replication except in cell lines where the gene is compensated for by stable integration into the genome. While all these nonreplicating viruses improve the safety profile, they would not be expected to be efficient for the purpose of tumor-directed gene therapy. It is my bias that a nonreplicating vector will never be sufficient to transduce enough cells within a human tumor as a systemically delivered vector to completely eradicate the tumor, even with a significant bystander effect. Our biodistribution study with psoralen/UV-inactivated virus supports this bias, where no measurable tumor β-galactosidase activity could be recovered compared to 10^6 RLU/mg protein with a replicating virus [24].

Our goal, therefore (as discussed above), is to use an efficient strain of vaccinia virus and attenuate the virus by inhibiting replication in nondividing cells, but maintaining replication in tumor cells. Also, it is possible that an enzyme/prodrug approach will inhibit viral replication and provide a switch for turning off infection prior to host toxicity. As discussed above, we have shown that treatment with 5FC prolongs survival in a model where mice are administered a lethal dose of vaccinia expressing the CD gene. This was the original design of

'suicide genes' and provides an additional safety switch which could potentially be very valuable and needs to be explored further [61].

Human Trials

The extent of experience with vaccinia over the years and its proven safety record should lead to acceptance of exploration of this vector in more novel delivery systems in terminal cancer patients. Vaccinia virus is being utilized in multiple clinical trials as vaccines for treatment of a variety of tumors as well as treatment of infectious diseases such as HIV (table 4). Replicating vaccinia virus has been delivered as subcutaneous, intramuscular, intratumoral, and intravesical (bladder) injections in clinical immunotherapy trials without significant vector related toxicity [51, 62, 63]. Doses of up to 10^9 pfu have been delivered safely. Intravenous injection of fowlpox virus has been performed with no significant toxicity, however, this species does not replicate in human cells. No systemic injection of a replicating vaccinia virus has been performed in human trials. Its use as a tumor-directed replicating oncolytic vector for cancer gene therapy has not been explored in clinical trials. The closest example of this is the intratumoral injection of replicating vaccinia expressing cytokine genes for the immunologic rejection of tumor.

Mastrangelo et al. [51] studied intratumoral injections of up to 2×10^7 pfu vaccinia expressing GmCSF in melanoma nodules in 7 revaccinated patients. In 5 of 7 patients they were able to see complete clearance of an injected lesion. These patients were immunized immediately prior to receiving the vector and it is certainly not clear whether viral replication had any effect in eradicating local tumor, but it gives hope that if systemic viral delivery could lead to viral gene expression within multiple tumor sites that this could lead to more global clearance of systemic tumor.

We have recently begun preclinical toxicology studies in the development of a tumor-directed vaccinia treatment delivered in an isolated perfusion model for intransit melanoma isolated to the limb. This model delivery system should allow for increased viral concentrations to be delivered to the tumor vasculature while theoretically avoiding preformed circulating antibodies. In addition, manipulations of the perfusion circuit, such as hyperthermia, may enhance tumor vascular leakiness and therefore viral infection.

Other Pox Viruses

Because of universally preformed immunity to vaccinia virus for all patients over the age of 25 who were vaccinated for smallpox, intense interest

Table 4. Clinical trials with vaccinia virus

Title	Principal investigator(s)	Institution
A phase I study of recombinant vaccinia that expresses prostate specific antigen in adult patients with adenocarcinoma of the prostate	A.P. Chen	National Naval Medical Center, Bethesda, Md.
Phase I study of recombinant CEA vaccinia virus vaccine with post vaccination CEA peptide challenge	D.J. Cole	Medical University of South Carolina, Charleston, S.C.
A phase I trial of recombinant vaccinia virus that expresses PSA in patients with adenocarcinoma of prostate	D.W. Kufe J.P. Eder	Dana-Farber Cancer Institute, Boston, Mass.
Phase I trial in patients with metastatic melanoma of immunization with a recombinant vaccinia virus encoding the MaART-1 melanoma antigen	S.A. Rosenberg	National Institute of Health, Bethesda, Md.
A phase I/II clinical trial evaluating the safety and biological activity of recombinant vaccinia-PSA vaccine in patients with serological recurrence of prostate cancer following radical prostatectomy	M.B. Sanda	University of Michigan Urology Clinics, Ann Arbor, Mich.
A pilot study of sequential vaccinations with ALVAC-CEA and vaccina-CEA with the addition of IL-2 and GM-CSF in patients with CEA expressing tumors	J.L. Marshall R.A. Peck	Georgetown University Medical Center, Washington, D.C. Sponsor: National Cancer Institute-Cancer Therapy Evaluation Program
A phase I trial of a recombinant vaccinia-CEA (180 kD) vaccine delivered by intradermal needle injection versus subcutaneous jet injection in patients with metastatic CEA expressing adenocarcinoma	R.M. Conry	University of Alabama at Birmingham, Birmingham, Ala. Sponsor: Drug Regulatory Affairs Branch, Cancer Therapy Evaluation Program (CTEP), Division of Cancer Treatment, Diagnosis and Centers, NCI, NIH

Table 4. (Continued)

Title	Principal investigator(s)	Institution
Phase I/II trial of antigen-specific immunotherapy in MUC-1 positive patients with adenocarcinoma of the prostate using vaccinia virus-MUC1-IL2 (TG 1031)	R. Figlin	University of California Los Angeles, Los Angeles, Calif. Sponsor: Transgene, SA
Phase I/II trial of antigen specific immunotherapy in MUC-1 positive patients with advanced non-small cell lung cancer using vaccinia-virus-MUC-1-IL-2	B.J. Gitlitz	University of California Los Angeles, Los Angeles, Calif. Sponsor: Transgene, SA
A phase I trial of recombinant vaccinia virus that expresses DF3/MUC1 in patients with metastatic adenocarcinoma	C.W. Kufe	Dana-Farber Cancer Institute, Boston, Mass.
A phase II randomized trial of recombinant fowlpox and recombinant vaccinia virus expressing PSA in patients with adenocarcinoma of the prostate	E.P. Eder	Dana-Farber Cancer Institute, Boston, Mass. Sponsor: NCI-CTEP
Immunization of patients with metastatic melanoma using recombinant fowlpox and vaccinia viruses encoding the tyrosinase antigen	S.L. Topalian	National Institutes of Health, Bethesda, Md.

Adapted from Rosenberg et al. [63, p 3067].
Other trials may exist which are not reported here.

exists in studying non-cross-reactive pox viruses. As discussed above, the non-cross-reacting species which have been reported thus far in this context do not replicate in human cells and therefore are not useful as an oncolytic virus. In review of the different genera, the yatapox viruses stand out as potential replicating vectors for human gene therapy. Monkey caretakers have developed cutaneous nodules after handling monkeys with similar lesions from a yatapox virus, and live virus could be recovered from these lesions [55]. This suggests that these viruses replicate in human cells, and could be used for tumor-directed gene delivery.

We have studied one member of this genus, the YLD virus, and found that it replicates efficiently in human cells, expresses genes at high levels

using vaccinia promoters, and it does not cross-react with vaccinia antibodies [64]. We have sequenced the YLD thymidine kinase gene and made recombinants into this locus expressing marker genes. We are in the process of studying this vector in vivo to see whether it has similar tumor tropism as the vaccinia virus. Unfortunately, unlike vaccinia it does not appear to replicate in murine cells, and therefore it will be more difficult to model tumor targeting with this vector compared to vaccinia. We are continuing investigation of this virus as a possible alternative pox virus for tumor-directed oncolytic gene therapy.

Conclusions

Vaccinia virus is an interesting gene expression vector which is worthy of continued exploration for tumor-directed gene therapy. It is an efficient, destructive virus with some element of baseline tumor specificity in murine, rat, and rabbit tumor models. Mutations leading to tumor-specific replication could potentially lead to selective tumor cell killing. Powerful transcription machinery can lead to very high levels of therapeutic gene expression within tumor cells, and its immunogenicity may lead to an immunologic bystander effect against tumor cells.

It is possible that a 3-pronged approach to vaccinia-mediated cancer gene therapy would be possible. This would involve the vaccinia replicative oncolytic effect, the toxic effect of the transgene expressed, as well as the immunologic clearance of the vector and stimulation of immune response against tumor-associated antigens. Because the vaccinia virus can include multiple genes, it would be possible to express toxic genes, multiple suicide genes, cytokine genes, costimulatory genes, HLA genes and tumor antigens. Reversible immunosuppression could lead to a period of time for the virus to replicate in cancer cells, followed by prodrug delivery at a time when the maximum number of cells within the tumor are expressing the enzyme gene. The reversal of immunosuppression would allow for immune clearance of the virus and bystander clearance of tumor cells. This versatility may be unique to vaccinia virus.

Compared to other replicating vectors such as herpes virus and adenovirus, the study of vaccinia as a tumor-directed suicide gene vector is in its infancy. I think over time the advantages of this vector will become more apparent, and its applicability may be more significant as the population ages and more cancer patients have not been vaccinated against smallpox. The complexity of the virus may allow for further manipulation to enhance the specificity and it is possible that further genetic manipulations can improve its ability to spread through tumor cells. Further understanding of the biology of the virus will

improve our ability to manipulate it to our advantage and enhance its potential as a vector for tumor-directed gene therapy.

References

1 Moss B: Poxviridae: The viruses and their replication; in Fields BN, Knipe DM, Howley PM (eds): Fields Virology. Philadelphia, Lippincott-Raven, 1996, pp 2637–2671.
2 Moss B: Vaccinia virus. A tool for research and vacccine development. Science 1991; 252:1662–1667.
3 Moss B, Carroll MW, Wyatt LS, Bennink JR, Hirsch VM, Goldstein S, Elkins WR, Fuerst TR, Lifson JD, Piatak M, Restifo NP, Overwijk W, Chamberlain R, Rosenberg SA, Sutter G: Host range restricted, non-replicating vaccinia virus vectors as vaccine candidates. Adv Exp Med Biol 1996; 397:7–13.
4 Gnant MF, Noll LA, Irvine KR, Puhlmann M, Terrill RE, Alexander HR Jr, Bartlett DL: Tumor-specific gene delivery using recombinant vaccinia virus in a rabbit model of liver metastases. J Natl Cancer Inst 1999;91:1744–1750.
5 Fenner F: Poxviruses; in Fields BN, Knipe DM, Howley PM (eds): Fields Virology. Philadelphia, Lippincott-Raven, 1996, pp 2673–2700.
6 Baxby D: Vaccinia virus; in Quinnan GV Jr (ed): Vaccinia Viruses as Vectors for Vaccine Antigens. New York, Elsevier, 1985, pp 3–8.
7 Fenner F, Wittek R, Dumbell KR: Vaccinia virus: The tool for smallpox eradication; in Fenner F, Wittek R, Dumbell K (eds): The Orthopoxviruses. New York, Academic Press, 1989, pp 143–170.
8 Goebel SJ, Johnson GP, Perkus ME, Davis SW, Winslow JP, Paoletti E: The complete DNA sequence of vaccinia virus. Virology 1990;179:247–266.
9 Antoine G, Scheiflinger F, Dorner F, Falkner FG: The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. Virology 1998;242: 365–396.
10 Smith GL, Vanderplasschen A: Extracellular enveloped vaccinia virus; in Enjuanes A (ed): Coronaviruses and Arteriviruses. New York, Plenum Press, 1998, pp 395–414.
11 Chung C-S, Hsiao J-C, Chang YS, Chang W: A27L protein mediates vaccinia virus interaction with cell surface heparan sulfate. J Virol 1998;72:1577–1585.
12 Hsiao J-C, Chung C-S, Chang W: Vaccinia virus envelope D8L protein binds to cell surface chondroitin sulfate and mediates the adsorption of intracellular mature virions to cells. J Virol 1999;73:8750–8761.
13 Moss B, Earl PL: Expression of proteins in mammalian cells using vaccinia viral vectors. In Ausubel M et al. (eds): Curr Prot Molec Biol 1998; Supplement 43, pp 16.15.1–16.19.11.
14 Blasco R, Sisler JR, Moss B: Dissociation of progeny vaccinia virus from the cell membrane is regulated by a viral envelope glycoprotein: Effect of a point mutation in the lectin homology domain of the A34R gene. J Virol 1998;67:3319–3325.
15 McIntosh AAG, Smith GL: Vaccinia virus glycoprotein A34R is required for infectivity of extravellular enveloped virus. J Virol 1996;70:272–281.
16 Broyles SS, Liu X, Zhu M, Kremer M: Transcription factor YY1 is a vaccinia virus late promoter activator. J Biol Chem 1999;274:35662–35667.
17 Rosales R, Sutter G, Moss B: A cellular factor is required for transcription of vaccinia viral intermediate-stage genes. Proc Natl Acad Sci USA 1994;91:3794–3798.
18 Smith GL: Vaccinia virus immune evasion. Immunol Lett 1999;65:55–62.
19 Shisler JL, Isaacs SN, Moss B: Vaccinia virus serpin-1 deletion mutant exhibits a host range defect characterized by low levels of intermediate and late mRNAs. Virology 1999;262:298–311.
20 Chung CS, Vasilevskaya IA, Wang SC, Bair CH, Chang W: Apoptosis and host restriction of vaccinia virus in RK13 cells. Virus Res 1997;52:121–132.
21 Ichihashi Y: Extracellular enveloped vaccinia virus escapes neutralization. Virology 1996;217: 478–485.

22 Vanderplasschen A, Hollinshead M, Smith GL: Antibodies against vaccinia virus do not neutralize extracellular enveloped virus but prevent virus release from infected cells and comet formation. J Gen Virol 1997;78:2041–2048.
23 Peplinski GR, Tsung K, Casey MJ, Meko JB, Fredrickson TN, Buller RM, Norton JA: In vivo murine tumor gene delivery and expression by systemic recombinant vaccinia virus encoding interleukin-1 beta. Cancer J Sci Am 1996;2:21.
24 Puhlmann M, Brown CK, Gnant M, Huang J, Libutti SK, Alexander HR, Bartlett DL: Vaccinia as a vector for tumor-directed gene therapy: Biodistribution of a thymidine kinase-deleted mutant. Cancer Gene Ther 2000;7:66–73.
25 McCart JA, Puhlmann M, Lee J, Hu Y, Libutti SK, Alexander HR, Bartlett DL: Complex interactions between the replicating oncolytic effect and the enzyme/prodrug effect of vaccinia-mediated tumor regression. Gene Ther 2000;7:1217–1223.
26 Brunner G, Reimbold K, Meissauer A, Schirrmacher V, Erkell LJ: Sulfated glycosaminoglycans enhance tumor cell invasion in vitro by stimulating plasminogen activation. Exp Cell Res 1998; 239:301–310.
27 Kohn S, Nagy JA, Dvorak HF, Dvorak AM: Pathways of macromolecular tracer transport across venules and small veins. Structural basis for the hyperpermeability of tumor blood vessels. Lab Invest 1992;67:596–607.
28 Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG: Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest 1998;78:1385–1394.
29 Buller RML, Chakrabarti S, Cooper JA, Twardzik DR, Moss B: Deletion of the vaccinia virus growth factor gene reduces virus virulence. J Virol 1988;62:866–874.
30 Buller RM, Smith GL, Cremer K, Notkins AL, Moss B: Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature 1985;317:813–815.
31 Whitman ED, Tsung K, Paxson J, Norton JA: In vitro and in vivo kinetics of recombinant vaccinia virus cancer-gene therapy. Surgery 1994;116:183–188.
32 McCart JA, Hu YK, Alexander HR, Libutti SK, Moss B, Bartlett DL: A combined thymidine kinase/vaccinia growth factor deleted vaccinia virus as a vector for cancer gene therapy. Proc Am Soc Gene Ther 1999;2:160.
33 Katz E, Wolffe EJ, Moss B: The cytoplasmic and transmembrane domains of the vacinia virus B5R protein target a chimeric human immunodeficiency virus type 1 glycoprotein to the outer envelope of nascent vaccinia virions. J Virol 1997;71:3178–3187.
34 Galmiche MC, Rindisbacher L, Wels W, Wittek R, Buchegger F: Expression of a functional single chain antibody on the surface of extracellular enveloped vaccinia virus as a step towards selective tumour cell targeting. J Gen Virol 1997;78:3019–3027.
35 Hsiao JC, Chung CS, Chang W: Vaccinia virus envelope D8L protein binds to cell surface chondroitin sulfate and mediates the adsorption of intracellular mature virions to cells. J Virol 1999;73:8750–8761.
36 Wyatt LS, Carroll MW, Czerny C-P, Merchlinsky M, Sisler JR, Moss B: Marker rescue of the host range restriction defects of modified vaccinia virus ankara. Virology 1998;251:334–342.
37 Perkus ME, Goebel SJ, Davis SW, Johnson GP, Limbach K, Norton EK, Paoletti E: Vaccinia virus host range genes. Virology 1990;179:276–286.
38 Ray C, Black RA, Kronheim SR, Greenstreet TA, Sleath PR, Salvensen GS, Pickup DJ: Viral inhibition of inflammation: Cowpox virus encodes an inhibitor of the interleukin-1beta converting enzyme. Cell 1992;69:597–604.
39 Kettle S, Blake NW, Law KM, Smith GL: Vaccinia virus serpins B13R (SPI-2) and B22R (SPI-1) Encode M_r 38.5 and 40K, intracellular polypeptides that do not affect virus virulence in a murine intranasal model. Virology 1995;206:136–147.
40 Rivas C, Gil J, Melkova Z, Esteban M, Diaz-Guerra M: Vaccinia virus E3L protein is an inhibitor of the interferon (IFN)-induced 2-5A synthetase enzyme. Virology 1998;243:406–414.
41 Kibler KV, Shors T, Perkins KB, Zeman CC, Banaszak MT, Biesterfeldt J, Langland JO, Jacobs BL: Double-stranded RNA is a trigger for apoptosis in vaccinia virus-infected cells. J Virol 1997; 71:1992–2003.

42 Davies MV, Elroy-Stein O, Jagus R, Moss B, Kaufman RJ: The vaccinia virus K3L gene product potentiates translation by inhibiting double-stranded-RNA-activated protein kinase and phosphorylation of the alpha subunit of eukaryotic initiation factor 2. J Virol 1992;66: 1943–1950.
43 Kettle S, Alcami A, Khanna A, Ehret R, Jassoy C, Smith GL: Vaccinia virus serpin B13R (SPI-2) inhibits interleukin-1 beta-converting enzyme and protects virus-infected cells from TNF- and Fas-mediated apoptosis, but does not prevent IL-1 beta-induced fever. J Gen Virol 1997;78: 677–685.
44 Moon KB, Turner PC, Moyer RW: SPI-1-dependent host range of rabbitpox virus and complex formation with cathepsin G is associated with serpin motifs. J Virol 1999;73:8999–9010.
45 Suminami Y, Nawata S, Kato H: Biological role of SCC antigen. Tumor Biol 1998;19:488–493.
46 Palumbo GJ, Higginbotham JN, Toland B, Ramsey J, Blaese RM: A replication competent recombinant vaccinia vector expressing HSV-TK for the treatment of tumors in vivo. Proc Am Soc Gene Ther 1998;1:169a.
47 Puhlmann M, Gnant M, Brown CK, Alexander HR, Bartlett DL: Thymidine kinase deleted vaccinia virus expressing purine nucleoside phosphorylase as a vector for tumor directed gene therapy. Hum Gene Ther 1999;10:649–657.
48 Gnant MFX, Puhlmann M, Alexander HR Jr, Bartlett DL: Systemic administration of a recombinant vaccinia virus expressing the cytosine deaminase gene and subsequent treatment with 5-fluorocytosine leads to tumor specific gene expression and prolongation of survival in mice. Cancer Res 1999;59:3396–3404.
49 Gnant M, Puhlmann M, Bartlett DL, Alexander HR: Regional verses systemic delivery of recombinant vaccinia virus as suicide gene therapy for murine liver metastases. Ann Surg 1999; 230:352–361.
50 Chakrabarti S, Sisler JR, Moss B: Compact, synthetic, vaccinia virus early/late promoter for protein expression. Biotechniques 1997;23:1094–1097.
51 Mastrangelo MJ, Maguire HC, Jr., Eisenlohr LC, Laughlin CE, Monken CE, McCue PA, Kovatich AJ, Lattime EC: Intratumoral recombinant GM-CSF-encoding virus as gene therapy in patients with cutaneous melanoma. Cancer Gene Ther 1998;6:409–422.
52 Wang M, Bronte V, Chen PW, Gritz L, Panicali D, Rosenberg SA, Restifo NP: Active immunotherapy of cancer with a nonreplicating recombinant fowlpox virus encoding a model tumor-associated antigen. J Immunol 1995;154:4685–4692.
53 Li Y, Hall RL, Moyer RW: Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses. J Virol 1997;71:9557–9562.
54 Scheiflinger F, Dorner F, Falkner FG: Construction of chimeric vaccinia viruses by molecular cloningand packaging. Proc Natl Acad Sci USA 1992;89:9977–9981.
55 Grace JT Jr, Mirand EA: Yaba virus infection in humans. Exp Med Surg 1965;23:213–216.
56 Fenner F, Wittek R, Dumbell KR: The pathogenesis, pathology, and immunology of orthopoxvirus infections; in Fenner F, Wittek R, Dumbell K (eds): The Orthopoxviruses. New York, Academic Press, 1989, pp 85–141.
57 Cutler ML, Shupert WL, Schlom J, Kantor J: Low-level transforming activity of an activated Ras gene under the control of a vaccinia virus p40 promoter is abrogated by truncation of the Ras cDNA. Vaccine 1999;17:2275–2283.
58 Dorozynski A, Anderson A: Deaths in vaccine trials trigger French inquiry. Science 1991; 252:501–502.
59 Blanchard TJ, Alcami A, Andrea P, Smith GL: Modified vaccinia virus Ankara undergoes limited replication in human cells and lacks several immunomodulatory proteins: Implications for use as a human vaccine. J Gen Virol 1998;79:1159–1167.
60 Tsung K, Yim JH, Marti W, Buller RM, Norton JA: Gene expression and cytopathic effect of vaccinia virus inactivated by psoralen and long-wave UV light. J Virol 1996;70:165–171.
61 Plautz G, Nabel EG, Nabel GJ: Selective elimination of recombinant genes in vivo with a suicide retroviral vector. New Biol 1991;3:709–771.
62 Lattime EC, Lee SS, Eisenlohr LC, Mastrangelo MJ: In situ cytokine gene transfection using vaccinia virus vectors. Semin Oncol 1996;23:88–100.

63 Rosenberg SA, Blaese RM, Brenner MK, Deisseroth AB, Ledley FD, Lotze MT, Wilson JM, Nabel GJ, Cornetta K, Economou JS, Freeman SM, Riddell SR, Oldfield E, Gansbacher B, Dunbar C, Walker RE, Schuening FG, Roth JA, Crystal RG, Welsh MJ, Culver K, Heslop HE, Simons J, Wilmott RW, Habib NA: Human gene marker/therapy clinical protocols. Hum Gene Ther 1999; 10:3067–3123.
64 Lee J, Hu Y, McCart JA, Moss B, Alexander HR, Bartlett DL: A novel replicating pox vector for cancer gene therapy (abstract). Soc Surg Oncol 2000.

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Replication-Competent, Oncolytic Newcastle Disease Virus for Cancer Therapy

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Introduction

Background Virology

Newcastle disease virus (NDV) is an avian virus belonging to the Paramyxoviridae, a family of enveloped, nonsegmented, negative-stranded RNA viruses [1]. NDV is an important pathogen for poultry and is widely distributed in naturally occurring bird populations [2]. There is a wide variation in the avian pathogenicity of NDV isolates, including naturally attenuated vaccine strains of proven safety and those that are highly pathogenic for chickens [3]. The virus name comes from the site of the first reported disease outbreak among chickens on a farm near Newcastle-upon-Tyne in England in 1926 [4, 5].

Historical Overview of NDV in the Treatment of Human Cancer

In 1994, a new age for therapeutic viruses was proposed with NDV considered a most promising agent [6]. The first hints of the potential anticancer benefit of this virus were made over 30 years ago. In 1964, Wheelock and Dingle [7] reported a significant reduction in leukemic blasts in a patient with myelogenous leukemia treated intravenously with NDV. In 1965, Cassel and Garrett [8] reported the effects of intratumoral NDV treatment on a patient with cervical cancer. Marked tumor shrinkage of the injected mass as well as the superclavicular lymph node metastasis was observed and the patient tolerated the treatment well. In 1971, Csatary [9] noted that a chicken farmer, shortly after a known exposure to NDV from his flock of infected chickens, had a spontaneous remission of his metastatic gastric cancer. In this same report, he noted

tumor regressions in 3 other patients, all of whom were intentionally inoculated with NDV. Since then, cytolytic strains of NDV virus have been shown to have a preference for replicating in and killing human cancer cells compared to normal cells [10–12]. NDV has additional properties which are potentially useful as an adjuvant for tumor vaccines [13] including the stimulation of an antitumor T-cell response by the presence of surface viral glycoproteins on NDV-infected tumor cells [14]. Furthermore, the virus is a strong inducer of cytokines such as interferon [15–17] and tumor necrosis factor-α (TNF-α); [18, 19]. Currently, the use of NDV for cancer therapy has taken on renewed scientific interest [20] and ProVirus, Inc., has initiated a phase-I intravenous trial in advanced cancer patients.

Features of Cytolytic NDV Exploited for Cancer Therapy

NDV is a fast growing RNA virus with signs of progeny virions first detectable within 3 h after infection [21]. Cytolytic strains of NDV rapidly and selectively replicate in human tumor cells with selective killing of malignant cells compared to normal cells [10]. After infecting a tumor cell, the virus can efficiently spread to neighboring tumor cells by means of syncytia formation [10]. Cytolytic strains of NDV have a high potency for killing tumor cells and one infectious virus leads to rapid death of at least 10,000 cancer cells in 2–3 days [10, 12]. Unlike many viral agents being developed for cancer, the general human population is seronegative to NDV [22, 23]. There is an extensive safety database for NDV primarily from low dose human tumor vaccine trials. NDV was well tolerated in humans in doses tested up to 3×10^9 infectious units by the intravenous route [7, 16] and tested up to 2.4×10^{12} infectious units by the intratumoral route [8]. Environmental safety of NDV is indicated by the absence of genetic recombination [24, 25], the lack of a carrier state [26], the genetic stability of naturally attenuated strains [3], the lack of antigenic drift [27], the absence of human-to-human transmission [28], and the extensive safety record of human tumor-passaged virus [29]. Cytolytic strains of NDV, therefore, have key features for development as replication-competent, oncolytic agents. The high potency and tumor selectivity are especially important for systemic administration to treat metastatic cancer. Also, from a practical standpoint, sufficient production for clinical use is achievable due to the growth of NDV to titers of $>10^9$ pfu/ml in embryonated chicken eggs [30].

Brief Overview of the Biology of NDV

Classification

NDV, also called avian paramyxovirus type 1 (avian PMV-1), is a member of the Paramyxoviridae, a family of enveloped, nonsegmented, negative-stranded

RNA viruses [1]. NDV falls within the Paramyxovirinae subfamily in the Rubulavirus genus (not to be confused with rubella virus) which also includes mumps virus and human parainfluenza virus types 2, 4a and 4b [31]. Recent complete nucleotide sequencing of the genome suggests that NDV is only distantly related to other members of the Rubulavirus genus and it is proposed as a member of a new genus [32].

Pathogenicity in the Natural Host

Strains with widely varying virulence have been identified since the first reports of Newcastle disease in 1926. There are three broad categories of NDV virulence [33]: velogenic, mesogenic and lentogenic. The highly pathogenic velogenic strains kill a large percentage of adult fowl and all young chickens [4]. Velogenic strains are further subdivided into (a) viscerotropic-velogenic strains which principally cause acute and lethal hemorrhagic lesions of the digestive tract, and (b) neurotropic-velogenic strains which principally cause an acute respiratory and nervous system infection in adult birds. Less virulent strains, isolated from birds that have mild respiratory symptoms or which are asymptomatic, have been used as naturally attenuated live vaccines and are classified as either mesogenic (of moderate virulence) or lentogenic (of low virulence).

Avian Infection and Spread

Over 200 species of birds have been shown to be infected with NDV [34]. Strains of low virulence appear to be enzootic in many parts of the world. Spread of the virus occurs by three major routes [34]: (1) movement of live domestic poultry or poultry products; (2) movement of pet birds (e.g., parrots), and (3) migration of birds within or between continents.

Control of the Disease in Chickens by Vaccination

Vaccination against NDV as a means of controlling the disease has been used by the poultry industry for more than 40 years and has employed either live attenuated or inactivated vaccines [3, 35]. Vaccination using one strain of NDV can confer protection against all other strains. Live vaccines have been used throughout the world (USA, Europe, Australia, Africa, Asia, the Middle East) and have included both mesogenic and lentogenic virus strains, each having a well-documented track record in terms of phenotypic stability [3, 35]. The minimum live NDV vaccine dose recommended by the USDA is 3×10^5 infectious units. The standard in the UK is 10^6 infectious units with 3×10^6 infectious units suggested as the preferred dose [36].

Physical Characteristics of NDV

NDV virions, like other virions in the Paramyxovirinae subfamily of Paramyxoviridae, are large, pleomorphic, membrane-enveloped virus particles

exhibiting spherical-to-rod shapes ranging in size from 150 to 400 nm [37]. Electron microscopy reveals an envelope covered with spikes of glycoproteins (HN and F) that are 8- to 12-nm-long. Contained inside the membrane is a long, coiled nucleocapsid of 18 nm diameter with 5.5 nm pitch with left-handed helical symmetry.

Genetic Characteristics of NDV

The genome of NDV is a single strand of negative-sense RNA that is complementary to mRNA which in turn codes for the viral proteins. The NDV genome is 15,186 nucleotides in length and the complete sequence of several strains has been recently reported [32, 38, 39]. The NDV genome contains 6 genes encoding for the following six gene products listed in order from the 3′ end: nucleocapsid protein (NP, 55 kD); phosphoprotein (P, 53 kD); matrix (M, 40 kD); fusion (F, 67 kD); hemagglutinin-neuraminidase (HN, 74 kD); and large protein (L, 200 kD). By means of an overlapping reading frame, the P gene encodes for an additional gene product, the V protein. In addition, the F glycoprotein is synthesized as an inactive precursor (F_0, 67 kD), which undergoes proteolytic cleavage to yield the biologically active protein consisting of the disulfide-linked chains F_1 (55 kD) and F_2 (12.5 kD).

Function of the Viral Proteins

Hemagglutinin-Neuraminidase

The HN glycoprotein (see table 1 for a list of NDV proteins), as the name implies, has two activities which are found both in virions and in the plasma membrane of NDV-infected cells [37]. The attachment or hemagglutinating activity mediates virion binding to sialic (neuraminic) acid-containing host cell receptors including those found on chicken erythrocytes. The neuraminidase activity presumably has a role in allowing budding virions to be released from the host cell by destroying local receptors and also a role in preventing virion clumping by destroying any sialic acid residues on viral glycoproteins [37]. In some lentogenic NDV strains (Ulster and Queensland V4), the HN glycoprotein is synthesized as an inactive precursor HN_0 (of 82 kD) which requires proteolytic removal of an 8 kD peptide from the exposed C terminus for activity [40–42].

Fusion Protein

In order for infectious progeny to be produced and multiple rounds of infection to occur, the F (fusion) protein is required to be proteolytically activated from a precursor F_0 (68 kD). Cleavage of F_0 forms the larger F_1 (55 kD) and the smaller F_2 (12 kD) fragments which are held together by disulfide bonds in a protein denoted as $F_{1,2}$ [37]. Cleaved F protein is required for the viral

Table 1. Main NDV proteins in order of size (largest to smallest) and their function

Protein	Abbreviation	Size kD	Function
Large	L	~200	RNA directed RNA polymerase
Hemagglutinin-neuraminidase	HN	74	Receptor binding
Fusion F_0 (uncleaved)	F_0	67	Precursor to cleaved F
Nucleocapsid	NP	55	Major structural component of nucleocapsid
Fusion F_1	F_1	55	Mediates fusion of virus and host cell membrane
Phosphoprotein (or nucleocapsid-associated protein)	P (also denoted as NAP)	53	Associates with the nucleocapsid; polymerase assessory function
Matrix	M	40	Organizes virus assembly
Fusion F_2	F_2	12	Smaller fragment of cleaved F protein which remains attached to F_1 via disulfide bonds

membrane to fuse with the host cell membrane and, therefore, for the infection to proceed. This activation of F is dependent upon both the virus strain and the host cell. Most avian and mammalian cells are capable of cleaving the F_0 of velogenic and mesogenic NDV strains through the presence of furin or furin-like proteases [43]. Exceptions include certain lymphoma cell lines that are deficient in this proteolytic [43]. Cleavage of the F_0 of lentogenic NDV strains is much more restricted, occurring only in embryonated avian eggs or in culture of avian chorioallantoic membrane cells [40, 41]. This highly restricted activation of the lentogenic F protein is believed to be at least part of the reason why lentogenic strains are very host cell restricted in their production of infectious progeny and less virulent compared to velogenic and mesogenic strains.

Matrix Protein

The M (matrix) protein is a highly basic, largely hydrophobic protein. This protein confers specificity relative to virus assembly with a high degree of exclusion of host cell proteins. The M protein binds selectively to viral membrane glycoproteins and to the nucleocapsid prior to budding [44]. The M protein is also thought to control the rate of RNA synthesis as indicated by in vitro experiments for other paramyxoviruses [44]. The majority of the M protein in NDV-infected cells is located within the nucleus [45], which is a surprising finding since RNA replication occurs in the cytoplasm.

Nucleocapsid Protein, Phosphoprotein, and Large Protein

Three proteins (NP, P and L) are associated with the nucleocapsid and all form a protein complex with RNA-dependent RNA transcriptase activity. NP is the most abundant viral protein in infected cells and in the virion [37]. As the main structural component of the nucleocapsid, the NP protein complexes with viral RNA rendering it RNase-resistant.

V Protein

The V protein, which is encoded for by the P gene, is found in virions of other rubulaviruses [31], however its function remains unknown. For Sendai virus, another member of the Paramyxovirinae subfamily, V protein expression is completely dispensable for in vitro replication, although it does contribute to in vivo pathogenesis [46].

Antitumor Activity of NDV

Overview

It is important to distinguish three different conceptual uses of NDV in cancer treatment (table 2) [10, 13, 47]: (1) certain NDV strains can be directly oncolytic to human and murine cancer cells without the need for immune effector cells or molecules; (2) NDV can serve as an immune adjuvant in cancer vaccines through active specific immunotherapy, and (3) NDV can cause nonspecific immune stimulation through the induction of cytokines (e.g., interferon, TNF-α, IL-6 and IL-1), and chemokines (e.g., RANTES, IP-10).

Oncolytic Activity of NDV

Tumor Cell Binding Followed by Rapid Virus Replication

NDV is a fast growing RNA virus. Binding of virions to tumor cells is rapid, occurring within minutes [48] and progeny virus is detectable as early as 3 h after infection [21]. Experiments using various neuraminidases to treat host cells have shown that sialic acid is a key component of the cell surface receptor [49]. The receptor must have a wide cellular distribution because of the diverse cell types that NDV can infect, including human tumor cells of neuroectodermal, mesenchymal, and epithelial origins [10]. When a high multiplicity of NDV infection is used, diverse human tumor cells but not normal fibroblasts exhibit rapid cell-to-cell fusion (in less than 1 h), indicating that differences in host cell membranes are recognized by NDV [50].

Cytolytic NDV strains selectively replicate in, and rapidly kill diverse human tumor cells [10, 12]. At the same multiplicity of infection, no effect is seen on normal cells. Plaques (macroscopic areas of cytolysis) in tumor cell

Table 2. Three uses of NDV in cancer treatment

Examples of direct oncolytic activity of NDV
Tumor-selective cytolysis
Syncytia formation in infected tumor cells
Durable complete responses induced in human tumor xenografts
Requirement for live virus for antitumor effects
Virus localization and replication in tumors
Examples of NDV as an immune adjuvant
Clinical trials of therapeutic tumor vaccines with NDV as a component
Stimulation of CTL and DTH responses after tumor vaccination
Example of NDV as a cytokine inducer
Interferon induction

monolayers are seen as early as 18 h after NDV infection of tumor cells [10]. Increased membrane permeability is seen when new virions are being released by budding at the cell surface and is suggested to play an important role in host cell death [51]. Dying tumor cells also display nuclear fragmentation after NDV infection [52]. Regarding apoptotic cell death, chicken embryo cells have been shown to undergo apoptosis after NDV infection [53, 54].

Cell-to-Cell Spread

After infecting a human tumor cell in vitro or in vivo, NDV can efficiently spread to neighboring cells by means of syncytia formation (cell-to-cell fusion); [10, 50]. Eight hours after NDV infection of human tumor cells in athymic mice, numerous multinucleated malignant cells can be seen in histological sections of tumor cells along with signs of tumor necrosis [10].

High Potency of NDVs Oncolytic Activity

NDV has a high potency for killing tumor cells; one infectious virus is able to kill tens of thousands of cancer cells in a monolayer within 2–3 days [10, 12].

Use of NDV as an Oncolytic Agent

In 1965, Cassel and Garrett [8] were the first to observe that certain strains of NDV can have an oncolytic effect on human tumors. They used NDV strain 73-T which had been generated by extensive passage of NDV in murine Ehrlich ascites tumor cells with 73 in vitro passages and 13 in vivo passages. Extensive tumor necrosis was observed following inoculation of 10^7 infectious units of

NDV strain 73-T into human adenocarcinoma xenografts grown in hamster cheek pouches. In addition, a patient with a cervical cancer was treated intratumorally with 2.4×10^{12} infectious units of this same NDV strain. Pronounced tumor sloughing and cessation of bleeding from the primary tumor and shrinkage of the superclavicular lymph node was noted and this patient tolerated the virus treatment well. Virus replication apparently occurred since the patient's urine sample became positive at 8 days after inoculation and continued to be positive for the next 3 days.

Schirrmacher et al. [47] tested the effects of a velogenic strain Italien on human Me-Wo-Met melanoma xenografts in athymic mice. Intratumoral injections of virus-infected allantoic fluid caused approximately 100% growth inhibition lasting 3 months, whereas continued tumor growth was seen in the animals treated with control allantoic fluid. Purified and concentrated virus was not tested. The noncytolytic (lentogenic) strain Ulster was reported as having no effect in this same tumor model.

Lorence et al. [11, 12] tested the effects of purified and concentrated NDV 73-T in human tumor xenograft models. After intratumoral injection of 10^7 pfu, complete regression was seen in 8 of 10 fibrosarcoma xenografts. Also noted was marked regression of $>80\%$ seen in 6 of 9 uncultured, primary and secondary sarcoma explants from 1 patient [12]. Transformation of human fibroblasts with either H-ras or N-ras oncogenes was associated with a 1,000-fold increase in sensitivity to NDV cytolysis [12].

Durable complete tumor regressions of subcutaneous IMR-32 human neuroblastoma xenografts were seen in 17 of 18 mice after a single intratumoral NDV injection [11]. The one tumor that showed partial regression showed complete regression after a second NDV treatment. Tumor responses were associated with selective replication of virus in tumor tissue. Virus levels increased more than 80-fold in virus-injected tumors while no infectious virus was recovered from normal muscle tissue after intramuscular injection [11].

Systemic injection of NDV was also shown to have a marked antitumor effect [55]. Lorence and Reichard [55] tested the effects of intraperitoneal injection of NDV strain 73-T on subcutaneous IMR-32 human neuroblastoma xenografts. This systemic NDV treatment caused complete tumor regression in 6 of 7 mice, with a partial tumor regression in the remaining mouse. Control vehicle had no effect.

Live Virus Is Required for the Oncolytic Effects of NDV

Several studies have indicated that the oncolytic activity of cytolytic NDV, including its ability to cause tumor regression, requires live virus. Lorence et al. [11] tested the effects of live versus UV-killed NDV strain 73-T in treating

subcutaneous human neuroblastoma xenografts by the intratumoral route in athymic mice. Live virus (10^7 pfu) caused complete regression in 6 of 7 mice. The remaining mouse had partial regression that became complete following a second dose on day 10. In marked contrast, rapid tumor growth occurred in all 9 mice treated with an equivalent amount of UV-inactivated virus and in all 7 mice treated with vehicle control.

Cassel and Garrett [8] noted a similar difference between live NDV and heat-inactivated virus. Six days after intraperitoneal inoculation of Ehrlich ascites tumor cells, mice were treated with live NDV (10^6 infectious units) or heat-inactivated virus. No ascites developed over a 65-day observation period in the animals treated with live NDV, while animals treated with inactivated virus were all dead by day 15.

The effectiveness of live NDV in contrast to the ineffectiveness of inactivated virus was also demonstrated by in vitro experiments. Reichard et al. [10] observed that live NDV caused plaques indicative of tumor cytolysis in a wide variety of human cancer cells, including bladder carcinoma, Wilm's tumor, fibrosarcoma, osteosarcoma, neuroblastoma and carcinoma lines, but not in 9 normal human fibroblast isolates. Heat-inactivated virus had no activity in any of these tumor cells. Additional studies by Reichard et al. [10] demonstrated a similar effect when administering NDV to the tumor cell inoculation site immediately after subcutaneous tumor cell injection in athymic mice. While tumors formed in 24 of 26 mice treated with heat-inactivated virus, no tumors grew in any of the 20 mice treated with live virus.

Use of NDV to Stimulate Antitumor Immunity

Studies Using Cytolytic NDV Strains

A recent, extensive review on the use of NDV as a biologic adjuvant to stimulate antitumor immunity of tumor vaccines is given by Schirrmacher et al. [13]. The concept of active specific immunotherapy is to have a vaccine component stimulate the immunogenicity of tumor-associated antigens. Cassel and Garrett [8, 56] were the first to observe this phenomenon. Mice cured of their ascites tumors by an oncolytic strain of NDV (73-T) were able to resist rechallenge with 2×10^7 cells from the same tumor line when given at either the same site (intraperitoneal) or a different site (subcutaneous) [8, 56].

Cassel et al. [29, 57] followed this observation with two clinical studies which enrolled 83 stage-III melanoma patients between 1975 and 1982. After therapeutic lymphadenectomy for palpable tumor in their lymph nodes, patients were treated at regular intervals with an oncolysate containing live NDV strain 73-T and consisting of autologous (or in some cases, allogeneic) melanoma cells lysed ex vivo by live NDV. Initial results were encouraging with only 12%

of the patients progressing to disseminated disease within 3 years compared to 95% of historical controls [57, 58]. Longer follow-up studies indicate that the patients treated with the NDV-oncolysate had a 63% 10-year survival rate [29] and 55% 15-year survival rate [59] compared to 6–15% 10-year survival in historical controls having had palpable lymph node dissemination [59, 60]. In a 1998 report [59], 34 of the original 83 patients continue to receive NDV oncolysates at 3- to 6-month intervals.

Studies Using Noncytolytic NDV Strains

Schirrmacher et al. [13] have preclinically and clinically tested the use of NDV as an immune adjuvant using Ulster, a lentogenic (noncytolytic) NDV strain [13]. While the Ulster strain of NDV cannot replicate in normal cells (except avian chorioallantoic cells), de novo expression of NDV antigens at high density was observed on the surface of all human cancer cell types tested including 33 established tumor cell lines, 40 primary cultures and more than 400 noncultured freshly isolated patient-derived tumor cells [13]. However, unlike cytolytic strains, new virions produced by the Ulster strain in tumor cells were noninfectious. Virus amplification in tumor cells was not dependent on cell proliferation as it occurred in gamma-irradiated tumor cells [13].

Preclinical testing examined the effects of NDV infection on antitumor immunity [13, 61]. NDV infection or HN transfection augmented the tumor-specific or antigen-specific cytotoxic T lymphocyte response [13, 61]. NDV infection of tumor cells increased lymphocyte binding and provided a T-cell costimulatory function [14, 48, 61]. Antibody inhibition and transfection experiments indicate that these phenomena were mediated by the NDV HN surface glycoprotein [14, 48, 61].

NDV infection can also overcome tumor anergy [14]. A patient-derived T4-helper lymphocyte clone was obtained that could not be stimulated by autologous melanoma cells and became anergic to subsequent stimulation even in the presence of costimulatory signals, such as anti-CD28 antibodies [14]. Upon NDV infection, autologous tumor cells were now able to stimulate IL-2 production and proliferation of T4-helper cells.

Schirrmacher et al. [47] also showed that infection of human melanoma cells with either the noncytolytic lentogenic strain Ulster or the cytolytic velogenic strain Italien had the ability to induce a bystander effect [47]. After injecting a mixture of uninfected and infected tumor cells at a ratio of 5 : 1 into athymic mice, the cells infected with strain Ulster were able to markedly suppress the growth of the uninfected cells. In contrast, using the same test system, the cytolytic strain Italien completely prevented tumor growth of the uninfected cells.

Several phase-II clinical trials (renal carcinoma, breast carcinoma, colon, carcinoma, ovarian carcinoma, melanoma and glioblastoma) have been completed

by Schirrmacher et al. [13] using NDV strain Ulster as a tumor vaccine component. Irradiated autologous tumor cells that are then infected with live NDV were used to prepare the vaccines. Early results of the ovarian cancer, melanoma, glioblastoma and breast cancer trials showed promise [13]. A follow-up phase-III European trial in breast cancer patients of medium risk for disease recurrence has recently been initiated [13, 20].

In phase-II colon carcinoma studies, Nelson [20] found that 86% of patients with Duke's stage C survived 5 years after treatment with NDV-infected tumor cell vaccines compared to 42% for historical controls. Delayed-type hypersensitivity (DTH) response was examined in these colon cancer patients. Forty percent experienced an increased DTH reactivity against autologous tumor cells following NDV tumor vaccination while only 17% showed reactivity to NDV antigens, autoantigens from the patients' normal liver or test antigens [62]. In another study, NDV-infected tumor cell vaccination was compared to tumor cell vaccine mixed with bacillus Calmette-Guérin (BCG) organisms in the immunotherapy of patients with resected colorectal carcinoma. The 2-year survival for patients treated with NDV tumor vaccines was 98 versus 67% for the patients treated with the BCG tumor vaccine and 74% for historical controls [63].

Use of NDV as a Nonspecific Immune Stimulant (e.g., for Cytokine Production)

Interferon Induction

NDV is a well-known interferon inducer in mammals including humans [15–17]. In a study using RC19 tumor cells grown intraperitoneally in mice, Gresser and Bourali [64] showed that NDV, when given 24 h after tumor cells by the same route, was able to increase survival. Since exogenous interferon by itself was able to increase survival to an even greater degree, it was postulated by the authors that interferon played at least a role in NDV's antitumor effects in this tumor model. A similar conclusion was reached by Bart et al. [65] using a subcutaneous B16 murine melanoma model and intraperitoneal treatment with NDV. Compared to nonirradiated mice, irradiated mice had a greater degree of tumor growth inhibition and also had a higher serum interferon level after NDV treatment.

Merigan et al. [16] performed a dose escalation of a single intravenous injection of NDV with the intent to induce interferon. Determinations of the dose-limiting toxicity and the maximal tolerated dose were not objectives in this 17 patient study. Patients received single intravenous injections of NDV strain 73-T ranging from 2.4×10^6 to 1.6×10^8 pfu. NDV quantities between 1.2×10^7 and 1.6×10^8 pfu induced interferon in direct proportion to the dose.

In patients receiving the highest doses, the following clinical signs and symptoms consistent with interferon release were observed: a temperature spike ranging from 1 to 3 °C above baseline, a transient drop in leukocyte count, and a mild, flu-like syndrome. Viremia was occasionally detected 12–18 h after virus inoculation. Overall, the treatment was well tolerated.

Induction of Tumor Necrosis Factor

Strains of NDV can stimulate human peripheral blood mononuclear cells and rat splenocytes to produce TNF-α [18, 19]. TNF-α, also called cachectin, has multiple activities including those which are antineoplastic (e.g., the ability to cause hemorrhagic necrosis of tumors and to augment the cytotoxicity of natural killer cells and macrophages) and those which are toxic (e.g., causing cachexia and mediating endotoxic shock). In in vitro experiments, NDV infection of human tumor cells can markedly increase their sensitivity to lysis by TNF-α [18].

Induction of Other Factors

NDV is known to induce a variety of other factors with a wide range of biological activity. Besides interferon and TNF-α, NDV stimulates synthesis of other proinflammatory cytokines such as IL-1 and IL-6 [66]. This proinflammatory cytokine response to NDV is thought to be dampened in mammals by NDV stimulation of endogenous glucocorticoids [67]. IL-1 released in response to NDV can stimulate ACTH release from the hypothalamus leading to adrenal glucocorticoid production and the stress response [68].

Chemokines induced by NDV such as RANTES and IP-10 [69] can lead to the recruitment of T lymphocytes and monocytes to the site of NDV infection [13]. NDV also induces nitric oxide synthase and which is known to be associated with increased macrophage antitumoral activity [70]. Tissue inhibitor of metalloproteinases, which can inhibit tumor invasion, is induced by NDV [71].

Treatment of Cancer Patients in Hungary

Csatary et al. [72] reported seven responses in 33 patients treated with twice-weekly inhalation of NDV strain MTH-68/N. Side effects in this study were limited to fever. Additional studies by the same group reported favorable effects in 4 patients and tumor shrinkage in a patient with glioblastoma [73, 74]. Over 4,000 cancer patients have been treated in Hungary primarily by inhalation [75]. However, many of these patients received other therapies rendering the interpretation of the data relative to efficacy and safety unclear [20, 75].

Molecular Engineering

The rescuing of infectious NDV from cloned cDNA was recently reported [76]. In general, although recently accomplished, the genetic manipulation of negative-stranded RNA viruses has lagged behind that of DNA viruses and positive-strand RNA viruses. This is, in part, because the naked viral RNA by itself is not infectious after transfection [77]. Regarding the insertion of foreign genes, the enveloped, negative-stranded RNA viruses do not have packaging constraints like encapsulated DNA viruses. The nucleocapsid of paramyxoviruses can accommodate additional genes (e.g., those encoding for chloramphenicol acetyltransferase or green fluorescent protein) with recovery of fully infectious virus, although at a virus yield inversely proportional to the size of the insert [46, 78–80].

Safety

Overview

There is an extensive safety database for NDV, primarily from low-dose human tumor vaccine trials [13]. NDV is well tolerated in humans in doses of at least 3×10^9 infectious units by the intravenous route [7, 16], and at least 4×10^{12} infectious units by the intratumoral route [8]. Complementing these clinical findings, animal safety data provide evidence of the low pathogenicity of NDV in mammals.

Safety in Mammals

NDV has been extensively tested by a variety of routes in mammals with minimal signs of pathogenicity. Safety has been documented in rabbits by the intracerebral (i.c.) and intravenous (i.v.) routes, guinea pigs by the i.c. and intraperitoneal (i.p.) routes, 1-day-old mice by the subcutaneous (s.c.) and i.c. routes, adult mice by the i.c. route and i.p., rats by the i.c. route, and hamsters by the i.c. route [8]. Baron and Buckler [15] demonstrated the safety of large amounts of even velogenic NDV given by the i.v. route to mice. Upton et al. [81] extensively evaluated 25 different strains of NDV by i.c. injection into weanling mice. They observed that vaccine strains of NDV, including lentogenic strains such as B1 and mesogenic strains such as NJ-Roakin had mild effects. In contrast, virulent velogenic strains produced moderate to severe neurological effects.

Immunodeficient mice can tolerate NDV injections well by the intravenous, intraperitoneal and intratumoral routes. Schirrmacher et al. [47] reported that velogenic strains of NDV are well tolerated upon repeat inoculation by the intratumoral and intravenous routes in immunodeficient athymic

mice. Lorence et al. [11, 12, 55] confirmed the safety of high doses of NDV in athymic mice injected by the intraperitoneal and intratumoral routes.

Testing of virulent velogenic strains in monkeys by intranasal, intradermal, and perineural peripheral routes had no effect [82]. However moderate to severe encephalitis developed after intracerebral injection. Since these strains are most virulent in chickens, it is not clear what effect vaccine strains of NDV would have in this experimental setting.

Accidental Exposure in Humans

There have been over 100 documented cases of conjunctivitis in people after accidental exposure. These cases include accidental eye inoculation of high-titered NDV into the eye by laboratory workers as well as chicken handlers in whom infected material was introduced into their eyes resulting in conjunctivitis 1–2 days later (see Kleiman [83] and Chang [28] for reviews). Human infections are mild, last 3–4 days, and result in inflammation of the ocular conjunctiva, most commonly unilateral, without affecting the cornea, visual axis or extraocular structures. There are rare reports of human systemic illness, especially an influenza-like illness following inhalation of virus-containing aerosol. These systemic symptoms include low-grade fever, chills and malaise. NDV infection in humans is always self-limiting without any lasting sequelae [28, 83]. No therapy is recommended [83, 84] and no quarantine or isolation of human patients is required [28]. Also there is no human-to-human transmission (see Environmental Safety).

Human Seropositivity

Two surveys indicate a low incidence of seropositivity among the general human population and a significantly higher percentage for the poultry-associated population. In a study in the United States by Miller and Yates [22], none of 100 people in the general population were seropositive for NDV antibodies by hemagglutination inhibition and only 7% were positive at low levels by plaque neutralization (all positive titers below 1 : 16). For 116 poultry workers, 29 and 17% were positive by these two methods, respectively. In India, Charan et al. [23] found 4% of 109 people in the general population were seropositive and 38% of 104 poultry workers were seropositive by hemagglutination inhibition.

Human Safety of Intentional NDV Injection

Intravenous Injection

Wheelock and Dingle [7] administered the Hickman strain, a velogenic NDV strain to a single patient with acute myelogenous leukemia using intravenous doses of 3.2×10^9, 1.3×10^{10}, 1.3×10^{10}, and 6.3×10^9 infectious units over 4 consecutive days. The patient was reported to tolerate the treatments

well with only a transient fever spike noted on the first day. An overall 74% reduction in leukemic blast count was observed. This patient had had prior i.v. treatment with other viruses and it is not clear how these previous treatments may have affected the safety profile.

Merigan et al. [16] administered NDV strain 73-T to 17 patients intravenously in single doses up to 1.6×10^8 pfu. Side effects included fever, ranging from 1 to 3°C above baseline, a transient drop in white blood count, and a mild, flu-like syndrome in patients receiving the higher doses. These side effects correlated to the induction of interferon. Viremia was occasionally detected 12–18 h after dosing.

Recently, Csatary and Bakacs [74] reported that NDV strain MTH-68/H was well tolerated in 3 patients with glioblastoma when given by the i.v. route and caused no neurotoxicity.

Intratumoral Injection

A dose of 2.4×10^{12} infectious units of strain 73-T was injected intratumorally in 1 patient and reported to be well tolerated [8]. In addition to pronounced tumor sloughing, virus replication apparently occurred since the patient's urine sample became positive 8 days after inoculation and continued for 3 more days.

Intramuscular Injection into AIDS Patients

Csatary and Massey [85] provided evidence that vaccine strains of NDV are well tolerated in severely immunocompromised AIDS patients. These investigators reported that 5 AIDS patients were given weekly intramuscular NDV injections. Although 4 of these patients had T4 counts below 75/ml, they all tolerated the treatment well with no report of detrimental effects.

Dosing by Inhalation

Csatary et al. [72] reported the results of a phase-II/B study in Hungary in which a total of 33 patients with diverse types of advanced cancer received NDV strain MTH-68/N by inhalation twice weekly for 6 months. The authors reported that the treatment was well tolerated and that fever, which was observed, did not cause any patient to withdraw from the study. In a later paper, they indicated that over 300 patients have tolerated treatment with NDV well [73], and a recent publication indicates that over 4,000 people have been treated in Hungary [75].

Intradermal Injection as a Tumor Vaccine Component

Recently, Schirrmacher et al. [13] have been testing live NDV as a component of tumor vaccines given intradermally to over 1,400 patients and reported

minimal side effects. The tolerability of these NDV tumor vaccines is best seen in one study, a direct comparison between NDV-infected tumor cell vaccination and BCG tumor vaccination [63]. The patients treated with the NDV-infected tumor cells experienced mild side effects consisting of erythema, swelling and induration at the injection site. A slight fever was noted in 17% of the patients and generalized lymph node swelling for 48 h observed in 15% of the patients. In contrast to these mild side effects, the BCG vaccine led to more serious side effects including long-lasting ulcers at the injection site in all patients, abscesses in 20% of the patients requiring surgical excision, and significant fatigue in 60% of the patients.

Human Safety Profile of Tumor-Passaged Virus by the Subcutaneous Route

NDV strain 73-T has been passaged in primary human tumor cells to generate oncolysates and administered in live form as a tumor vaccine component to melanoma patients as follows: (1) autologous melanoma cells from each patient were isolated, expanded in vitro and then infected with NDV; (2) the virus was allowed to amplify at least 3 orders of magnitude in these human tumor cells, and (3) this human tumor-passaged virus in the form of infected tumor cells was then safely and repeatedly inoculated by an s.c. route on a weekly schedule into 163 patients [29]. Over 11,000 total doses of human tumor-passaged NDV were safely administered to 163 patients at least four times per year for up to 18 years without any adverse reactions [29].

Although the above human safety profile of tumor-passaged virus occurred in tumor vaccine studies, these safety studies are relevant to the use of NDV as an oncolytic agent since oncolysis requires the in vivo amplification of the virus in the tumor.

Environmental Safety

Overview

NDV is enzootic in many parts of the world and can be easily isolated from chickens at poultry markets in the United States. The environmental safety of NDV is indicated by the absence of genetic recombination, the lack of a carrier state, the genetic stability of naturally attenuated strains, the lack of antigenic drift, and the absence of human-to-human transmission. The extensive safety record of human tumor-passaged virus (as outlined above) is also an important environmental issue indicating the stable properties of the virus with passage in tumor cells.

Absence of Recombination, Gene Reassortment, and Nucleic Acid Integration

In numerous studies, deliberate attempts to cause recombination between strains of NDV and between paramyxoviruses have been negative [24, 25, 86]. In addition, there is no evidence for viral recombination in nature. Toyoda et al. [87] analyzed the sequences of the HN and F genes of multiple strains of NDV isolated over a period of 50 years. They concluded that no gene exchange by recombination had occurred in the generation of three lineages which were stable even after having cocirculated in nature for a considerable time.

Unlike Orthomyxoviridae and Reoviridae, there is no risk for genetic reassortment with Paramyxoviridae like NDV, since they have a nonsegmented RNA genome. There is no evidence for integration of viral nucleic acid into the host genome, presumably because NDV is a RNA virus for which nucleic acid replication occurs in the cytoplasm without the expression of a reverse transcriptase and without a DNA intermediate [13].

Genetic Stability of NDV and Lack of Antigenic Drift

Live poultry vaccine strains of NDV, including both mesogenic and lentogenic strains, have been in widespread use for over 40 years and have been shown to be stable in terms of virulence parameters [3, 35]. Multiple strains of NDV have coexisted in nature for a considerable time with individual strains being genetically stable and distinct [87, 88].

In contrast to influenza, NDV strains do not display antigenic drift. Compared to influenza, there is much less variation in the surface glycoproteins proteins (HN and F) for a paramyxovirus species in general, as shown, for example, by the lifelong immunity against all strains of mumps virus conferred by infection with mumps virus [86]. Sakaguchi et al. [27] also concluded that there was no antigenic drift in NDV by analyzing the sequence of the HN gene for 13 NDV strains. Although there was minor variation in the 13 HN gene sequences, it did not appear that these changes were cumulative or directional. These findings are in complete agreement with the vaccination results from over 60 years of field testing throughout the world which indicated that there is no significant antigenic variation between NDV strains and that vaccines from one strain protect against all other strains [1, 4, 35].

Lack of a Carrier State

In a study by Clancy et al. [26] chickens infected with a mesogenic vaccine strain of NDV cleared the infection. The absence of a carrier state was indicated in this study by the lack of seroconversion of naive chickens housed with poultry inoculated with NDV 6 weeks earlier.

No Known Human-to-Human Transmission

Accidental human NDV infection causing conjunctivitis is reported in a review by Chang [28] as not being transmitted from one person to another. An epidemiological study by Nelson et al. [89] indicated the lack of human-to-human transmission. In this study, workers on an eviscerating line in a poultry processing plant in a small town in Minnesota were intensively exposed to a chicken virulent (velogenic) strain of NDV in infected poultry carcasses. Forty cases of NDV conjunctivitis were noted in 90 workers on this eviscerating line. Only mild cases occurred without any corneal involvement and this effect was reversible with no constitutional symptoms and no lasting sequelae. There were no secondary cases reported among their 210 coworkers at the plant nor among their family contacts or 1,500 community members. This lack of transmission occurred despite inadequate hygienic measures including minimal use of limited hand-washing facilities in the plant.

In a study of workers in their laboratory at Johns Hopkins in the 1950s, Bang and Foard [90] also observed the lack of human-to-human transmission of NDV. With repeated exposure to high titer virus without the use of biosafety cabinets, 60% of the individuals seroconverted. All members of their laboratory not directly exposed to NDV were seronegative.

Previous testing of live NDV in over 1,400 patients, mainly in vaccine trials, have also indicated the absence of horizontal transmission in humans. Most noteworthy is the study by Cassel and Murray [29, personal commun.] in which 11,000 doses of between 10^5 and 10^7 infectious units of live human tumor-passaged NDV as a component of tumor vaccines were administered to 163 patients with no detectable spread of the virus over an 18-year follow-up period.

Presence of NDV in the Environment

King and Seal [91] demonstrated the ease of isolating NDV from chickens at poultry markets in the USA. NDV is enzootic in many parts of the world and can be isolated from many free-living birds [34].

Human Trials of Oncolytic Newcastle Disease Virus

PV701, a naturally attenuated strain of NDV, was selected for clinical development by Pro-Virus, Inc., because of its preclinical safety and efficacy profile. PV701 is currently in phase-I testing by Pro-Virus, Inc. Advanced cancer patients with a wide variety of tumor types have been enrolled in this study using the intravenous route with dose escalation. This route of administration was chosen based on preclinical efficacy and the potential advantage of systemic treatment for a systemic disease like metastatic cancer. Results of this phase-I trial are pending.

Conclusion

The use of NDV for cancer therapy has taken on renewed scientific interest [20]. NDV has several properties that help differentiate it from other viruses for cancer therapy: (1) high potency for oncolysis; (2) rapid virus replication in tumors; (3) tumor cell selectivity; (4) syncytia formation with efficient spread of infection from tumor cell to tumor cell; (5) high titer growth; (6) seronegativity of the general human population; (7) genetic stability; (8) ability to stimulate immune specific antitumor effects in humans; (9) extensive human safety database, and (10) environmental safety.

In conclusion, cytolytic strains of NDV have key features as replication-competent, oncolytic agents. Their high oncolytic potency and tumor selectivity are especially important for systemic administration which is being explored in a current phase-I intravenous trial of advanced cancer patients using PV701, a cytolytic NDV strain.

References

1 Alexander DJ: Newcastle disease virus – An avian paramyxovirus; in Alexander DJ (ed): Newcastle Disease. Boston, Kluwer Academic, 1988, pp 11–22.
2 Spradrow PB: Geographical distribution; in Alexander DJ (ed): Newcastle Disease. Boston, Kluwer Academic, 1988, pp 247–255.
3 Hitchner SB: Control of Newcastle disease in the United States by vaccination; in Hanson RP (ed): Newcastle Disease Virus. Madison, University of Wisconsin Press, 1964, pp 85–98.
4 Alexander DJ: Historical aspects; in Alexander DJ (ed): Newcastle Disease. Boston, Kluwer Academic, 1988, pp 1–10.
5 Doyle TM: A hitherto unrecorded disease of fowls due to a filter-passing virus. J Comp Pathol Ther 1927;40:144–169.
6 Kenney S, Pagano JS: Viruses as oncolytic agents: a new age for 'therapeutic' viruses? J Natl Cancer Inst 1994;86:1185–1186.
7 Wheelock EF, Dingle JH: Observations on the repeated administration of viruses to a patient with acute leukemia. N Engl J Med 1964;271:645–651.
8 Cassel WA, Garrett RE: Newcastle disease virus as an antineoplastic agent. Cancer 1965;18:863–868.
9 Csatary LK: Viruses in the treatment of cancer. Lancet 1971;ii:825.
10 Reichard KW, Lorence RM, Cascino CJ, Peeples ME, Walter RJ, Fernando MB, Reyes HM, Greager JA: Newcastle disease virus selectively kills human tumor cells. J Surg Res 1992;52:448–453.
11 Lorence RM, Reichard KW, Katubig BB, Reyes HM, Phuangsab A, Mitchell BR, Cascino CJ, Walter RJ, Peeples ME: Complete regression of human neuroblastoma xenografts in athymic mice after local Newcastle disease virus therapy. J Natl Cancer Inst 1994;86:1228–1233.
12 Lorence RM, Katubig BB, Reichard KW, Reyes HM, Phuangsab A, Sassetti MD, Walter RJ, Peeples ME: Complete regression of human fibrosarcoma xenografts after local Newcastle disease virus therapy. Cancer Res 1994;54:6017–6021.
13 Schirrmacher V, Ahlert T, Probstle T, Steiner HH, Herold-Mende C, Gerhards R, Hagmuller E: Immunization with virus-modified tumor cells. Semin Oncol 1998;25:677–696.
14 Haas C, Ertel C, Gerhards R, Schirrmacher V: Introduction of adhesive and costimulatory immune functions into tumor cells by infection with Newcastle disease virus. Int J Oncol 1998;13:1105–1115.

15 Baron S, Buckler CE: Circulating interferon in mice after intravenous injection of virus. Science 1963;141:1061–1063.
16 Merigan TC, De Clercq E, Finkelstein MS, Clever L, Walker S, Waddell DJ: Clinical studies employing interferon inducers in man and animals. Ann NY Acad Sci 1970;173:746–759.
17 Brehm G, Kirchner H: Analysis of the interferons induced in mice in vivo and in macrophages in vitro by Newcastle disease virus and by polyinosinic-polycytidylic acid. J Interferon Res 1986; 6:21–28.
18 Lorence RM, Rood PA, Kelley KW: Newcastle disease virus as an antineoplastic agent: Induction of tumor necrosis factor-alpha and augmentation of its cytotoxicity. J Natl Cancer Inst 1988;80: 1305–1312.
19 Zorn U, Dallmann I, Grosse J, Kirchner H, Poliwoda H, Atzpodien J: Induction of cytokines and cytotoxicity against tumor cells by Newcastle disease virus. Cancer Biother 1994;9: 225–235.
20 Nelson NJ: Scientific interest in Newcastle disease virus is reviving. J Natl Cancer Inst 1999;91:1708–1710.
21 Huppert J, Gresland L, Rosenbergova M: Newcastle disease virus: Virus particle and RNA synthesis in different host cells and at different temperatures. J Gen Virol 1974;22:129–142.
22 Miller LT, Yates VJ: Reactions of human sera to avian adenoviruses and Newcastle disease virus. Avian Dis 1971;15:781–788.
23 Charan S, Mahajan VM, Agarwal LP: Newcastle disease virus antibodies in human sera. Indian J Med Res 1981;73:303–307.
24 Bratt MA, Hightower LE: Genetics and paragenetic phenomena of paramyxoviruses; in Fraenkel-Conrat H, Wagner RR (eds): Regulation and Genetics. Genetics of Animal Viruses. New York, Plenum Press, 1977, vol 9, pp 457–533.
25 Pringle CR: The genetics of paramyxoviruses; in Kingsbury DW (ed): The Paramyxoviruses. New York, Plenum Press, 1991, pp 1–39.
26 Clancy CF, Cox HR, Bottorff CA: Laboratory experiments with living Newcastle disease vaccine. Poultry Sci 1949;28:58–62.
27 Sakaguchi T, Toyoda T, Gotoh B, Inocencio NM, Kuma K, Miyata T, Nagai Y: Newcastle disease virus evolution. I. Multiple lineages defined by sequence variability of the hemagglutinin-neuraminidase gene. Virology 1989;169:260–272.
28 Chang PW: Newcastle disease; in Steele JH (ed): CRC Handbook Series in Zoonoses, Section B. Boca Raton, CRC Press, 1981, vol II, pp 261–274.
29 Cassel WA, Murray DR: A ten-year follow-up on stage II malignant melanoma patients treated postsurgically with Newcastle disease virus oncolysate. Med Oncol Tumor Pharmacother 1992; 9:169–171.
30 Baron SB: Relationship of interferon and temperature to virulence of Newcastle disease virus; in Hanson, RP (ed): Newcastle Disease Virus. Madison, University of Wisconsin Press, 1964, pp 205–220.
31 Lamb RA, Kolafsky D: *Paramyxoviradae*: The viruses and their replication; in Fields BN (ed): Fields Virology. Philadelphia, Lippincott-Raven, 1996, pp 1177–1204.
32 de Leeuw O, Peeters B: Complete nucleotide sequence of Newcastle disease virus: Evidence for the existence of a new genus within the subfamily *Paramyxovirinae*. J Gen Virol 1999;80: 131–136.
33 Hanson RP, Brandly CA: Identification of vaccine strains of Newcastle disease virus. Science 1955;122:156–157.
34 Kaleta EF: Newcastle disease in free-living and pet birds; in Alexander DJ (ed): Newcastle Disease. Boston, Kluwer Academic, 1988, pp 197–246.
35 Meulemans G: Control by vaccination; in Alexander DJ (ed): Newcastle Disease. Boston, Kluwer Academic, 1988, pp 318–332.
36 Thornton DH: Quality control of vaccines; in Alexander DJ (ed): Newcastle Disease. Boston, Kluwer Academic, 1988, pp 347–365.
37 Samson ACR: Virus structure; in Alexander DJ (ed): Newcastle Disease. Boston, Kluwer Academic, 1988, pp 23–44.

38 Phillips RJ, Samson AC, Emmerson PT: Nucleotide sequence of the 5′-terminus of Newcastle disease virus and assembly of the complete genomic sequence: Agreement with the 'rule of six'. Arch Virol 1998;143:1993–2002.
39 Krishnamurthy S, Samal SK: Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beudette C and completion of the entire genome sequence. J Gen Virol 1998;79:2419–2424.
40 Nagai Y, Klenk HD, Rott R: Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. Virology 1976;72:494–508.
41 Nagai Y, Klenk HD: Activation of precursors to both glycoproteins of Newcastle disease virus by proteolytic cleavage. Virology 1977;77:125–134.
42 Schuy W, Garten W, Linder D, Klenk HD: The carboxyterminus of the hemagglutinin-neuraminidase of Newcastle disease virus is exposed at the surface of the viral envelope. Virus Res 1984;1:415–426.
43 Sakaguchi T, Fujii Y, Kiyotani K, Yoshida T: Correlation of proteolytic cleavage of F protein precursors in paramyxoviruses with expression of the fur, PACE4 and PC6 genes in mammalian cells. J Gen Virol 1994;75:2821–2827.
44 Peeples ME: Newcastle disease virus replication; in Alexander DJ (ed): Newcastle Disease. Boston, Kluwer Academic, 1988, pp 45–78.
45 Coleman NA, Peeples ME: The matrix protein of Newcastle disease virus localizes to the nucleus via a bipartite nuclear localization signal. Virology 1993;195:596–607.
46 Nagai Y: Paramyxovirus replication and pathogenesis. Reverse genetics transforms understanding. Rev Med Virol 1999;9:83–99.
47 Schirrmacher V, Ahlert T, Heicappell R, Appelhans B, von Hoegen P: Successful application of non-oncogenic viruses for antimetastatic cancer immunotherapy. Cancer Rev 1986;5:19–49.
48 Schirrmacher V, Haas C, Bonifer R, Ertel C: Virus potentiation of tumor vaccine T-cell stimulatory capacity requires cell surface binding but not infection. Clin Cancer Res 1997;3:1135–1148.
49 Reichard KW, Lorence RM, Katubig BB, Peeples ME, Reyes HM: Retinoic acid enhances killing of neuroblastoma cells by Newcastle disease virus. J Pediatr Surg 1993;28:1221–1225; discussion 1225–1226.
50 Reichard KW, Lorence RM, Cascino CJ: Selective replication of Newcastle disease virus (NDV) in cancer cells is associated with virus-induced cell fusion. Proc Am Assoc Cancer Res 1992; 33:521.
51 Katzman J, Wilson DE: Newcastle disease virus-induced plasma membrane damage. J Gen Virol 1974;24:101–113.
52 Bankowski RA: Cytopathogenicity of Newcastle disease virus; in Hanson RP (ed): Newcastle Disease Virus. Madison, University of Wisconsin Press, 1964, pp 231–246.
53 Lam KM, Vasconcelos AC, Bickford AA: Apoptosis as a cause of death in chicken embryos inoculated with Newcastle disease virus. Microb Pathog 1995;19:169–174.
54 Lam KM: Apoptosis in chicken embryo fibroblasts caused by Newcastle disease virus. Vet Microbiol 1995;47:357–363.
55 Lorence RM, Reichard KW: Methods of treating and detecting cancer using viruses, Patent Cooperation Treaty (PCT), 1994, WO1994US0004732.
56 Cassel WA, Garrett RE: Tumor immunity after viral oncolysis. J Bacteriol 1966;92:792.
57 Cassel WA, Murray DR, Phillips HS: A phase II study on the postsurgical management of stage II malignant melanoma with a Newcastle disease virus oncolysate. Cancer 1983;52: 856–860.
58 Cassel WA, Murray DR: Treatment of stage II malignant melanoma patients with Newcastle disease virus oncolysate. Nat Immun Cell Growth Regul 1988;7:351–352.
59 Batliwalla FM, Bateman BA, Serrano D, Murray D, Macphail S, Maino VC, Ansel JC, Gregersen PK, Armstrong CA: A 15-year follow-up of AJCC stage III malignant melanoma patients treated postsurgically with Newcastle disease virus (NDV) oncolysate and determination of alterations in the CD8 T cell repertoire. Mol Med 1998;4:783–794.
60 Cassel WA, Olkowski ZL, Murray DR: Immunotherapy in malignant melanoma. J Clin Oncol 1999;17:1958F.

61 Ertel C, Millar NS, Emmerson PT, Schirrmacher V, von Hoegen P: Viral hemagglutinin augments peptide-specific cytotoxic T cell responses. Eur J Immunol 1993;23:2592–2596.
62 Schlag P, Manasterski M, Gerneth T, Hohenberger P, Dueck M, Herfarth C, Liebrich W, Schirrmacher V: Active specific immunotherapy with Newcastle disease virus-modified autologous tumor cells following resection of liver metastases in colorectal cancer. Cancer Immunol Immunother 1992;35:325–330.
63 Ockert D, Schirrmacher V, Beck N, Stoelben E, Ahlert T, Flechtenmacher J, Hagmuller E, Buchcik R, Nagel M, Saeger HD: Newcastle disease virus-infected intact autologous tumor cell vaccine for adjuvant active specific immunotherapy of resected colorectal carcinoma. Clin Cancer Res 1996;2:21–28.
64 Gresser I, Bourali C: Exogenous interferon and inducers of interferon in the treatment of Balb-c mice inoculated with RC19 tumour cells. Nature 1969;223:844–845.
65 Bart RS, Kopf AW, Vilcek JT, Lam S: Role of interferon in the anti-melanoma effects of poly (I).poly (C) and Newcastle disease virus. Nat New Biol 1973;245:229–230.
66 Guha-Thakurta N, Majde JA: Early induction of proinflammatory cytokine and type I interferon mRNAs following Newcastle disease virus, poly [rI:rC], or low-dose LPS challenge of the mouse. J Interferon Cytokine Res 1997;17:197–204.
67 Besedovsky HO, del Rey A: Mechanism of virus-induced stimulation of the hypothalamus-pituitary-adrenal axis. J Steroid Biochem 1989;34:235–239.
68 Dunn AJ, Vickers SL: Neurochemical and neuroendocrine responses to Newcastle disease virus administration in mice. Brain Res 1994;645:103–112.
69 Fisher SN, Vanguri P, Shin HS, Shin ML: Regulatory mechanisms of MuRantes and CRG-2 chemokine gene induction in central nervous system glial cells by virus. Brain Behav Immun 1995;9:331–344.
70 Umansky V, Shatrov VA, Lehmann V, Schirrmacher V: Induction of NO synthesis in macrophages by Newcastle disease virus is associated with activation of nuclear factor-kappa B. Int Immunol 1996;8:491–498.
71 Gewert DR, Coulombe B, Castelino M, Skup D, Williams BR: Characterization and expression of a murine gene homologous to human EPA/TIMP: A virus-induced gene in the mouse. EMBO J 1987;6:651–657.
72 Csatary LK, Eckhardt S, Bukosza I, Czegledi F, Fenyvesi C, Gergely P, Bodey B, Csatary CM: Attenuated veterinary virus vaccine for the treatment of cancer. Cancer Detect Prev 1993;17:619–627.
73 Csatary LK, Moss RW, Beuth J, Torocsik B, Szeberenyi J, Bakacs T: Beneficial treatment of patients with advanced cancer using a Newcastle disease virus vaccine (MTH-68/H). Anticancer Res 1999;19:635–638.
74 Csatary LK, Bakacs T: Use of Newcastle disease virus vaccine (MTH-68/H) in a patient with high-grade glioblastoma. JAMA 1999;281:1588–1589.
75 Position of the Scientific Medical Council on the antitumor studies conducted in Hungary on the Newcastle disease virus. Orv Hetil 1998;139:2903–2905.
76 Peeters BP, de Leeuw OS, Koch G, Gielkens AL: Rescue of Newcastle disease virus from cloned cDNA: Evidence that cleavability of the fusion protein is a major determinant for virulence. J Virol 1999;73:5001–5009.
77 Pekosz A, He B, Lamb RA: Reverse genetics of negative-strand RNA viruses: closing the circle. Proc Natl Acad Sci USA 1999;96:8804–8806.
78 Bukreyev A, Camargo E, Collins PL: Recovery of infectious respiratory syncytial virus expressing an additional, foreign gene. J Virol 1996;70:6634–6641.
79 He B, Paterson RG, Ward CD, Lamb RA: Recovery of infectious SV5 from cloned DNA and expression of a foreign gene. Virology 1997;237:249–260.
80 Sakai Y, Kiyotani K, Fukumura M, Asakawa M, Kato A, Shioda T, Yoshida T, Tanaka A, Hasegawa M, Nagai Y: Accommodation of foreign genes into the Sendai virus genome: Sizes of inserted genes and viral replication. FEBS Lett 1999;456:221–226.
81 Upton E, Hanson RP, Dow D, Brandly CA: Studies of intracerebral inoculation of Newcastle disease virus (NDV) into mice. I. Response of weanling mice to 25 strains of NDV. J Infect Dis 1953;92:175–182.

82 Wenner HA, Monley A, Todd RN: Studies of Newcastle disease virus encephalitis in rhesus monkeys. J Immunol 1950;64:305–321.
83 Kleiman MB: Newcastle disease virus; in Lennette EH (ed): Laboratory Diagnosis of Viral Infections. New York, Dekker, 1992, pp 567–571.
84 Pavan-Langston D: Viral disease of the cornea and external eye; in Albert DM, Jakobeic FA (eds): Principles and Practice of Ophthalmology. Philadelphia, Saunders, 1994, vol 1, pp 117–161.
85 Csatary LK, Massey RJ: Method of treating viral diseases with attenuated virus. United States Patent Office, 1993, 5,215,745.
86 Kingsbury DW: Paramyxoviridae and their replication; in Fields BN, Knipe DM, Chanock RM, Melnick JL, Hirsch MS, Monath TP, Roizman B (eds): Virology. New York, Raven Press, 1990, vol 1, pp 945–962.
87 Toyoda T, Sakaguchi T, Hirota H, Gotoh B, Kuma K, Miyata T, Nagai Y: Newcastle disease virus evolution. II. Lack of gene recombination in generating virulent and avirulent strains. Virology 1989;169:273–282.
88 Seal BS, King DJ, Bennett JD: Characterization of Newcastle disease virus vaccines by biological properties and sequence analysis of the hemagglutinin-neuraminidase protein gene. Vaccine 1996; 14:761–766.
89 Nelson CB, Pomeroy BS, Schrall K, Park WE, Lindeman RJ: An outbreak of conjunctivitis due to Newcastle disease virus (NDV) occurring in poultry workers. Am J Public Health 1952;42: 672–678.
90 Bang FB, Foard M: The serology of Newcastle disease virus infection. III. Prevalence of antibody against Newcastle in individuals closely exposed to the virus and in the absence of conjunctivitis. J Immunol 1956;76:352–356.
91 King DJ, Seal BS: Biological and molecular characterization of Newcastle disease virus isolates from surveillance of live bird markets in the northeastern United States. Avian Dis 1997;41: 683–689.

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