Edited by LOUIS M. WEISS AND KAMI KIM

Toxoplasma gondii

THE MODEL APICOMPLEXAN: PERSPECTIVES AND METHODS



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The Model Apicomplexan: Perspectives and Methods

Edited by Louis M. Weiss and Kami Kim



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Cover photograph: Tachyzoites of ME49 (Type II strain) *T. gondii* in the primary murine astrocytes *in vitro*. Electron microscopy performed at the Albert Einstein College of Medicine, Bronx, New York, USA in 1995. Photomicrograph courtesy of Dr Sandra Halonen, Montana State University

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This book is dedicated to Elmer Pfefferkorn PhD, Dartmouth College. Elmer's work paved the way for the explosion in molecular biology, cell biology, and genomic research associated with this organism. Elmer's intellectual rigor and deep thinking has had a significant influence on current researchers on Toxoplasma gondii, and we are all indebted to his generosity of spirit and profound insights into this pathogen. This page intentionally left blank

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Preface

Toxoplasma gondii is a ubiquitous, apicomplexan parasite of warm-blooded animals, and is one of the most common parasitic infections of humans. Infection can result in encephalitis in immunecompromised hosts, chorioretinitis in immunecompetent hosts or congenital transmission with fetopathy if a seronegative pregnant women becomes infected. It has been estimated that, in the absence of effective antiretroviral therapy and immune reconstitution, the risk for development of toxoplasmosis in a patient with AIDS with positive serologic findings for Toxoplasma is as high as 30 percent. Waterborne outbreaks of acute infection with chorioretinitis and an association of infection with increased mortality rates in California sea otter are emerging epidemiologic trends due to T. gondii infection.

The Apicomplexa are parasites that cause a wide variety of diseases in animals. *Toxoplasma gondii* has become a model organism for the study of the Apicomplexa, as it is the most experimentally tractable organism in this important group of intracellular parasites that includes *Plasmodium*, *Eimeria, Cryptosporidium, Neospora*, and *Theileria*. Currently *T. gondii* remains the apicomplexan species most readily amenable to genetic manipulation, with refined protocols for both classic and reverse genetics. Transient transfection efficiency is high (routinely over 50 percent), and expression

of epitope tags, reporter constructs, and heterologous proteins is relatively uncomplicated. Because of the difficulties in genetic manipulation of most Apicomplexa, T. gondii has been used as an expression system for these parasites. T. gondii has also been used for testing the biological or biochemical function of proteins that for one reason or another cannot be readily expressed in other organisms. The pathogenic stages of T. gondii are easily propagated and quantified in the laboratory; the mouse animal model is well-established; and reagents for study of the host response as well as basic biology of the parasite are widely available. Because of these experimental advantages, T. gondii has emerged as a major model organism for the study of apicomplexan biology.

Immunity to *T. gondii* is a complex process involving innate and adaptive immune responses. *T. gondii* has been a useful model system pathogen for understanding the immune response to an intracellular pathogen, including studies on macrophage function, cell-mediated immunity, dendritic cells, and the gut-associated immune response. The ease with which it can be cultured *in vitro*, availability of reporter parasite lines, and its pathogenicity in mouse models has facilitated genetic studies of the immune response to this organism.

The availability of genome sequences has revolutionized the study of microbial pathogens.

Genome sequences for the Apicomplexa are in various stages of completion. The *T. gondii* genome is ≈ 65 Mb, and has been sequenced for a type II strain (ME49), at 12X (http://www.toxodb.org, http://www.apidb.org): A type I (GT-I) and type III (VEG) stain have also been sequenced. These data have been integrated with genetic mapping data. Plans are in place for sequencing other strains of interest as well.

In general, *T. gondii* genes are much more intronrich than those of *Plasmodium* or *Cryptosporidium*. This has made gene prediction more problematic, but recent gene models have been devised which address these problems and have permitted proteomic studies on *T. gondii*. Both genetic and proteomic studies have resulted in rapid advances in our understanding of the composition of the various organelles in this organism and how these specialized structures interact to allow successful intracellular parasitism by this organism.

This book is an outgrowth of discussions held at the Seventh International Congress on Toxoplasmosis in Tarrytown, New York, in 2003, and the publication of papers and review articles from this congress in March 2004 in the *International Journal for Parasitology* (volume 34, number 3, pages 249–432). It was evident at this congress and confirmed at the Eighth International Congress in 2005 (Corsica, France) that the field of study of this pathogen had matured considerably since the First International Congress occurred in 1990 at Dartmouth University (Hanover, NH). This has been paralleled by attendance at this congress, which has grown from an initial group of 26 investigators to over 150 participants, and the increasing number of laboratories working on this organism.

There has been no recently published unified source for information on the biology, ultrastructure, genetics, immunology, and animal models of this pathogen. We believe the current book fills this unmet need. Authors were encouraged to review older literature comprehensively so that their chapters could serve as free-standing reference articles. Many chapters include summary tables and provide key reference material, including photomicrographs and data from the literature. We hope that the chapters can serve as summaries of the current state of the literature, providing an easy access point for studies on this organism.

The enthusiastic participation of the research community was critical in making this project a reality. We hope that the final product will serve as a key reference material for researchers who want to study *T. gondii* or use it as a model eukaryotic pathogen.

LMW and KK Bronx, NY

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We would like to thank our families – Lisa, Hannah, Talia, and Oren; Tom, Clayton, and Vaughan – for their patience, understanding and tolerance during the completion of this book. In addition, we want to thank members of the *Toxoplasma* research community for their enthusiasm and contributions to this project. The *Toxoplasma* research community is legendary for its generosity toward colleagues and new investigators, and it has been a unique pleasure to be involved with such a welcoming and intellectually stimulating group of researchers.

There have been many key research groups and individual researchers who have contributed to the development of the critical knowledge base required for progress on this pathogen. This book is a testament to these researchers.

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The History and Life Cycle of *Toxoplasma gondii*

J.P. Dubey

1.1 Introduction
 1.2 The etiological agent
 1.3 Parasite morphology and life cycle
 1.4 Transmission
 1.5 Toxoplasmosis in humans
 1.6 Toxoplasmosis in other animals

1.7 Diagnosis1.8 Treatment1.9 Prevention and control Acknowledgements References

1.1 INTRODUCTION

Infections by the protozoan parasite *Toxoplasma gondii* are widely prevalent in humans and other animals on all continents. There are many thousands of references to this parasite in the literature, and it is not possible to give equal treatment to all authors and discoveries. The objective of this chapter is, rather, to provide a history of the milestones in our acquisition of knowledge of the biology of this parasite.

1.2 THE ETIOLOGICAL AGENT

Nicolle and Manceaux (1908) found a protozoan in tissues of a hamster-like rodent, the gundi,

Ctenodactylus gundi, which was being used for leishmaniasis research in the laboratory of Charles Nicolle at the Pasteur Institute in Tunis. They initially believed the parasite to be Leishmania, but soon realized that they had discovered a new organism and named it Toxoplasma gondii, based on the morphology (mod. L. toxo = arc or bow, plasma = life) and the host (Nicolle and Manceaux, 1909). Thus, its complete designation is Toxoplasma gondii. In retrospect, the correct name for the parasite should have been Toxoplasma gundii; Nicolle and Manceaux (1908) had incorrectly identified the host as Ctenodactylus gondi. Splendore (1908) discovered the same parasite in a rabbit in Brazil, also erroneously identifying it as Leishmania, but he did not name it.

1.3 PARASITE MORPHOLOGY AND LIFE CYCLE

The life cycle of *Toxoplasma gondii* is illustrated in Figure 1.1.

1.3.1 Tachyzoites

The tachyzoite (Frenkel, 1973) is lunate (Figure 1.2A), and is the stage that Nicolle and Manceaux (1909) found in the gundi. This stage has also been called trophozoite, the proliferative form, the feeding form, and endozoite. It can infect virtually any cell in the body. It divides by a specialized process called endodyogeny, first described by Goldman *et al.* (1958). Gustafson *et al.* (1954) first studied the ultrastructure of the tachyzoite. Sheffield and Melton (1968) provided a complete description of endodyogeny when they fully described its ultrastructure.

1.3.2 Bradyzoite and tissue cysts

The term 'bradyzoite' (Gr. brady = slow) was proposed by Frenkel (1973) to describe the stage encysted in tissues. Bradyzoites are also called cystozoites. Dubey and Beattie (1988) proposed that cysts should be called tissue cysts (Figures 1.2B, 1.2C) to avoid confusion with oocysts. It is difficult to determine from the early literature who first identified the encysted stage of the parasite (Lainson, 1958). Levaditi et al. (1928) apparently were the first to report that T. gondii may persist in tissues for many months as 'cysts'; however, considerable confusion between the terms 'pseudocysts' (group of tachyzoites) and 'tissue cysts' existed for many years. Frenkel and Friedlander (1951) and Frenkel (1956) characterized cysts cytologically as containing organisms with a subterminal nucleus and periodic acid Schiff (PAS)-positive granules (Figure 1.2C) surrounded by an argyrophilic cyst



FIGURE 1.1 Life cycle of T. gondii.



FIGURE 1.2 Life-cycle stages of *T. gondii*.

(A) Tachyzoites (arrowhead) in smear. Giemsa stain. Note nucleus dividing into two nuclei (arrow).

- (B) A small tissue cyst in smear stained with Giemsa and a silver stain. Note the silver-positive tissue cyst wall (arrowhead) enclosing bradyzoites that have a terminal nucleus (arrow).
- (C) Tissue cyst in section, PAS. Note PAS-positive bradyzoites (arrow) enclosed in a thin PAS-negative cyst wall (arrowhead).
- (D) Unsporulated oocysts in cat feces, unstained.
- This figure is reproduced in color in the color plate section.

wall (Figure 1.2B). Wanko *et al.* (1962) first described the ultrastructure of the *T. gondii* cyst and its contents. Jacobs *et al.* (1960a) first provided a biological characterization of cysts when they found that the cyst wall was destroyed by pepsin or trypsin, but the cystic organisms were resistant to digestion by gastric juices (pepsin-HCl) whereas tachyzoites were destroyed immediately. Thus, tissue cysts were shown to be important in the life cycle of *T. gondii* because carnivorous hosts can become infected by ingesting infected meat. Jacobs *et al.* (1960b) used the pepsin digestion procedure to isolate viable *T. gondii* from tissues of chronically infected animals. When *T. gondii* oocysts were discovered in cat feces in 1970,

oocyst shedding was added to the biological description of the cyst (Dubey and Frenkel, 1976).

Dubey and Frenkel (1976) performed the first indepth study of the development of tissue cysts and bradyzoites, and described their ontogeny and morphology. They found that tissue cysts formed in mice as early as 3 days after their inoculation with tachyzoites. Cats shed oocysts (Figure 1.2D) with a short prepatent period (3–10 days) after ingesting tissue cysts or bradyzoites, whereas after they ingested tachyzoites or oocysts the prepatent period was longer (\geq 18 days), irrespective of the number of organisms in the inocula (Dubey and Frenkel, 1976; Dubey, 1996, 2001, 2006). Prepatent periods of 11–17 days are thought to result from the ingestion of transitional stages between tachyzoite and bradyzoite (Dubey, 2002, 2005).

Wanko *et al.* (1962) and Ferguson and Hutchison (1987) reported on the ultrastructure of the development of *T. gondii* tissue cysts. The biology of bradyzoites, including morphology, development in cell culture in vivo, conversion of tachyzoites to bradyzoites and vice versa, tissue cyst rupture, and distribution of tissue cysts in various hosts and tissues, was reviewed critically by Dubey *et al.* (1998).

1.3.3 Enteroepithelial asexual and sexual stages

Asexual and sexual stages (Figures 1.3, 1.4) were reported in the intestine of cats in 1970 (Frenkel, 1970). Dubey and Frenkel (1972) described the asexual and sexual development of *T. gondii* in enterocytes of the cat, and designated the asexual enteroepithelial stages as types A through E (Figures 1.3, 1.4) rather than as generations conventionally known as schizonts in other



FIGURE 1.3 Asexual and sexual stages of *T. gondii* in sections of small intestine of cats fed tissue cysts. H&E stain.

- (A) Type C (arrow) schizont with a residual body and a type B schizont with a hypertrophied host cell nucleus (arrowhead), 52 hours p.i.
- (B) Heavily infected small intestine with schizonts in and gamonts in the epithelium, 5 days p.i.
- (C) Types D and E schizonts (a, d), a mature female gamont (e), a young female gamont (b), and two male gamonts (c) in the epithelium.
- (D) Tachyzoites in the lamina propria (arrows). Types B and D schizonts are below the enterocyte nucleus and often cause hypertrophy of the parasitized cell, whereas types D and E schizonts are always above the enterocyte nucleus and do not cause hypertrophy of the host cell even in hyperparasitized cases. Tachyzoites are found in the lamina propria of the cat intestine.

This figure is reproduced in color in the color plate section.



FIGURE 1.4 Smears of intestinal epithelium of a cat 7 days after feeding tissue cysts (Giemsa stain).(A) Note different sizes of merozoites (a–c), schizont with three nuclei (d), schizont with six or more nuclei and merozoites budding from the surface

(e), and a multinucleated schizont (f). (B) Four biflagellated microgametes (arrows) and

merozoites (arrowhead) for size comparision. This figure is reproduced in color in the color plate

section.

coccidian parasites. These stages were distinguished morphologically from tachyzoites (Figure 1.3D) and bradyzoites, which also occur in cat intestine. The challenge was to distinguish different stages in the cat intestine, because there was profuse multiplication of *T. gondii* 3 days post-infection (Figure 1.4A). The entire cycle was completed by 66 hours after feeding tissue cysts to cats (Dubey and Frenkel, 1972). There are reports on the ultrastructure of schizonts (Sheffield, 1970; Piekarski *et al.*, 1971; Ferguson *et al.*, 1974), gamonts (Ferguson *et al.*, 1974, 1975; Speer and Dubey, 2005), and oocysts and sporozoites (Christie *et al.*, 1978; Ferguson *et al.*, 1979a, 1979b; Speer *et al.*, 1998). In 2005, Speer and Dubey described the ultrastructure of asexual enteroepithelial types B through E and distinguished their merozoites.

1.4 TRANSMISSION

1.4.1 Congenital

The mechanism of transmission of T. gondii remained a mystery until its life cycle was discovered in 1970. Soon after the initial discovery of the organism, it was found that the C. gundi were not infected in the wild and had acquired T. gondii infection in the laboratory. Initially transmission by arthropods was suspected, but this was never proven (Frenkel, 1970, 1973). Congenital T. gondii infection in a human child was initially described by Wolf et al. (1939a, 1939b) and later found to occur in many species of animals, particularly sheep, goats, and rodents. Congenital infections can be repeated in some strains of mice (Beverley, 1959), with infected mice producing congenitally infected offspring for at least 10 generations. Beverley discontinued his experiments because of high mortality in some lines of congenitally infected mice, and because the progeny from the last generation of infected mice were seronegative and presumed not to be infected with T. gondii. Jacobs (1964) repeated these experiments and found that congenitally infected mice may be infected, but not develop antibodies because of immune tolerance. Dubey et al. (1995a) isolated viable T. gondii from seronegative naturallyinfected mice. These findings are of epidemiological significance.

1.4.2 Carnivorism

Congenital transmission occurs too rarely to explain widespread infection in man and animals worldwide. Weinman and Chandler (1954) suggested that transmission might occur through the ingestion of undercooked meat. Jacobs *et al.* (1960a) provided evidence to support this idea by demonstrating the resistance to proteolytic enzymes of *T. gondii* derived from cysts. They found that the cyst wall was immediately dissolved

by such enzymes but the released bradyzoites survived long enough to infect the host. This hypothesis of transmission through the ingestion of infected meat was experimentally tested by Desmonts et al. (1965) in an experiment with children in a Paris sanatorium. They compared the acquisition rates of T. gondii infection in children before and after admission to the sanatorium. The 10 percent yearly acquisition rate of T. gondii antibody rose to 50 percent after adding two portions of barely cooked beef or horse meat to the daily diet, and to a 100 percent yearly rate after the addition of barely cooked lamb chops. Since the prevalence of T. gondii is much higher in sheep than in horses or cattle, this illustrated the importance of carnivorism in transmission of T. gondii. Epidemiological evidence indicates it is common in humans in some localities where raw meat is routinely eaten. In a survey in Paris, Desmonts et al. (1965) found over 80 percent of the adult population sampled had antibodies to T. gondii. Kean et al. (1969) described toxoplasmosis in a group of medical students who had eaten undercooked hamburgers.

1.4.3 Fecal-oral

While congenital transmission and carnivorism can explain some of the transmission of T. gondii, it does not explain the widespread infection in vegetarians and herbivores. A study in Bombay, India, found the prevalence of T. gondii in strict vegetarians to be similar to that in non-vegetarians (Rawal, 1959). Hutchison (1965), a biologist at Strathclyde University in Glasgow, first discovered T. gondii infectivity associated with cat feces. In a preliminary experiment, Hutchison (1965) fed T. gondii cysts to a cat infected with the nematode Toxocara cati and collected feces containing nematode ova. Feces floated in 33% zinc sulfate solution and stored in tap water for 12 months induced toxoplasmosis in mice. This discovery was a breakthrough, because until then both known forms of T. gondii (i.e. tachyzoites and bradyzoites) were killed by water. Microscopic examination of feces revealed only T. cati eggs and Isospora oocysts. In Hutchison's report, T. gondii infectivity was not attributed to oocysts or T. cati eggs. He repeated the experiment with two T. cati-infected and two T. cati-free cats. T. gondii was transmitted only in association with T. cati infection. On this basis, Hutchison (1967) hypothesized that T. gondii was transmitted through nematode ova. He suspected transmission of T. gondii through the eggs of the nematode Toxocara, similar to the transmission of the fragile flagellate Histomonas through Heterakis eggs. Hutchison initially wanted to test the nematode theory using Toxocara canis and T. gondii transmission in the dog, but decided on the cat and Toxocara cati model because there was no place to house dogs (1965, J.P. Dubey, personal communication). Transmission of T. gondii by Toxocara canis eggs made more sense because of the known zoonotic potential of T. canis; Toxocara cati was not at that time known to infect humans, but T. canis was. Discovery of the life cycle of T. gondii would have been delayed had Hutchinson worked with dogs instead of cats.

Hutchison's (1965) report stimulated other investigators to examine fecal transmission of T. gondii through T. cati eggs (Dubey, 1966, 1968; Jacobs, 1967; Hutchison et al., 1968; Frenkel et al., 1969; Sheffield and Melton, 1969). The nematode egg theory of transmission was discarded after Toxoplasma infectivity was dissociated from T. cati eggs (Frenkel et al., 1969) and Toxoplasma infectivity was found in feces of worm-free cats fed T. gondii (Frenkel et al., 1969; Sheffield and Melton, 1969). Finally, in 1970, knowledge of the T. gondii life cycle was completed by discovery of the sexual phase of the parasite in the small intestine of the cat (Frenkel et al., 1970). T. gondii oocysts, the product of schizogony and gametogony, were found in cat feces and characterized morphologically and biologically (Dubey et al., 1970a, 1970b).

Several group of workers independently and at about the same time found *T. gondii* oocysts in cat feces (Hutchison *et al.*, 1969, 1970, 1971; Frenkel *et al.*, 1970; Dubey *et al.*, 1970a, 1970b; Sheffield and Melton, 1970; Overdulve, 1970; Weiland and Kühn, 1970; Witte and Piekarski, 1970). The discovery of *T. gondii* oocysts in cat feces, and its implications, has been reviewed by Frenkel (1970, 1973) and Garnham (1971).

In retrospect, the discovery and characterization of the T. gondii oocyst in cat feces was delayed because (1) T. gondii oocysts were morphologically identical to oocysts of the previously described coccidian parasite of cats and dogs (Dubey et al., 1970a), and (2) until 1970 coccidian oocysts were sporulated in 2.5% potassium dichromate. Chromation of the oocysts wall interfered with excystation of the sporozoites when oocysts were fed to mice, and thus the mouse infectivity titer of the oocysts was lower than expected from the number of oocysts administered (Dubey et al., 1970a). These findings led to the use of 2% sulfuric acid as the best medium for sporulation and storage of T. gondii oocysts. Unlike dichromate, which was difficult to wash off the oocysts, sulfuric acid could be easily neutralized and the oocysts could be injected without washing into mice (Dubey et al., 1972). Unlike other coccidians, T. gondii oocysts were found to excyst efficiently when inoculated parenterally into mice and thus alleviated the need for oral inoculation for bioassay of oocysts (Dubey and Frenkel, 1973).

Ben Rachid (1970) fed *T. gondii* oocysts to gundis, which died 6–7 days later from toxoplasmosis. This knowledge about the life cycle of *T. gondii* probably explains how gundis became infected in the laboratory of Nicolle. At least one cat was present in the Pasteur laboratory in Tunis (Dubey, 1977, 2006).

Of the many species of animals experimentally infected with T. gondii, only felids shed T. gondii oocysts (Janitschke and Werner, 1972; Jewell et al., 1972; Miller et al., 1972; Polomoshnov, 1979). Oocysts shed into the environment have caused several outbreaks of disease in humans (Teutsch et al., 1979; Benenson et al., 1982; Bowie et al., 1997; de Moura et al., 2006). T. gondii oocysts found in the feces of naturally infected cougars (Aramini et al., 1998) were epidemiologically linked to the largest recorded waterborne outbreak of toxoplasmosis in humans (Bowie et al., 1997). Seroepidemiological studies on isolated islands in the Pacific (Wallace, 1969), Australia (Munday, 1972), and the USA (Dubey et al., 1997) have shown an absence of Toxoplasma on islands without cats, confirming the important role of the cat in the natural

transmission of *T. gondii*. Vaccination of cats with a live mutant strain of *T. gondii* on eight pig farms in the USA reduced the transmission of *T. gondii* infection in mice and pigs (Mateus-Pinilla *et al.*, 1999), thus supporting the role of the cat in natural transmission of *T. gondii*.

Historically, before the discovery of the coccidian cycle of T. gondii, coccidian parasites were considered to be host- and site-specific, and to be transmitted by the fecal-oral route. After the discovery of the sexual cycle of T. gondii, several other genera (e.g. Sarcocystis, Besnoitia) were found to be coccidian. Although T. gondii has a wide host range, it has retained the definitive-host specificity restricted to felids. Dr J.K. Frenkel deserves the credit for initiating testing of many species of animals, including wild felids, for oocysts shedding, under difficult housing conditions (it was not easy handling bobcats and ocelots in cages). Only the felids were found to shed T. gondii oocysts (Frenkel et al., 1970; Miller et al., 1972). Although T. gondii can be transmitted in several ways, it has adapted to be transmitted most efficiently by carnivorism in the cat and by the fecal-oral (oocysts) route in other hosts. Pigs and mice (and presumably humans) can be infected by ingesting even one oocyst (Dubey et al., 1996), whereas 100 oocysts may not infect cats (Dubey, 1996). Cats can shed millions of oocysts after ingesting only a single bradyzoite, while ingestion of 100 bradyzoites may not infect mice orally (Dubey, 2001, 2006). This information has proved very useful in conducting epidemiological studies and for the detection by feeding to cats of low numbers of T. gondii in large samples of meat (Dubey et al., 2005).

After the discovery of the life cycle of *T. gondii* in the cat, it became clear why Australasian marsupials and New World monkeys are highly susceptible to clinical toxoplasmosis. The former evolved apparently in the absence of the cat (there were few or no cats in Australia and New Zealand before settlement by Europeans), and the latter live on tree tops and are not exposed to cat feces. In contrast, marsupials in America and Old World monkeys are resistant to clinical toxoplasmosis (Dubey and Beattie, 1988).

1.5 TOXOPLASMOSIS IN HUMANS

1.5.1 Congenital toxoplasmosis

Three pathologists - Wolf, Cowen, and Paige, from New York, USA - first conclusively identified T. gondii in an infant girl who was delivered full term by Caesarean section on 23 May 1938 at Babies' Hospital, New York (Wolf et al., 1939a, 1939b). The girl developed convulsive seizures at 3 days of age, and lesions were noted in the maculae of both eyes through an ophthalmoscope. She died when a month old, and an autopsy was performed. At post mortem, brain, spinal cord, and right eye were removed for examination. Free and intracellular T. gondii were found in lesions of encephalomyelitis and retinitis of the girl. Portions of cerebral cortex and spinal cord were homogenized in saline and inoculated intracerebrally into rabbits and mice. These animals developed encephalitis, T. gondii was demonstrated in their neural lesions, and T. gondii from these animals was successfully passaged into other mice.

Wolf, Cowen, and Paige reviewed in detail their own cases and those reported by others, particularly Jankû (1923) and Torres (1927), of *T. gondii*-like encephalomyelitis and chorioretinitis in infants (Wolf and Cowen, 1937, 1938; Wolf *et al.*, 1939a, 1939b, 1940; Cowen *et al.*, 1942; Paige *et al.*, 1942). Joseph Jankû (1923), an ophthalmologist from Czechoslovakia, was credited earlier with finding a *T. gondii*-like parasite in a human eye (Jankû, 1923). The following description of the case of Jankû is taken from the English translation published by Wolf and Cowen (1937):

The patient was born with left microphthalus and became blind at the age of 3 months, and had hydrocephalus. The child died when 11 months old. The eyes and brain were removed at autopsy. Grossly, the child had internal hydrocephalus but the brain was not available for histopathological examination. Chorioretinitis was present in both eyes and cyst-like structures [termed sporocysts by Jankû] were seen in the right eye.

Jankû (1923, reprinted 1959) thought that this parasite was *Encephalitozoon* (a microsporidium).

The material from this case is thought to have been destroyed in World War II bombing, and so confirmation of these findings is not possible. Torres (1927) found protozoa in lesions of encephalitis in a 2-day-old infant in Rio de Janeiro, Brazil. Numerous organisms were seen, but these were thought to be a new species of *Encephalitozoon*. This patient also had myocarditis and myositis. In the Netherlands, de Lange (1929) found protozoa in sections of the brain of a 4-month-old child that was born with hydrocephalus. These sections were reexamined by Wolf and Cowen, and a full account was reviewed by Sabin (1942).

Sabin (1942) summarized all that was known of congenital toxoplasmosis in 1942, and proposed typical clinical signs of congenital toxoplasmosis: hydrocephalus or microcephalus, intracerebral calcification, and chorioretinitis. These signs helped in the clinical recognition of congenital toxoplasmosis. Frenkel and Friedlander (1951) published a detailed account of five fatal cases of toxoplasmosis in infants that were born with hydrocephalus; T. gondii was isolated from two. They described the pathogenesis of internal hydrocephalus as a blockage of the aqueduct of Sylveus due to ventriculitis resulting from a T. gondii antigen-antibody reaction. This lesion is unique to human congenital toxoplasmosis, and has never been verified in other animals (Dubey, unpublished). This report was the first in-depth description of lesions of congenital toxoplasmosis not only in the central nervous system but also in other organs. Hogan (1951) also provided the first detailed clinical description of ocular toxoplasmosis.

1.5.2 Acquired toxoplasmosis

1.5.2.1 Children

Sabin (1941) reported toxoplasmosis in a 6-year-old boy from Cincinnati, Ohio. An asymptomatic child (initials RH) was hit with a baseball bat on 22 October 1937. He developed a headache 2 days later and convulsions the day after. He was admitted to hospital on the seventh day, but without obvious clinical signs. Except for lymphadenopathy and an enlarged spleen, nothing abnormal was found. He then developed neurological signs and died on the thirtieth day of illness. The brain and spinal cord were removed for histopathological examination and bioassay. Because of the suspicion of polio virus infection, a homogenate of cerebral cortex was inoculated into mice. T. gondii was isolated from the inoculated mice, and this isolate was given the initials of the child, becoming the famous RH strain. Only small lesions of nonsuppurative encephalitis were found microscopically in the brain of this child; neither gross lesions nor any viral or bacterial infections were found. This child most likely had acquired T. gondii infection recently, and the blow to the head was coincidental and unrelated to the onset of symptoms. It is noteworthy that some mice infected with the original RH strain did not die until day 21 post-inoculation, but by the third passage mice died 3-5 days after inoculation. The RH strain of T. gondii has since 1938 been passaged in mice in many laboratories. After this prolonged passage, its pathogenicity for mice has been stabilized (Dubey, 1977) and it has lost the capacity to produce oocysts in cats (Frenkel et al., 1976).

1.5.2.2 Toxoplasmosis in adults

Pinkerton and Weinman (1940) identified *T. gondii* in the heart, spleen, and other tissues of a 22-year-old patient who died in 1937 in Lima, Peru. The patient exhibited fever and concomitant *Bartonella* sp. infection. Pinkerton and Henderson (1941) isolated *T. gondii* from the blood and tissues of two individuals (aged 50 and 43) who died in St Louis, Missouri. Recorded symptoms included rash, fever, and malaise. These were the first reports of acute toxoplasmosis in adults without neurological signs.

Lymphadenopathy Siim (1956) drew attention to the fact that lymphadenopathy is a frequent sign of acquired toxoplasmosis in adults and these findings were confirmed by Beverley and Beattie (1958), who reported on the cases of 30 patients. A full appreciation of the clinical symptoms of acquired toxoplasmosis was achieved when outbreaks of acute toxoplasmosis were reported in adults in the USA (Teutsch *et al.*, 1979) and in Canada (Bowie *et al.*, 1997).

Ocular disease Before 1950, virtually all cases of ocular toxoplasmosis were considered to result from congenital transmission (Perkins, 1961). Wilder (1952) identified T. gondii in eyes that had been enucleated. The significance of this finding lies in the way this discovery was made. These eyes were suspected of being syphilitic, tuberculous, or of having tumors. Wilder was a technician in the registry of Ophthalmic Pathology at AFIP, and she routinely microscopically examined the sections that she prepared. She put enormous effort into identifying microbes in these 'tuberculous' eyes, but never identified bacteria or spirochetes by special staining. Then she found T. gondii in the retinas of these eyes. She subsequently collaborated with Jacobs and Cook and found most of these patients with histologically confirmed T. gondii infection had low levels of dye test antibodies (a titer of 1:16), and in one patient antibodies were demonstrable only in undiluted serum (Jacobs et al., 1954a). Jacobs et al. (1954b) made the first isolation of T. gondii from an eye of a 30-year-old male hospitalized at the Walter Reed Army Hospital. The eye had been enucleated because of pain associated with elevated intraocular pressure. A group of ophthalmologists from southern Brazil initially discovered ocular toxoplasmosis in siblings. Among patients with postnatally acquired toxoplasmosis who did not have retinochoroidal scars before, 8.3 percent developed retinal lesions during a 7-year followup (Silveira et al., 1988, 2001). Ocular toxoplasmosis was diagnosed in 20 of 95 patients with acute toxoplasmosis associated with the Canadian waterborne outbreak of toxoplasmosis in 1995 (Burnett et al., 1998; see also Holland, 2003).

AIDS epidemic Before the epidemic of the acquired immunodeficiency syndrome (AIDS) in adults in the 1980s, neurological toxoplasmosis in adults was rarely reported and was essentially limited to patients treated for tumors or those given transplants. Luft *et al.* (1983) reported acute toxoplasmosis-induced encephalitis that was fatal if not treated. In almost all cases, clinical disease occurred as result of reactivation of chronic infection initiated by the depression of intracellular immunity due to HIV infection. Initially, many of

these cases of toxoplasmosis in AIDS patients were thought to be lymphoma.

1.6 TOXOPLASMOSIS IN OTHER ANIMALS

Mello (1910), in Turin, Italy, first reported fatal toxoplasmosis in a domestic animal (a 4-month-old dog) that died of acute visceral toxoplasmosis. Over the next 30 years, canine toxoplasmosis was reported in Cuba, France, Germany, India, Iraq, Tunisia, the USSR, and the USA (Dubey and Beattie, 1988). Campbell *et al.* (1955) found that most cases of clinical toxoplasmosis were in dogs infected with canine distemper virus (CDV). Even vaccination with live attenuated CDV vaccine can trigger clinical toxoplasmosis in dogs (Dubey *et al.*, 2003a). The incidence of clinical toxoplasmosis in dogs decreased dramatically after vaccination with CDV vaccine became routine practice.

Strangely enough, the first case of toxoplasmosis was not reported in a cat until 1942, when Olafson and Monlux found it in a cat from Middletown, New York, USA. In the 1950s and 1960s, Galuzo and Zasukhin published (in Russian) their own studies and those of other researchers on many species of animals from the former USSR. This information was made available to scientists in other countries when their book was translated into English by Plous Jr and edited by Fitzgerald (1970). Jirá and Kozojed (1970, 1983) published the most comprehensive bibliography of toxoplasmosis, listing more than 12 000 references and categorizing them by hosts and topics. This work proved useful for literature searches prior to electronic databases.

Toxoplasmosis in sheep deserves special attention because of its economic impact. William Hartley, a well-known veterinary pathologist from New Zealand, and his associates, J.L. Jebson and D. McFarlane, discovered *T. gondii*-like organisms in the placentas and fetuses of several unexplained abortions in ewes in New Zealand. They called it New Zealand type II abortion. Hartley and Marshall (1957) finally isolated *T. gondii* from aborted fetuses. Hartley (1961) and Jacobs and Hartley (1964) experimentally induced toxoplasmic abortion in ewes. The identification of *T. gondii* abortion in ewes was a landmark discovery in veterinary medicine; prior to that, protozoa were not recognized as a cause of epidemic abortion in livestock. Subsequently, Jack Beverley and Bill Watson recognized epidemics of ovine abortion in the UK (Beverley and Watson, 1961). Dubey and Towle (1986) and Dubey and Beattie (1988) summarized all that was known about toxoplasmosis in sheep and its impact on agriculture. Millions of lambs are still lost throughout the world due to this infectious disease.

Sanger and Cole (1955) were first to isolate T. gondii from a food animal. Dubey and Beattie (1988) reviewed the worldwide literature on toxoplasmosis in humans and other animals. The discovery and naming of two new organisms, Neospora caninum (Dubey et al., 1988) and Sarcocystis neurona (Dubey et al., 1991), which were previously thought to be T. gondii resulted in new information on the host distribution of T. gondii. We now know that cattle and horses are resistant to clinical T. gondii, that N. caninum is a common cause of abortion in cattle worldwide (Dubey, 2003), and that S. neurona is a common cause of fatal encephalomyelitis in horses in the Americas (Dubey et al., 2001). There have been no confirmed cases of clinical toxoplasmosis in either cattle or horses (Dubey, unpublished).

The finding of *T. gondii* in marine mammals deserves special mention. Before the discovery of the *T. gondii* oocyst, no-one would have suspected that the marine environment would be contaminated with *T. gondii* and that fish-eating marine mammals would be found infected with *T. gondii* (Dubey *et al.*, 2003b; Conrad *et al.*, 2005). Thomas and Cole (1996) and Cole *et al.* (2000) isolated viable *T. gondii* from sea otters in the United States. Several reports have now appeared that confirm that *T. gondii* can occur in marine mammals.

1.7 DIAGNOSIS

1.7.1 Sabin–Feldman dye test

Development of a novel serologic test, the dye test, in 1948 by Albert Sabin and Harry Feldman was perhaps the greatest advancement in the field of toxoplasmosis (Sabin and Feldman, 1948). The dye test is highly sensitive and specific, with no evidence for false results in humans. Even titers as low as 1:2 are meaningful for the diagnosis of ocular disease. The ability to identify *T. gondii* infections based on a simple serological test opened the door for extensive epidemiological studies on the incidence of infection. It became clear that *T. gondii* infections are widely prevalent in humans in many countries. It also demonstrated that the so-called tetrad of clinical signs considered indicative of clinical congenital toxoplasmosis occurred in other diseases and assisted in the differential diagnosis (Sabin and Feldman, 1949; Feldman and Miller, 1956).

1.7.2 Detection of IgM antibodies

Remington *et al.* (1968) first proposed the usefulness of the detection of IgM antibodies in cord blood or infant serum for the diagnosis of congenital toxoplasmosis, since IgM antibodies do not cross the placenta whereas IgG antibodies do. Remington (1969) modified the indirect fluorescent antibody test (IFAT) and the ELISA (Naot and Remington, 1980) to detect IgM in cord blood. Desmonts *et al.* (1981) developed a modification of IgM-ELISA, combining it with the agglutination test (IgM-ISAGA) to eliminate the necessity for an enzyme conjugate. Although IgM tests are not perfect, they have proved useful for screening programs (Remington *et al.*, 2001).

1.7.3 Direct agglutination test

The development of a simple direct agglutination test has aided tremendously in the serological diagnosis of toxoplasmosis in humans and other animals. In this test, no special equipment or conjugates are needed. This test was initially developed by Fulton (1965), and was improved by Desmonts and Remington (1980) and then by Dubey and Desmonts (1987), who called it the modified agglutination test (MAT). The MAT has been used extensively for the diagnosis of toxoplasmosis in animals. The sensitivity and specificity of MAT has been validated by comparing serologic data and isolation of the parasite from naturally- and experimentally-infected pigs (Dubey *et al.*, 1995b; Dubey, 1997) and naturally-infected chickens (Dubey, unpublished).

1.7.4 Detection of T. gondii DNA

Burg *et al.* (1989) first reported detection of *T. gondii* DNA from a single tachyzoite, using the B1 gene in a polymerase chain reaction (PCR). Several subsequent PCR tests have been developed using different gene targets. Overall, this technique has proven very useful in the diagnosis of clinical toxoplasmosis.

1.8 TREATMENT

Sabin and Warren (1942) reported the effectiveness of sulfonamides against murine toxoplasmosis, and Eyles and Coleman (1953) discovered the synergistic effect of combined therapy with sulfonamides and pyrimethamine; the latter is the standard therapy for toxoplasmosis in humans (Remington *et al.*, 2001). Garin and Eyles (1958) found spiramycin to have antitoxoplasmic activity in mice. Since spiramycin is non-toxic and does not cross the placenta, it has been used prophylactically in women during pregnancy to reduce transmission of the parasite from mother to fetus (Desmonts and Couvreur, 1974a).

1.9 PREVENTION AND CONTROL

1.9.1 Serologic screening during pregnancy

Georges Desmonts initiated studies in Paris, France, in the 1960s, looking at seroconversion in women during pregnancy and the transmission of *T. gondii* to the fetus. Blood was obtained at the first visit, at 7 months, and at the time of parturition. Desmonts initiated prophylactic treatment of women who seroconverted during pregnancy. Results of the 15-year study demonstrated the following:

1. Infection acquired during the first two trimesters was most damaging to the fetus.

- 2. Not all women that acquired infection transmitted it to the fetus.
- 3. Women seropositive prior to pregnancy did not transmit infection to the fetus.
- 4. Treatment with spiramycin reduced congenital transmission but not clinical disease in infants (Desmonts and Couvreur, 1974a, 1974b).

At about the same time, Otto Thalhammer initiated a similar screening program for pregnant women in Austria (see Thalhammer, 1973, 1978). In addition to scientific knowledge, these screening programs have helped to disseminate information for the prevention of toxoplasmosis.

A neonatal serological screening and early treatment for congenital *T. gondii* infection was initiated in Massachusetts, USA, in the 1980s (Guerina *et al.*, 1994). The efficacy of treatment of *T. gondii* infection in the fetus and newborn is not fully delineated, and many issues related to the cost and benefit of screening and treatment in pregnancy and in newborns remain to be examined.

1.9.2 Hygiene measures

After the discovery of the life cycle of *T. gondii* in 1970, it became possible to advise pregnant women and other susceptible populations regarding avoiding contact with oocysts (Frenkel and Dubey, 1972). Studies were conducted to construct thermal curves showing temperatures required to kill *T. gondii* in infected meat by freezing (Kotula *et al.*, 1991), cooking (Dubey *et al.*, 1990), and gamma irradiation (Dubey *et al.*, 1986). These data are now used by regulatory agencies to advise consumers about the safety of meat. Freezing of meat overnight in a household freezer before human or animal consumption remains the easiest and most economical method of reducing transmission of *T. gondii* through meat.

1.9.3 Animal production practices

Extensive epidemiological studies on pig farms in the USA in the 1990s concluded that keeping cats out of the pig barns and raising pigs indoors can reduce *T. gondii* infection in pigs (Dubey *et al.*, 1995a; Weigel *et al.*, 1995). As a result of changes in pig husbandry, the prevalence of viable *T. gondii* in pigs is reduced to <1 percent (Dubey *et al.*, 2005). Because ingestion of infected pork is considered to be the main meat source of *T. gondii* for humans (at least in the United States), hopefully this will also reduce the prevalence of *T. gondii* in humans.

1.9.4 Vaccination

Vaccination of sheep with a live cystless strain of *T. gondii* reduces neonatal mortality in lambs, and this vaccine is available commercially (Wilkins and O'Connell, 1983; Buxton and Innes, 1995). To date, there is no vaccine suitable for human use.

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The Ultrastructure of *Toxoplasma gondii*

D.J.P. Ferguson and J.F. Dubremetz

2.1 Introduction

- 2.2 Invasive stage ultrastructure and genesis
- 2.3 Coccidian development in the definitive host2.4 Development in the intermediate host *References*

2.1 INTRODUCTION

This chapter reviews the electron microscopic data on *Toxoplasma gondii* and its life-cycle stages. Corresponding light microscopy of these stages can be found in Chapter 1.

2.2 INVASIVE STAGE ULTRASTRUCTURE AND GENESIS

2.2.1 Basic ultrastructural morphology

There are four invasive forms of *T. gondii*: the tachyzoite, bradyzoite, merozoite, and sporozoite. Tachyzoites and bradyzoites are associated with the intermediate host, and merozoites and sporozoites

with the definitive host. Tachyzoites and merozoites are responsible for the expansion of the population within a host, while the bradyzoites and sporozoites are capable of environmental transmission to new hosts.

All of the infectious stages have the same basic morphology, with only minor variations. The standard features will be described in this section, and are based mainly on observations of tachyzoites. The tachyzoite is the most extensively studied stage in the *T. gondii* life cycle because of the ease with which large numbers can be obtained both *in vivo* and *in vitro*. These invasive stages are crescent-shaped cells (approximately $2 \times 7 \mu m$) with a slightly more pointed anterior end (the anterior being defined by the direction of motility) (Figure 2.1A). They are comprised of a unique cytoskeleton (subpellicular microtubules, conoid), secretory organelles (rhoptries, micronemes, dense



FIGURE 2.1 Toxoplasma gondii tachyzoite ultrastructure.

- (A) Sagittal section of an intravacuolar tachyzoite. A, apicoplast; C, conoid; DG, dense granule; er, endoplasmic reticulum; G, Golgi body; HCN, host-cell nucleus; MN, micronemes; Mi, mitochondria; N, nucleus; nu, nucleolus; PV, parasitophorous vacuole; R, rhoptry; ac, acidocalcisome; tvn, tubo vesicular network. Bar = 1 μ m.
- (B) Enlargement of the Golgi area, showing the apicoplast, A, surrounded by four membranes (arrows). Bar = $0.5 \ \mu$ m.

granules), endosymbiontic derived organelles (mitochondrion, apicoplast), eukaryotic universal organelles (nucleus, endoplasmic reticulum, Golgi apparatus, ribosomes), and specific structures (acidocalcisomes), all enclosed by a complex membranous structure termed the pellicle.

The cytoskeleton comprises:

1. Two apical rings located beneath the plasma membrane at the apical tip of the parasite.

They are uncharacterized at the molecular level, but both are made of a thin ring of electrondense material; the upper one is 160 nm, the posterior one 200 nm in diameter (200 nmD).

- The conoid, which is a hollow truncated cone consisting of fibers wound into a spiral, like a compressed spring, 400 nm in diameter at the base and 250 nm high. It is made of tubulin organized in a unique fashion, consisting of asymmetrical filaments of about 9 protofilaments, very different from typical microtubules (Hu *et al.*, 2002a).
- 3. Two polar rings that encircle the top of the resting conoid (Figure 2.2A). The outer ring consists of dense material covering the anterior rim of the inner membrane complex (IMC, see below). The inner ring is formed of material which anchors the 22 subpellicular microtubules that extend underneath the IMC for approximately two-thirds of the body length (Nichols and Chiappino, 1987). These microtubules are classical 22-nm diameter hollow tubes, comprising 13 protofilaments made of tubulin (Hu *et al.*, 2002a).
- 4. A pair of adjacent intraconoidal microtubules, extending for a short distance (less than 1 μm) into the apical cytoplasm and ending anteriorly next to an apical vesicle of 40 nm that adheres to the plasma membrane (Hu *et al.*, 2002a).

The pellicle is a distinctive membrane complex that encloses the infectious stages. It consists of an outer unit membrane (plasmalemma) that completely encloses the organism, and an inner layer of two closely applied unit membranes found at a fixed distance (approx 15 nm) from the plasmalemma. The inner membrane complex (IMC) consists of fused plates formed from flattened vesicles derived from the ER-Golgi system (Vivier and Petitprez, 1969). The inner layer is interrupted by circular apertures at the anterior end (outer polar ring), where the conoid protrudes, and at the posterior end. The organization of the IMC has been essentially unravelled by EM freeze fracture (Porchet and Torpier, 1977; Morrissette et al., 1997). It is made of an apical plate, which is a single truncated cone, approximately 1 µm high, on

INVASIVE STAGE ULTRASTRUCTURE AND GENESIS



FIGURE 2.2 Ultrastructural details of bradyzoite and tachyzoite.

- (A) Apical area of a bradyzoite showing the two apical rings (a1, a2), and the two polar rings (p1, p2) above and around the conoid. Bar = $0.1 \mu m$.
- (B) Upper picture: micropore showing the invagination of the zoite plasmalemma (arrow) through an opening and indentation (arrowhead) of the inner membrane complex (imc). Lower picture: uptake of PV vesicular material through the micropore. Bar = 0.1 μm.
- (C) Freeze-fracture image of the pellicle of a tachyzoite (taken from Morrissette *et al.*, 1997). The three successive membranes are shown: Pe, protoplasmic face of the plasmalemma; Em, exoplasmic face of the external layer of the inner membrane complex; Pi, protoplasmic face of the inner layer of the inner membrane complex. Arrowheads point at lines of higher IMP density corresponding to underlying subpellicular microtubules. Bar = $0.1 \mu m$.

which six longitudinal rows of rectangular plates are attached. The rows end at the posterior end of the tachyzoite in triangular plates. The rows can extend straight or be twisted helically. The protoplasmic faces on both sides of the IMC are covered with lines of intramembranous particles (IMPs), with 22 lines of higher density corresponding to the underlying subpellicular microtubules (Figure 2.2B). IMPs have a 32-nm longitudinal periodicity, and are lined approximately 30 nm apart (Morrissette et al., 1997). The organization of IMPs in the apical plate is markedly different from that in the other plates, suggesting a distinct molecular structure in this apical area. An additional organized structure associated with the inner side of the IMC has been described, by negative staining after detergent extraction, as a network of 8-10-nm filaments containing two novel proteins with extended coiled-coiled domains that may play a role in determining cell shape (Mann and Beckers, 2001). The precise correlation between this network and the IMP alignments has not been defined.

The pellicle has an additional adaptation termed the micropore, which is located in the apical half of the cell normally just anterior to the nucleus. The single micropore consists of a circular (approximately 150 nm in diameter) invagination of the plasmalemma through a break in the inner membrane complex. The latter infolds to form an electrondense collar around the invagination (Figure 2.2C). These structures are present throughout development, and increase in number during endopolygeny and gametogony. They are thought to act as a cytostome-like structure with extensions of the invaginated plasmalemma budding off, resulting in the uptake of material (Figure 2.2C) (Nichols et al., 1994). This process has been clearly shown to be important in the malaria parasite, where the micropore is responsible for the ingestion of the erythrocyte hemoglobin (Aikawa et al., 1966).

Three distinct secretory organelles have been identified, which can vary in numbers and shape between the invasive stages (see below) (Figures 2.1A, 2.2A). First are small, rod-shaped micronemes (250×50 nm), located in the most apical area of the parasite, behind the conoid. They are homogeneously electron-dense. Second are the rhoptries, organized

as a group of elongated, club-shaped organelles that extend from within the conoid toward the nucleus. They show a long, narrow neck up to 2.5 μ m in length, and a sac-like body about 0.25 \times 1 μ m in the posterior area. The contents are electron-dense except in the widened part, where the structure can be either labyrinthine or electron-dense in appearance depending on the specific stage. The third type, found throughout the cell but mostly in the posterior part of the parasite, are spherical-shaped (0.3- μ m diameter) structures with electron-dense contents, which have been termed the dense granules.

Immuno-electron microscopy has played an important role in our understanding of the functions of these organelles. With the development of antibodies to specific proteins, it has been possible to begin to identify proteins specifically located in the different organelles. It has also been possible to identify proteins (MIC proteins) that are only present in the micronemes or proteins located in the dense granules (GRA proteins). Indeed, in the case of the rhoptries it has been possible not just to identify proteins located in the rhoptries, but also to differentiate between those located in the bulbous region (the ROP proteins) and those specific to the neck region (the RON proteins) (Bradley *et al.*, 2005).

The nucleus occupies a central or basal location, depending on the invasive stage (see below). It is often flattened on the upper side, where the Golgi apparatus is located. It contains a central nucleolus and small clumps of electrondense heterochromatin scattered throughout the nucleoplasm. The nuclear envelope has numerous nuclear pores, and is covered on its external side with ribosomes, except on the upper face, where the Golgi apparatus is located (Figures 2.1A, 2.1B, 2.23C). The nuclear envelope is in continuity with sheets of rough endoplasmic reticulum that extend into the cytoplasm of the tachyzoite.

On the upper surface of the nucleus a layer of clear vesicles of 70 nm diameter, some of which can be seen budding from the nuclear envelope, is topped by three or four flat Golgi cisternae, on top of which numerous vesicle of various contents and size can be observed (Figures 2.1A, 2.1B, 2.23B, 2.23C).

Using certain preparative technique, one or two vesicles of approximately 200 nm containing one or several electron-dense droplets or crystals of various sizes in a clear background are found near the nucleus or in the posterior part of tachyzoites (Figure 2.1A). These have been termed the acidocalcisomes, and the dark contents are believed to be calcium bound to pyrophosphate and polyphosphates (Luo *et al.*, 2005).

Several mitochondrial profiles of 0.5-µm width and various lengths can usually be observed at various locations above and below the nucleus (Figures 2.1A, 2.1B). These represent sections through a single branched and elongated mitochondrion. They show the typical apicomplexan structure, with bulbous cristae.

Above the Golgi is the apicoplast (Figure 2.1B). This organelle, limited by multiple membranes, has been identified morphologically since the early 1960s (Ogino and Yoneda, 1966; Sheffield and Melton, 1968; Vivier and Petitprez, 1969), but was only recently shown to be a typical plastid (Kohler et al., 1997). Since it appears to be a feature of all members of the Apicomplexa, with the exception of Cryptosporidium sp., the term 'apicoplast' was proposed. In the infectious stage it is relatively uniform in shape, up to 500 nm in diameter, bounded by possibly four membranes, and filled with granular and filamentous content in which ribosomes can be observed. The origin of the four membranes is still a matter of debate but could result from a secondary phagocytosis of an alga already containing an endosymbiont (Kohler, et al., 1997). It has recently been proposed that the 4-membrane structure could result from the extensive invagination of the inner membrane of a double membraned organelle but this requires confirmation (Kohler, 2005).

2.2.2 Comparison of the invasive stages

The infectious stages consisting of the tachyzoite, bradyzoite, merozoite, and sporozoite differ from

Life-cycle stage	Nucleus	Micronemes	Rhoptries number	Appearance	Dense granules	Polysaccharide granules
Tachyzoite	Central	Few	5–12	Labyrithine	Numerous	Few
Bradyzoite	Basal	Numerous	5-10	Solid	Numerous	Numerous
Merozoite	Central	Few	3–5	Solid	Few	Absent
Sporozoite	Basal	Numerous	5–10	Labyrithine	Numerous	Numerous

TABLE 2.1 Summary of the morphological differences between stages of T. gondii

each other in the number of apical organelles, the shape and electron-density of the rhoptries, the location of the nucleus, and the presence or absence of polysaccharide granules. The nucleus is more centrally located in the tachyzoite (Figure 2.1A) and merozoite (Figure 2.11B) and more basally located in the bradyzoite (Figure 2.23B) and sporozoite (Figure 2.21B). An additional cytoplasmic structure not described above is the polysaccharide granule. The polysaccharide granules are ovoid structures (250-180 nm) of variable electron-density located in both the apical and basal cytoplasm. They contain an unusual form of carbohydrate which is biochemically more similar to plant amylopectin than animal glycogen (Coppin et al., 2005). These granules are rarely found in tachyzoites or merozoites, but are present in large numbers in sporozoites and bradyzoites (Figures 2.21B, 2.23B). The granules appear to represent a stored energy source, which would be consistent with a possible requirement for the long-term survival of the bradyzoites and sporozoites or the extra energy needs during transmission between hosts. The most marked variations are in the apical organelles (see review by Dubey et al., 1998). There are relatively few micronemes in the tachyzoite and merozoite, but these are more numerous in the bradyzoite and sporozoite. In the case of the dense granules, these are numerous (5-12) in the tachyzoite and sporozoite, with fewer in the bradyzoite and very few (2-3) in the merozoite. In the case of the rhoptries, there are differences in the number, shape, and electron-density between stages. The number

of rhoptries is relatively similar (5–12) for the tachyzoite, bradyzoite, and sporozoite, with fewer in the merozoite (3–5). The rhoptries in the tachyzoite and sporozoite appear to have an elongated swelling with a labyrithine appearance, in comparison with the more bulbous and electron-dense swelling in the merozoite and bradyzoite. These differences are summarized in Table 2.1.

2.2.3 Host-cell invasion

Observations of T. gondii invasion have been performed in cell cultures and on red blood cells (which appear to be a possible, although unusual, abortive host-cell for this parasite) (Michel et al., 1979). Invasion is operated by a moving junction, which has the same morphological features as the one described for Plasmodium knowlesi (Aikawa et al., 1981), both in thin section and in freeze fracture. Interestingly, T. gondii makes the same junction with nucleated cells and with red blood cells (Porchet-Hennere and Torpier, 1983). It is a very close apposition of the parasite and host plasma membrane (Figures 2.3B, 2.4B), with thickening of the host side, and an accumulation of rhomboidally organized intramembranous particles on the protoplasmic face of the host plasma membrane (Figure 2.3C). This forms a very tight junction, which excludes small electron-dense tracers such as Ruthenium Red. The molecular organization of the moving junction is still unclear, but recent data have shown that it involves proteins derived from the rhoptry neck in association with



FIGURE 2.3 Host-cell invasion in vitro.

- (A) Serial section though the apical area of a tachyzoite at an early stage of Hela cell invasion. The moving junction is covering the apex of the tachyzoite (arrow); an empty rhoptry (eR) has exocytosed its contents in the neighboring host-cell cytoplasm as small vesicles (v). Bar = 0.1 μm.
- (B) Freeze-fracture image of the apical area of an invading tachyzoite at a stage corresponding to Figure 2.3A. The typical structure of the moving junction in the protoplasmic face (Pv) of the host-cell plasmalemma (which will turn into the parasitophorous vacuole membrane) is visible (arrows) below the parasite apical exoplasmic plasmalemmal face (Ee). HC, host cell. Arrow lower right represents angle of shadowing. Bar = $0.1 \mu m$.
- (C) Freeze-fracture image of the apex of an invading tachyzoite at a similar stage of invasion as Figure 2.3B, but corresponding to the complementary fracture faces, showing the pit (arrow) in the parasitophorous vacuole membrane (Ev, exoplasmic face) covering the site of rhoptry exocytosis in the tachyzoite plasmalemma (Pe, protoplasmic face). Arrow lower right represents angle of shadowing. Bar = 0.2 µm.



FIGURE 2.4 Host-cell invasion in vitro.

- (A) Freeze fracture of an invading tachyzoite showing the parasitophorous membrane (Ev), a clump of membrane whorls that may correspond to material exocytosed from the rhoptries (asterisk), and the plasmalemma of the tachyzoite (Pe). HC, host cell. Arrow lower left represents angle of shadowing. Bar = 0.5 µm.
- (B) Section through an invading tachyzoite showing the moving junction (arrows) and the continuity and the difference in electron density between the host-cell plasmalemma (HCM) and the parasitophorous vacuole membrane (PVM). N, nucleus. Bar = 0.2 µm.

the microneme protein AMA1 (Alexander *et al.*, 2005; Lebrun *et al.*, 2005).

Microneme exocytosis has never been clearly visualized, although it is thought to occur during both gliding motility and invasion; what has been shown is the accumulation of alignment of small, clear vesicles inside the conoid in conditions of chemically triggered microneme exocytosis, as though these dense, rod-like organelles gave rise to these small vesicles before or after exocytosis (Carruthers and Sibley, 1999). The docking site for microneme exocytosis is not known.

Rhoptry exocytosis is easily documented upon invasion, as an apical opening in continuity with the parasite plasma membrane, facing the developing parasitophorous vacuole membrane (PVM) (Nichols *et al.*, 1983). Freeze fracture shows an open pit in the PVM at that location, suggesting continuity between rhoptry contents and PVM or even host-cell cytoplasm (Figure 2.3A). The role of the apical vesicle and that of the apical rosette of intramembranous particles located at the rhoptry exocytosis site has never been elucidated, but the rhoptries open precisely at this location and the IMP rosette disappears, just as reported for trichocyst exocytosis in *Paramecium* sp. (Beisson *et al.*, 1976).

At very early stages of invasion, when the moving junction forms, small vesicles can be seen budding from the developing vacuole or laying in the host-cell cytoplasm (Figures 2.3A, 2.4A). At this stage, empty rhoptries are already observed. Therefore, these vesicles correspond to the physiological counterpart of the evacuoles, which are the product of frustrated rhoptry exocytosis in the host-cell cytoplasm when invasion is blocked by cytochalasin D (Hakansson *et al.*, 2001).

The membrane of the developing vacuole is completely devoid of intramembranous particles (Figure 2.4A) (Dubremetz, *et al.*, 1993), reflecting the selective exclusion of the intramembranous host-cell proteins at the moving junction. However, it will acquire IMPs during the first hour of development (Porchet-Hennere and Torpier, 1983), likely due to parasite contribution, especially from dense-granule protein translocation in the PVM (Dubremetz *et al.*, 1993). Progression of the moving junction along the zoite is sometimes (but not always) correlated with parasite constriction.

2.2.4 Parasitophorous vacuole, intracellular development

Within minutes after closure of the parasitophorous vacuole, the posterior part of the parasite invaginates and the tubulo-vesicular network (TVN) starts developing in this invagination (Sibley *et al.*, 1995). The origin of the TVN is not fully understood: it contains dense granule proteins that are exocytosed from the anterior end of the parasite, this exocytosis beginning before the completion of the invasion process (Dubremetz *et al.*, 1993). The origin of the tubular material itself, which is likely made of phospholipids, has never been elucidated; what is known is that the GRA2 protein is required to organize this network (Mercier *et al.*, 2002), and that these tubules are in direct continuity with the PVM, although these two structures contain distinct dense-granules derived proteins (Cesbron-Delauw, 1994).

Immediately after invasion, host-cell mitochondria and endoplasmic reticulum surround the PV and persist throughout the intracellular development (see Figure 2.8). The rhoptry protein ROP2, exocytosed during invasion, has been shown to anchor the host mitochondria to the PVM (Sinai and Joiner, 2001). The host ER is devoid of ribosomes on the side facing the vacuole. The distance between these organelles and the PV is highly conserved, and is about 12 nm and 18 nm for mitochondria and ER respectively (Sinai *et al.*, 1997). The PVM-associated mitochondria may look normal but sometimes show morphological changes, with the cristae becoming larger and irregular in shape and the stroma becoming electron-dense.

The parasitophorous vacuole described above is formed by actively invading parasites and is characterized by the absence of the fusion of the host-cell lysosomes, thus protecting the parasite during intracellular development (Jones and Hirsch, 1972; Jones *et al.*, 1972). This probably relates to the exclusion of the host-cell intramembranous proteins from the parasitophorous vacuole membrane during invasion. In contrast, parasites within vacuoles formed by host-cell phagocytosis exhibit lysosome fusion, and the parasites are broken down in typical phagolysosomes (Jones and Hirsch, 1972).

2.2.5 Endodyogeny

The tachyzoite is unique in it ability to undergo indefinite proliferation by a distinctive process termed 'endodyogeny', which involves parasite growth and division to form two daughters. Despite grossly resembling binary fission, endodyogeny is a highly complex event, related to the structural complexity of the formation of polarized daughters. In contrast with the canonical asexual division mode of most Apicomplexa and even the coccidian stages of *T. gondii*, the tachyzoite retains the apical complex until the end of the endodyogeny. Although both events occur simultaneously, we will describe mitosis and daughter formation successively.

2.2.5.1 Mitosis

There have been few descriptions of T. gondii mitosis at the ultrastructural level, and what has been observed can be interpreted by comparison with more detailed studies in related Apicomplexa, especially Eimeria spp. (Dubremetz, 1973). One unique feature of apicomplexan mitosis is the retention of an intact nuclear membrane throughout the process of division. Coccidian-type centrioles (150 nm diameter) consist of nine short tubules (100 nm long) centered on a central tubule. Centrosomes or spindle pole bodies are made of two centioles oriented in parallel (Figure 2.5B). Centrosomes are always found associated with centrocones or mitotic spindle poles, usually on the apical side of the nucleus. The earliest stage of mitosis is a transnuclear funnel containing fibrous material, corresponding to an invagination of the nuclear envelope opened on both sides towards the cytoplasm (Figure 2.5A). The mitotic spindle most likely polymerizes in this funnel, which then opens in the nucleoplasm in its middle part, whereas the poles give rise to the centrocones. The centrocones are at first subspherical invaginations of the nuclear envelope opened towards the centrosomes and through which the spindle microtubules extend. The intranuclear spindle is usually very short and transient, and has rarely been described. What occurs most likely is that the kinetochores are separated immediately after the funnel opening, and assemble on the nucleoplasmic side of the centrocones. Indeed, in Coccidia, caryokinesis does not depend on mitotic spindle elongation. Centrocones soon become conical evaginations of the nuclear envelope, opened on

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FIGURE 2.5 Tachyzoite endodyogeny.

- (A) Early stage of mitosis: the mitotic spindle elongates into a cytoplasmic funnel through the nucleus (arrow), between the centrioles (Ce). G, golgi body; Mi, mitochondrion. Bar = 0.5 μm.
- (B) Early stage of daughter zoite genesis where a dense fiber (arrow) extends between the centrosome (Ce, centriole) and the newly formed conoid, (C). The apical part of the inner membrane complex (imc) and subpellicular microtubules have started developing. Bar = $0.2 \mu m$.
- (C) Centrocone (ct) budding off the nuclear envelope (Ne) between a centriole (Ce) and three kinetochores (arrows) in an early stage of daughter zoite formation. R, rhoptry. Bar = 0.2 µm.

the centrosomes, and covered on the nucleoplasmic side with a layer of multilayered structures corresponding to the kinetochores (Figure 2.5C). What is specific to this stage is that each round of mitosis occurs simultaneously with the development of two daughter individuals.

2.2.5.2 Zoite biogenesis

Soon after the centrosomes separate and centrocones are formed, the future apical complex of each daughter tachyzoites starts to develop adjacent to each centrosome. The details of this biogenesis have not been studied as thoroughly as in *Eimeria* spp.(Dubremetz, 1975), but follow the same scheme (Vivier and Petitprez, 1969; Vivier, 1970; Hu *et al.*, 2002b). A very early stage of development shows a bent fiber originating between the pair of centrioles and joining an area where the conoid is being assembled (Figure 2.5B). The inner membrane complex and underlying subpellicular microtubules array appears to form around the conoid and, in a coordinated manner, starts to grow posteriorly (Figures 2.5C, 2.6A). This occurs within the mother-cell cytoplasm rather than in association with the mother-cell plasmalemma





- (A) Early stage of endodyogeny showing two developing daughters (arrows), with early rhoptries (R). The Golgi body (G) has divided. Only one nuclear pole (ct) and centriole (ce) is present in the plane of the section. A, apicoplast; D, dense granule; imc, inner membrane complex; Mi, mitochondrion, N, nucleus. Bar = 0.5 µm.
- (B) Later stage of endodyogeny where the daughter nuclei (N) have entered the developing zoites. Imc, inner membrane complex. Bar = 1 μ m.

that is characteristic of daughter formation in classical schizogony undergone by most Apicomplexa. Early stages are short, flattened cones above the centrocones (Figure 2.6A), which later elongate into the grossly cylindrical shape that will eventually surround the mature organism (Figure 2.6B).

The Golgi apparatus divides concomitantly with spindle formation, with each newly formed Golgi body being found on the upper nuclear envelope, near each centrocone (Figures 2.5A, 2.6A) (Pelletier et al., 2002). The apicoplast elongates and appears to divide during daughter formation, with a portion entering each daughter. Rhoptry precursors are observed at this time as heterogeneous, irregularly shaped vesicles of about 0.3 µm near the Golgi bodies and within the inner membrane complex (Figure 2.6A). As development proceeds, the nucleus become U-shaped and the developing inner complex elongates and engulfs the daughter nuclei (Figure 2.6B), while additional organelles (rhoptry precursors and then micronemes) are formed anterior to the Golgi bodies. The rhoptry contents condense to eventually acquire their mature labyrinthine appearance, while the rhoptry ducts appear and elongate towards the conoid.

As the daughters grow, the inner membrane complex of the mother cell breaks down along with the anterior organelles. The fully formed daughters fill much of the mother-cell cytoplasm, and their inner membrane complex comes in contact with the mother-cell plasmalemma to form the daughter pellicle (Figures 2.7A, 2.7B). This is initiated at the anterior end and results in the daughters remaining connected via a small portion of residual cytoplasm before finally separating. Repeated rounds of division lead to accumulation of tachyzoites within the vacuole, which may adopt a typical rosette appearance when grown in flat cell (such as human foreskin) fibroblasts (Figure 2.8A).

In certain cases, each of the daughters, while remaining attached by their posterior ends, can undergo a new cycle of endodyogeny (Figure 2.8B). There is evidence for the synchronized division of the tachyzoites within a vacuole (Figures 2.8A, 2.8B). In quantitative studies it was observed that



FIGURE 2.7 Tachyzoite endodyogeny.

- (A) Early budding stage where one of the daughter tachyzoites is protruding out of the mother cell while being enclosed by the mother plasmalemma (arrow). A, apicoplast; G, golgi body; N, nucleus; R, rhoptry. Bar = 0.5 µm.
- (B) Late stage of daughter budding, where the remnants of the mother cell apical complex are still visible (arrow) while the two daughters are almost completely formed. G, golgi body; N, nucleus. Bar = $0.5 \mu m$.

this is more common *in vivo* for avirulent parasites compared to virulent parasites (Ferguson and Hutchison, 1981). This is only observed for tachyzoites but is not seen during division of bradyzoites in tissue cysts.

Endoyogeny is the exclusive form of asexual division undergone within the intermediate host (during tachyzoite and bradyzoite formation), and differs from the processes undergone in the definite host (merozoite formation) or within the oocyst (sporozoite formation) (see later sections).



FIGURE 2.8 Tachyzoites endodyogeny. (A) Typical rosette of intracellular tachyzoite multiplication in adherent cells grown *in vitro* where division occur in one single plane. The vacuole and tubulo vesicular network (tvn) surround the parasites, all of which are in an early stage of endodyogeny (arrows). Host cell mitochondria (arrowheads) surround the parasitophorous vacuole membrane. HCN, host cell nucleus. Bar = 1 μ m. (B) The next round of endodyogeny (arrowheads) may begin before the end of the previous one, when daughter tachyzoites are not fully separated (arrow).

2.3 COCCIDIAN DEVELOPMENT IN THE DEFINITIVE HOST

2.3.1 Host–parasite relationship

Coccidian development is limited to the epithelial cells of the small intestine of the cat (the definitive host). In all stages undergoing coccidian development, the parasites are located within a tight-fitting,



FIGURE 2.9 *Toxoplasma gondii* developing in enterocytes of the cat intestine.

- (A) Early developmental stage located in a thickwalled, tight-fitting parasitophorous vacuole (PV). N, nucleus. Bar = $1 \mu m$.
- (B) Enlargement showing the laminated structure of the electron-dense membrane (PVM) limiting the parasitophorous vacuole. Note a conical structure protruding into the membrane (arrow). P, parasite pellicle. Bar = $0.1 \mu m$.
- (C) Tangential section through the membrane of the parasitophorous vacuole illustrating the circular shape of the conical protrusion in to the parasitophorous vacuole membrane (arrows). Bar = $0.1 \,\mu$ m.
- (D) Detail in which the membrane of the vacuole can be resolved into three unit membranes (arrowheads). Bar = $0.1 \,\mu$ m.
- (E) Mid-stage schizont with a number of nuclei (N), and a centrally located elongated apicoplast (A). Mi, mitochondrion. Bar = $1 \mu m$.
- (F) Detail showing the double membranes enclosing the mitochondrion (Mi) and nucleus (N), compared to the multiple membranes enclosing the apicoplast (A). Bar = $0.5 \mu m$.

thick walled parasitophorous vacuole (Figure 2.9A) (Ferguson et al., 1974; Ferguson, 2004). At higher power the wall has a laminated appearance, which in certain areas can be seen to consist of three closely applied unit membranes (Figure 2.9D). In addition there are a number of conicalshaped, dense structures impinging on the luminal surface of the PV (Ferguson, 2004) which, in certain cases, appear to connect the surface of the parasite to the parasitophorous vacuole membrane (Figures 2.9B). In contrast to the host-parasite relationship of the tachyzoite, there is no evidence of formation of the tubular structure within the PV or the congregation of the host-cell mitochondrion or strands of rER around the periphery of the PV (Figure 2.9A). These structural differences correlate with the lack of expression of the majority of dense granule proteins. Of the GRAs 1-8 and NTPase identified in the tachyzoite, only GRA7 and NTPase are expressed by the gut stages (Ferguson et al., 1999a, 1999b; Ferguson, 2004). This laminated, thickwalled PV is similar to that observed for certain Isospora species (Ferguson et al., 1980) to which T. gondii is closely related, but differs from those of the genus Eimeria, which are limited by a singleunit membrane (Ferguson et al., 1976).

2.3.2 Asexual development

During coccidian development only a single asexual process has been observed, which has unique structural features and has been termed endopolygeny (Piekarski et al., 1971). This term had been used previously to describe an abnormal type of development observed for the tachyzoite (Vivier, 1970). However, the abnormal tachyzoite development described did not represent an internal budding process. Therefore, because of the accuracy of the description and its usage over the years, it would appear appropriate to retain the term for the description of the asexual multiplication of the coccidian stages. In studies of coccidian development of both type I and type II strains of T. gondii occurring between 4 and 10 days p.i. only a single process was observed, although there are marked variations in the number of daughters formed. The process involves growth of the parasite and repeated nuclear divisions (Figure 2.9E) employing an excentric intranuclear spindle, as described during endodyogeny. There is also a marked increase in the size of the mitochondria, which are located predominately around the periphery. In addition it is possible to observe multiple profiles of the apicoplast (limited by four membranes), but these were more centrally located and, from immunocytochemistry, appeared to consist of a single branched structure (Ferguson et al., 2005). These three organelles can be differentiated by their ultrastructural features (Figure 2.9F). The number of nuclear divisions varies between parasites, which has a direct effect on the number of daughters formed. It is the presence of this proliferative phase prior to daughter formation that distinguishes endopolygeny from endodyogeny. It is not clear how the number of nuclear divisions is controlled, but it does not appear to relate to parasite size or a given number of nuclear divisions, since these can vary markedly between parasites (cf Figures 2.10A, 2.10C).

The trigger for the end of the proliferative phase and the initiation of the differentiation phase (daughter formation) is unclear. It is at the end of the proliferative phase that the elongated apicoplast divides simultaneously into a number of fragments equal to the number of nuclei (Ferguson et al., 2005). Daughter formation can occur at any time between the 4- and approximately 20-nuclei stage, and is initiated during or just after the final nuclear division (Figures 2.10A, 2.10B). The first evidence of the initiation of daughter formation is the appearance of a conical structure formed by a number of flattened vesicles each with underlying longitudinally running microtubules and with the conoid in the apex (Figure 2.10B). The initiation of daughter formation is synchronized with all daughters forming at the same time (Figures 2.10A, 2.10C). The mechanism of daughter formation is similar to that observed for the two daughters formed during endodyogeny of the tachyzoite or bradyzoite. Since this occurs at a multi-nucleated stage, numerous daughters are formed – thus the appropriateness of the term endopolygeny. The simultaneous formation of a large number of daughters requires an extremely well-coordinated process to ensure that all daughters receive a full complement



- (A) Small schizont with few nuclei (N) but showing the initiation of daughter formation (D). Bar = $1 \mu m$.
- (B) Detail of a schizont showing the plate-like structures of the inner membrane complex representing the initiation of daughter formation (arrows). Ce, centriole; N, nucleus; NP, nuclear pole; G, Golgi body. Bar = $0.5 \,\mu$ m.
- (C) Low-power image of a large schizont with a number of nuclei showing the formation of a larger number of daughters. Bar = 1 μ m.
- (D) Detail showing the posterior growth on the inner membrane complex of the daughter to partially enclose the apicoplast (Å), and nucleus (N). Note the anlagen of the rhoptry (R), and the conoid (C), in the apex of the daughter. Bar = $0.5 \,\mu$ m.

of organelles and are therefore viable. As daughter formation progresses by the posterior growth of the inner membrane complex, it encloses a nucleus, apicoplast, and mitochondrion (Figure 2.10D). In the apical cytoplasm, one or two electron-dense spherical structures representing the nascent rhoptries (Figure 2.10D) and a number of cigar-shaped micronemes appear. The merozoites have relatively few dense granules, and these appear to form late in daughter development. This posterior growth continues until the merozoite is fully formed and contains the full complement of organelles. In the apical cytoplasm there is maturation of the rhoptries, with the development of the duct leading to the conoid. In contrast to the tachyzoite and bradyzoites, the bulbous end of the rhoptry remains spherical. At this point the daughters fill the mother-cell cytoplasm but are still enclosed in the schizont plasmalemma. The final stage is the invagination of the mother-cell plasmalemma, starting at the anterior of the daughter and progressing posteriorly to form the outer membrane of the pellicle of each daughter (Figure 2.11A). A single micropore is formed in the pellicle just anterior to



FIGURE 2.11

- (A) Late schizont showing the daughters filling the mother cell cytoplasm and the partial invagination of the plasmalemma around the daughters (arrows). N, nucleus; R, rhoptry; MN, microneme. Bar = $1 \mu m$.
- (B) Mature schizont with fully formed merozoites containing the characteristic apical organelles. N, nucleus; C, conoid; R, rhoptry; DG, dense granule. Bar = 1 μm.
- (C) Scanning electron micrograph of a fracture through an intestinal villus from an infected cat. A number of small trophozoites (Tr) and two mature schizonts with crecentic shaped merozoites (arrows) can be seen within the epithelial cells. Bar = $2 \mu m$.

the nucleus. The merozoites often remain attached to a small amount of residual cytoplasm at the posterior end. These banana-shaped daughters can be seen forming fan-like structures (Figures 2.11B, 2.11C). The merozoites are released from the host cell into the lumen, where they can reinvade enterocytes. However, this process has not been observed by electron microscopy.

Unlike many *Eimeria* species, there does not appear to be the distinct sequential generations of schizogony, which differ from each other in their size and number of daughters formed. However, in studies of the early stages of infection (1-3 days), additional asexual processes have been described (Speer and Dubey, 2005). It has been reported that certain developing parasites have a similar host-parasite relationship and undergo endodyogeny and repeated endodyogeny (type B schizonts), while others appeared intermediate (type C schizonts). The type B schizonts have a similar relationship to that described for parasites invading the small intestine of the intermediate host (Dubey, 1997; Speer and Dubey, 1998). These stages appear to be rare, and could represent examples where the initial invading bradyzoite failed to undergo conversion to coccidian development but underwent conversion to tachyzoite development, as seen in the intermediate host. It is known that the cat can act as an intermediate host as well as the definitive host.

2.3.3 Sexual development

After an unknown number of asexual cycles, certain merozoites on entering a new enterocyte can develop into either male (microgametocyte) or female (macrogametocyte) gametocytes. In microgametogony this results in the formation of multiple (15–30) male gametes (microgametes) and in macrogametogony the formation of a single female gamete (macrogamete). The trigger for the conversion from asexual to sexual development is unknown; nor is it known what is responsible for deciding whether an invading merozoite develops into either a microgametocyte or macrogametocyte.

The initiation of gametocyte formation appears to be less controlled in *T. gondii* than in other species of Coccidia. In the majority of *Eimeria* spp. there is a fixed number of sexual cycles followed by the vast majority of merozoites simultaneously developing into sexual stages. In *T. gondii* there does not appear to be a distinct conversion, with a mixture of both asexual and sexual stages observed throughout enteric development. There is no ultrastructural feature that could identify the merozoites that will develop into sexual stages, nor are there any differences in the host–parasite relationship or parasitophorous vacuole.

On entering the host cell the merozoite becomes more spherical and loses the majority of its apical organelles, such as the rhoptries and dense granules, although the conoid and a few micronemes remain. This stage (trophozoite) begins to grow and there appears to be an increase in the size of the mitochondrion/mitochondria, which are located round the periphery. However, at this stage it is not possible to differentiate between organisms that will develop into micro- and macro-gametocytes.

2.3.3.1 *Microgametogony and the microgamete*

There are relatively few descriptions of microgametogony (Colley and Zaman, 1970; Pelster and Piekarski, 1971; Ferguson et al., 1974; Dubey et al., 1998). Initially is impossible to differentiate between the proliferative phase of endopolygeny and microgametogony, with both processes involving continued growth and repeated nuclear divisions. It has been reported that the earliest stage allowing differentiation between schizogony and microgametogony is based on differences in the distribution of the nuclear chromatin (Ferguson et al., 1974). During schizogony the electron-dense heterochromatin remains dispersed throughout the nuclei (Figure 2.9E), whereas in the later stages of microgametogony the heterochromatin condenses into electron-dense masses at the periphery of the nucleus (Figure 2.12A). In microgametogony, the nuclei move to the periphery of the cell with two centrioles and a dense plaque (perforatorium) located between the nuclei and the plasmalemma (Figure 2.12A). The centrioles become

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FIGURE 2.12 Electron micrographs illustrating various stages in the process of microgametogony.

- (A) Mid-stage microgametocyte showing the peripherally located nuclei containing areas of condensed chromatin (arrows). The centriole (Ce) and the plate-like perforatorium (P) can be seen located between the nuclei (N) and the plasmalemma. Bar = $1 \mu m$.
- (B) Detail showing the protrusion the flagella (F) plus the dense portion of the nucleus and a mitochondrion (Mi) into the PV. Bar = $0.5 \mu m$.
- (C) Late stage showing a number of microgametes forming in the PV while still attached to the mother cell (arrows). N, nucleus; F, flagellum. Bar = $1 \mu m$.
- (D) Detail from Figure 2.12C showing elongating nucleus (N) and mitochondrion (Mi) of the microgamete still connected to the mother cell (arrows). F, flagellum. Bar = $0.5 \,\mu$ m.

the basal bodies for the developing flagella, which begin to grow by protruding into the parasitophorous vacuole (Figure 2.12B). Interestingly, although the centrioles differ from metazoan centrioles, the flagella have the typical nine peripheral doublet tubules with the two central microtubules. As this flagellar growth occurs, the chromatin condensation continues at the side of the nucleus closest to the centrioles, with the other part of the nucleus having a more electron-lucent appearance. In addition, a mitochondrion is located adjacent to each nucleus (Figure 2.12B). There is no significant development in the apicoplast during this process (Ferguson et al., 2005). Microgamete development continues with flagellar growth and protrusion of a portion of cytoplasm containing the basal bodies, the electron-dense portion of the nucleus, and a mitochondrion into the lumen of the PV (Figures 2.12B, 2.12C, 2.12D). As this occurs there is division of the nucleus, with the electron-dense portion separating from the electron-lucent portion. The electron-dense portion enters the developing microgamete, and the lucent portion remains within the mother cell as a residual nucleus. The microgametocyte of T. gondii produces relatively few (15-30) microgametes (Figure 2.12C). The immature microgametes are still attached to the mother cell by a narrow cytoplasmic isthmus (Figures 2.12C, 2.12D).

Maturation continues with each microgamete becoming elongated in appearance and consisting of an electron-dense nucleus with a mitochondrion located between the nucleus and the basal bodies from which the two very long flagella run toward the posterior (Figures 2.13A, 2.13B). In addition there is an electron-dense plate termed the perforatorium in the apex and a number (four) of longitudinally running microtubules (Ferguson *et al.*, 1974). Once fully formed the microgametes detach from the microgametocyte, leaving a large residual cytoplasmic body.

2.3.3.2 *Macrogametogony and the macrogamete*

The development of the macrogametocyte has been described in a few studies (Colley and Zaman, 1970; Pelster and Piekarski, 1972; Ferguson *et al.*, 1975). It is associated with growth of the trophozoite and the appearance of a large nucleus with



FIGURE 2.13 The structure of the mature microgamete.

- (A) Longitudinal TEM section through a microgamete showing the electron-dense nucleus (N) and the anterior mitochondrion (Mi) and the basal bodies of the two flagella (F). Bar = 1 μ m.
- (B) SEM of a microgamete illustrating the nucleus (N) and the two very long, posteriorly pointing flagella (F). Bar = 1 μ m.

dispersed chromatin and a large nucleolus but no nuclear division. As the macrogametocyte grows there is a marked increase in the size of the peripherally located mitochondrion and the centrally located apicoplast (Figure 2.14A). In addition, a number of Golgi bodies are distributed throughout the cytoplasm. The first distinct organelle of macrogametogony is the appearance of flocculent material condensed within dilatations of the rER (Figures 2.14B, 2.14D). This material represents the initiation of formation of the wall-forming body type 2 (WFB2), such bodies being so called because of their role in the formation of the oocyst wall (see below). A Golgi body is often associated with the membrane of ER surrounding the wall-forming bodies type 2. As maturation continues, there is an increase in size and number of the wall-forming bodies type 2, and a number of electron-dense membrane-bound granules appear to form from vesicles produced by the Golgi bodies (Figure 2.14C). These are of various sizes, and are termed wall-forming bodies type 1 (WFB1) (Figure 2.14D). However, it has been



FIGURE 2.14 Various stages in the development of the macrogametocyte.

- (A) Early macrogametocyte characterized by the central nucleus (N) with a large nucleolus (Nu). The cytoplasm contains peripheral profile of an enlarged mitochondrion (Mi) and an enlarged Golgi body (G). A few polysaccharide granules (PG) and lipid droplets were present in the cytoplasm. Bar = 1 μ m.
- (B) Mid-stage macrogametocyte showing increased numbers of polysaccharide granules (PG) and lipid droplets (L), the appearance of wall-forming bodies type 1 (W1) and type 2 (W2) in the cytoplasm, and an increase in size of the apicoplast, A. Bar = 1 μ m.
- (C) Mature macrogamete showing the centrally located nucleus with adjacent apicoplast, A. The cytoplasm contains numerous wallforming bodies type 1 (W1) and a few type 2 (W2), plus numerous polysaccharide granules (PG) and lipid droplets (L). Bar = 2 μm.
- (D) Detail of the cytoplasm of a mature macrogamete showing the numerous dense granules representing the wall-forming bodies type 1 (W1). The wall-forming body type 2 (W2) is located within the rough endoplasmic reticulum. PG, polysaccharide granule; L, lipid droplet. Bar = 0.5 μm.

possible, using immuno-electron microscopy, to identify two populations of membrane-bound electron-dense granules (Ferguson *et al.*, 2000). One population appears to be involved in the formation of the outer veil, and consists of what are termed the veil-forming bodies (VFBs). These were originally termed wall-forming bodies type 1a, but, with the identification of similar granules in the macrogametocyte of Eimeria maxima, it is proposed that VFBs may be a more appropriate term (Ferguson et al., 2003). The wall-forming bodies type 1 appear slightly larger than the veilforming bodies. As the veil- and wall-forming bodies are being synthesized, there is also the synthesis of numerous polysaccharide granules and lipid droplets and an expansion of the apicoplast (Figure 2.14C). When fully developed, the macrogametocyte can be considered to be a mature macrogamete (Figure 2.14C). This is not a sharp division but one of convenience to differentiate the developing stage from the mature gamete.

2.3.4 Oocyst wall formation

The oocyst wall is a multi-layer structure which is extremely resistant to physical and chemical insults. As such, it is fundamental to the survival of the parasite. Without this wall, the parasite could not survive for extended periods in the external environment required for transmission between hosts resulting from fecal contamination. The oocyst wall is a complex structure consisting of distinct layers (Ferguson et al., 1975; Speer et al., 1998), and is synthesized while the macrogamete is still within the host cell. In reviewing these data with reference to later observations for both T. gondii and Eimeria spp., the wall can be divided into three zones. The first is a loose outer veil consisting of two or three membranes (termed layers 1-3; Ferguson et al., 1975), which is formed by the fusion of the veil-forming bodies with the macrogamete plasmalemma and release of their contents (Ferguson et al., 2000). This occurs during the maturation of the macrogamete. This is followed by the triggered secretion of the WFB1, which occurs simultaneously in the mature macrogamete to form the outer layer of the oocyst wall, (Figure 2.15A) (layer 4; Ferguson et al., 1975). This initially forms a thick layer that undergoes polymerization to form a 30-70-nm electron-dense layer. Finally, the contents of the WFB2 are released



FIGURE 2.15

- (A) Early stage of oocyst wall formation showing the outer veil (V) and partial formation of the outer layer of the oocyst wall (arrows). Note this is associated with the loss of the VFB and the WFB1 from the macrogamete cytoplasm, while the WFB2 (W2) remain. L, lipid droplet; PG, polysaccharide granule; N, nucleus. Bar = 1 μ m.
- (B) Newly released oocyst showing the outer veil (V) and fully formed oocyst wall (OW) enclosing a cytoplasmic mass containing polysaccharide granules (PG) and lipid droplets (L). Bar = $1 \mu m$.
- (C) SEM showing a number of microgametes (Mi) apparently attached to a macrogamete/ oocyst (Ma) with two adjacent merozoites (Me). Bar = 1 µm.
- (D) Detail of the oocyst wall consisting of the outer veil (V) plus the thin, electron-dense outer layer (O) and the thicker inner layer (I), which is separated from the plasmalemma (P) of the cytoplasmic mass. Bar = 100 nm.

and coalesce to form the electron-lucent inner layer of the oocyst wall (layer 5; Figures 2.15B, 2.15D) (Ferguson *et al.*, 1975). The cytoplasmic mass loses the WFBs during oocyst wall formation and is characterized by a central electron-lucent nucleus and cytoplasm packed with polysaccharide granules and lipid droplets (Figure 2.15B).

This process is identical to that described for the closely related genus Eimeria (Ferguson et al., 2003). For correct formation of the oocyst wall there is a requirement for tight control and sequential secretion of the veil-forming bodies and the wall-forming bodies 1 and 2. From the available data for Coccidia it would be most accurate to consider the outer veil as part of the early development, as it is lost by the time oocysts are released with the feces. The oocyst proper can be considered as a double-layered structure (reviewed by Belli et al., 2006). The outer electrondense layer is thinner in the T. gondii oocyst (Figure 2.15D) than in those of Eimeria spp. (Belli et al., 2006). The formation and polymerization of the inner layer has a dramatic effect on the ability to process the oocyst for ultrastructural examination. To date, no technique has been developed that will allow the oocysts of T. gondii or any other coccidian oocyst to be examined by electron microscopy. Over the past 30 years, numerous attempts, using many electron microscopic fixatives and embedding protocols, have resulted in failure. The two layers provide different structural and chemical protection. The outer layer contains mostly proteins and carbohydrate, and appears to provide structural strength. In contrast, the inner layer has a high lipid content and appears to provide the protection from chemical insult by its impervious nature (even to electron-microscopy reagents). Work on the properties of the oocyst is continuing in the closely related genus Eimeria (Belli et al., 2006).

2.3.5 Fertilization

It would appear logical that if sexual development takes place, there will be fusion between a microgamete and a macrogamete to form a fertilized zygote. However, this process has never been visualized. It could be expected that the mature microgametes and macrogametes are released from the host cells and fertilization takes place in the lumen. Indeed, macrogametes/oocysts with attached microgametes have been observed on rare occasions (Figure 2.15C) (Ferguson, 2002). However, as has been described above, oocyst wall formation is initiated prior to release of the macrogamete from the host cell. An additional anomaly in T. gondii is the formation of very few microgametocytes, so there are relatively few microgametes in relation to the number of macrogametes. It is a universal feature of plants and animals that there is a vast excess of male gamete formation because of the importance of ensuring maximum fertilization of the female gametes. That fertilization can occur has been proven from the identification of cross-fertilized parasites (Pfefferkorn and Pfefferkorn, 1980). However, T. gondii, unlike most other metazoans, is normally haploid, and whether this will affect the necessity for fertilization is open to question (Ferguson, 2002).

2.3.6 Oocyst and extracellular sporulation

The oocyst is the only stage of T. gondii that is capable of undergoing extra-cellular development - all other development processes can only occur within viable nucleated host cells. The oocysts are excreted in an unsporulated form with a single undifferentiated cytoplasmic mass - the primary sporoblast (Figure 2.16A). In the external environment asexual development (sporulation) occurs, which finally results in the formation of two sporocysts, each of which contains four sporozoites. Initial attempts to study this process were unsuccessful because of the inability to process the oocyst for ultrastructural examination. It was only possible to overcome this problem by developing a technique that involved freezing and cryosectioning of the oocysts prior to processing for electron microscopy (Birch-Andersen et al., 1976). The aim was to fracture the oocyst wall without destroying the cytoplasmic mass within. This technique is inefficient, with destruction of a large proportion of oocysts; however, a few oocysts remain intact and these are used to examine the ultrastructural changes associated with sporulation. Due to these difficulties, studies have been limited to a series of papers on the sporulation of E. brunetti (Ferguson et al., 1978a, 1978b) as a model for the genus Eimeria and T. gondii



- (A) Section through an unsporulated oocyst (zero hours) showing the central nucleus (N) with cytoplasm containing a Golgi body, mitochondria and a number of polysaccharide granules (PG) and lipid droplets (L). OW, oocyst wall. Bar = 1 μm.
- (B) Early stage in sporulation, with the cytoplasmic mass containing a number of nuclei (N). PG, polysaccharide granules; L, lipid droplets. Bar = 1 μm.
- (C) Section through an oocyst in which the cytoplasmic mass has started to divide (arrows) to form the two secondary sporoblasts. Note the two nuclei (N) in one of the forming sporoblasts. PG, polysaccharide granules. Bar = $1 \,\mu m$
- (D) Section through the two secondary sporoblasts. N, nucleus; L, lipid droplet; PG, polysaccharide granule. Bar = $1 \mu m$.

(Ferguson *et al.*, 1979a, 1979b, 1979c). The quality of the ultrastructural observations was limited due to the technical difficulties, but the developmental process could be followed.

The original central cytoplasmic mass, termed the primary sporoblast, was similar to that of the macrogamete (*cf* Figures 2.15B and 2.16A). The cytoplasm contains a single large nucleus plus numerous polysaccharide and lipid droplets admixed with mitochondria and rER enclosed by a unit membrane (Figure 2.16A). In *T. gondii*, the nucleus underwent two rounds of division giving rise to four nuclei (Figure 2.16B). The cytoplasmic mass then underwent elongation and became limited by two additional membranes. This was followed by cytokinesis of the cytoplasmic mass with the formation of centrally located infoldings of the limiting membranes (Figure 2.16C), which finally fused, thus dividing the primary sporoblast into two spherical secondary sporoblasts each with two nuclei (Figure 2.16D). This process is summarized diagrammatically in Figure 2.17.

As each secondary sporoblast develops it becomes more elongated or cigar-shaped and develops into the sporocyst, which is characterized by the formation of the sporocyst wall (Figures 2.18A, 2.18D, 2.18E).

In *T. gondii* the wall of the sporocyst appears to be formed by material secreted from the cytoplasm, which condenses on one of the limiting membranes. It forms a distinctive structure comprised of four plates (Figures 2.18D, 2.18E) joined by specialized sutures with an overlaying thin layer of electrondense material (Figures 2.18B, 2.19A). This wall has various banded striations which could be consistent with organized repetitive protein structures



FIGURE 2.17 A diagrammatic representation of the changes observed during the development of the zygote and formation of the sporoblasts. Ce, centriole; ER, rough endoplasmic reticulum; G, Golgi body; L, lipid droplet; MI, mitochondrion; MP, micropore; N, nucleus; NP, nuclear pole; NU, nucleolus; OW, oocyst wall; PG, polysaccharide granule; V, vacuole. Reproduced from Ferguson *et al.*, 1979a, with permission.

that probably provide structural strength and increase resistance to external insult (Figure 2.19B).

Within the cytoplasm of the developing sporocyst a nucleus was observed at either end of the elongated sporocyst, with the majority of the cytoplasm containing polysaccharide granules and lipid droplets (Figure 2.18A). It was observed that the anlagen of two daughters formed adjacent to the plasmalemma above each nucleus at either end of the sporocyst (Figure 2.18C). The process of daughter formation was similar to that observed during endodyogeny, with the nucleus appearing to divide during the posterior growth of the inner membrane complex of each daughter and two daughters forming at either end of the sporocyst (Figure 2.18C). This inner membrane growth continued until the daughters were fully formed and enclosed a nucleus, apicoplast, and mitochondrion, and the apical organelles (micronemes, rhoptries, and dense granules). This formation of the daughters adjacent to the sporocyst plasmalemma differs from the internal formation associated with endodyogeny or endopolygeny. In this situation, it was observed that the plasmalemma invaginated with the growth of the inner membrane complex to form the sporozoite pellicle, and in this respect is similar to classical schizogony. This resulted in the formation of four daughters (two from each end). A small residual cytoplasmic mass remained within each sporocyst. The process of sporulation is represented diagrammatically in Figure 2.20.

2.3.7 Excystation

There have been few ultrastructural studies on the process of excystation (Christie *et al.*, 1978; Ferguson *et al.*, 1979d; Speer *et al.*, 1998). In certain studies the oocyst wall was broken mechanically by grinding, although it has been reported that reasonable excystation can occur without this process (Speer *et al.*, 1998). Excystation is stimulated by incubation in a mixture of trypsin and bile salts (sodium taurocholate). This excystation fluid



- (A) Early development of the sporocyst showing the elongated appearance with a nucleus (N) located at either end of the sporocyst and the cytoplasm containing polysaccharide granules (PG) and lipid droplets (L). Bar = 1 μ m.
- (B) Cross-section through the sporocyst wall which consists of a thin, continuous outer layer (O) and an inner layer (I) consisting of four plates. There is a swelling of the plates of the inner layer at the junction where they are joined by an intermediate strip (IS) of material. Bar = 100 nm.
- (C) Enlargement of part of a sporoblast showing the nucleus (N) and the two dense plaques (arrows) representing the initiation of daughter formation. Bar = 1 μ m.
- (D) Advanced stage of sporozoite formation (SP) showing the nucleus becoming enclosed by the inner membrane complex of the daughters. The junction between the four plates of the sporocyst wall can be seen (arrows). Bar = $1 \mu m$.
- (E) Scanning electron micrograph illustrating the raised junctions between the plates (arrows). Bar = $1 \mu m$.

appears to act on the sporocyst wall, causing increased tension, which results in an infolding of the edges of the plates along the suture lines (Figures 2.21A, 2.21B). At the sutures there is a separation of the inner aspect of the inner layer of the sporocyst wall, which initially remains attached at the outer edge (Figure 2.21C). This connection eventually ruptures, and with it the outer membrane of the sporocyst wall (Figure 2.21D). There appears to be rapid separation of the plates and infolding to form scroll-like structures, allowing the sporozoites to escape (Figure 2.21E).

2.4 DEVELOPMENT IN THE INTERMEDIATE HOST

2.4.1 Tachyzoite development

When an intermediate host is infected by ingestion of tissue cysts or oocysts, the bradyzoites and sporozoites are released into the lumen of the small intestine. These invade the enterocyte or intra-epithelial lymphocytes of the small intestine, or pass through into the lamina propria and invade cells there. The process of invasion has not been observed, but is likely to be similar to that described previously for tachyzoites (see section 2.2.3). In either case, the parasite (bradyzoite or sporozoite) defaults to tachyzoite development with formation of the characteristic parasitophorous vacuole and undergoes multiplication by endodyogeny (see section 2.2.5). This process has been described in detail by Speer and Dubey (Dubey et al., 1998; Speer and Dubey, 1998). From in vitro studies it was originally proposed that the sporozoite entered a host cell and formed an enlarged PV, which it then left to form a second vacuole within which it underwent tachyzoite development (Speer et al., 1995). However, this was not observed in their in vivo studies, and could represent an in vitro artefact (Dubey et al., 1997). Thereafter, the tachyzoites disseminated systemically via the vascular system to all organs of the body. In the various organs they undergo proliferation by endodyogeny in many cell types, and are present initially in large numbers in the lungs and spleen, but by 6-10 days have invaded



FIGURE 2.19 A diagrammatic representation of (A) the cross-sectional and (B) the three-dimensional appearance of the sporocyst wall at the junction between the four plates which form the wall. The suture consists of a swelling of the adjacent plates forming the inner layer of the sporocyst wall (I) separated by a thin strip of intervening material (IS) which is connected to the plates by electron dense material toward the outer aspect (arrows). The suture is covered by the continuous thin outer layer (O) of the sporocyst wall. Reproduced from Ferguson *et al.*, 1979b, with permission.

all organs including the brain. However, in immunocompetent mice (genetically resistant to *Toxoplasma*) the number of lesions and tachyzoites peaked at about 12 days, and by 21 days it was difficult to identify tachyzoites in any organ, including the brain, even using immunocytochemistry.

2.4.2 Stage conversion: tachyzoite to bradyzoite

There appears to be very marked tissue tropism in relation to the organs where the majority of tissue cysts are formed. The two tissues where the



FIGURE 2.20 A diagrammatic representation of the changes observed during development of the sporocysts and formation of the sporozoites. C, conoid; ER, rough endoplasmic reticulum; G, Golgi body; L, lipid droplet; MI, mitochondrion; MN, microneme; MP, micropore; N, nucleus; NP, nuclear pole; P, plaque representing the inner membrane complex of the developing daughter; PL, pellicle; PG, polysaccharide granule; R, rhoptry; RB, residual body of cytoplasm; SW, sporocyst wall; V, vacuole. Reproduced from Ferguson *et al.*, 1979c, with permission.



- (A) Scanning electron micrograph of a sporocyst undergoing excystation, showing the separation of the plates of the sporocyst wall (arrows). Bar = $1 \mu m$.
- (B) Transmission electron micrograph through an excysting sporocyst, showing separation and infolding of the plates of the sporocyst wall (arrows). The sporozoites (SP) contain a posteriorly located nucleus (N) and numerous micronemes (MN) and polysaccharide granules (PG). C, conoid. Bar = 1 μ m.
- (C) Early stage in excystation showing inward curling (arrow) of the sporocyst wall at the junction of the plates of the inner layer (I), resulting in a separation of the plates and the intermediate strip (IS). Bar = 100 nm.
- (D) More advanced stage of excystation showing separation of the the inner plates and rupture of the outer layer (arrow). Bar = 200 nm.
- (E) Example of the continued curling of the plates of the sporocyst wall to form tightly wound, scroll-like structures. Bar = 100 nm.

majority of tissue cysts are observed are striated muscles, including the heart and the central nervous system. However, this can vary between species. For example, the majority of tissue cysts are located in the musculature in pigs (Dubey, 1986), but predominantly in the brains of mice. This again contrasts with the *in vitro* situation, where almost any cell type can act as a host cell during stage conversion. This again emphasizes the need for caution when extrapolating from *in vitro* results.

Stage conversion has been examined in detail in mice. At 12-15 days following oral infection, lesions are observed in the brain that consist of numbers of parasites undergoing tachyzoite development admixed with parasites forming early tissue cysts (Figure 2.22A). It is observed that only a small subpopulation of the tachyzoites underwent conversion. The lesions consist of numbers of extracellular tachyzoites plus a few intracellular organisms located in typical tachyzoite-like PVs (Figures 2.22B, 2.22D). These can often be identified as inflammatory cells, which form part of the lesion. However, it is also possible to identify a number of early tissue cysts (Figures 2.22C, 2.22E). A number of cysts are seen within the lesion, and indeed it is possible to observe two cysts forming within the one host cell. These can be differentiation from tachyzoite-like vacuoles by the distinctive structure of the PV and PMV enclosing parasites, which could be identified at the one- to two-cell stage (Figure 2.22E). The distinctive PVs appear to form at the time of invasion. This is also consistent with the immunocytochemical results using the stagespecific antibodies (SAG1 and ENO2 for the tachyzoites and BAG1 and ENO1 for bradyzoites). The parasitophorous vacuoles of early tissue cyst are characterized by their tight fit and being limited by a membrane with numerous irregular shallow invaginations (Figure 2.22E). These vacuoles lack the tubular network, but possess a thin layer of amorphous material. In addition, there is no congregating of the host-cell mitochondrion or rER around the vacuole. When examined by immunoelectron microscopy, it is observed that the material beneath the membrane reacts positively to the cyst wall protein recognized by the antibody CC2. It has been reported that a small subpopulation of the tachyzoites from peritoneal exudates contained lucent cytoplasmic granules which were positive with CC2 (Gross et al., 1996).



- (A) Low magnification of a section through the brain of a mouse at 15 days post-infection showing a lesion containing parasites undergoing stage conversion. Note the formation of a number of early tissue cysts (Cy) and the group of tachyzoite-like organisms (T). Bar = 5 μ m.
- (B) Part of a cell in the brain showing a tachyzoite (T) within a typical tachyzoite parasitophorous vacuole with its tubular network (TN). Bar = 1 μ m.
- (C) Example of a very early tissue cyst containing two parasites within a tight-fitting vacuole (PV). N, nucleus. Bar = $1 \mu m$.
- (D) Detail from Figure 2.22B showing the strand of rough endoplasmic reticulum (rER) associated with the parasitophorous vacuole membrane characteristic of a tachyzoite containing vacuole. Bar = 100 nm.
- (E) Detail from Figure 2.22C showing the undulating parasitophorous vacuole membrane (arrows) and the absence of host-cell organelles associated with the membrane consistent with early cyst formation. HC, host cell. Bar = 100 nm.

It is interesting to speculate that there is a subpopulation of tachyzoites which, on reaching the correct environment, may be pre-programmed and are able directly to initiate tissue cyst formation. These tissue cysts continue to enlarge over the next three weeks with the bradyzoites dividing by endodyogeny (Figure 2.23A). Initially a large number of the parasites are undergoing endodyogeny, but the proportion of dividing organisms reduces during the first 28 days post-infection, and from 3 months onward very few dividing parasites are seen (Ferguson and Hutchison, 1987a). During this early development the cyst enlarges and the depth of the invaginations increases, and a more distinct underlying amorphous layer forms (Figure 2.23E). Within the early tissue cyst the bradyzoites still have similar ultrastructural features to the tachyzoites, particularly rhoptries, with a labyrinthine appearance (Ferguson and Hutchison, 1987b). This shows that although the structure is that of a tissue cyst and the expression of marker molecules (BAG1, ENO1, LDH2) is that of bradyzoites, the specific ultrastructural features lag behind. It is often 21-28 days before typical bradyzoites can be identified. In addition, many of the dividing parasites had more electron-lucent cytoplasm with few organelles (Figure 2.23A).

2.4.3 Structure of the tissue cyst and bradyzoite

The structure of the mature tissue cyst observed from 3 to 24 months following infection (approximately the lifespan of the mouse) remained relatively unchanged (Figure 2.23D). The first important observation is that throughout this period the tissue cysts are retained within a viable host cell (Figures 2.23D, 2.23E). It had originally been thought that the mature cysts are extracellular; however, on ultrastructural examination a thin rim of host-cell cytoplasm could be observed enclosing the tissue cysts (Ferguson and Hutchison, 1987b). This may explain the lack of an immune response to the tissue cysts; they are masked by the host cell. With the limited hostcytoplasm available, it is difficult to identify the cell type; however, in the majority of cases the host



- (A) Part of the periphery of an early tissue cyst (21 days post-infection) showing the cyst wall (CW) and the thin rim of host-cell (HC) cytoplasm. The cyst contains a number of bradyzoites (Br) and electron-lucent organisms undergoing endodyogeny (arrows). Bar = $1 \mu m$.
- (B) Longitudinally sectioned bradyzoite showing the posteriorly located nucleus (N), numerous polysaccharide granules (PG), plus dense granules (DG), rhoptries (R), micronemes (MN), and conoid (C). Bar = 1 μm.
- (C) Detail of the region just anterior to the nucleus of the bradyzoite in Figure 2.23B, showing the Golgi body (G), and the apicoplast (A). PG, polysaccharide granule. Bar = 100 nm.
- (D) Low-power image of a mature tissue cyst (1 year post-infection) showing a large number of bradyzoites (Br) enclosed by a cyst wall (CW) retained within a host cell (HC). Bar = $10 \mu m$.
- (E) Detail from the periphery of the cyst in Figure 2.23D, showing the cyst wall (CW) with deep invaginations of the limiting membrane of the cyst into the underlying granular material. Note that the host cell (HC) can be definitively identified as a neuron because of the presence of synapses (S). Bar = 100 nm.

cells could be identified as neurons because of the defining presence of synapses (Figure 2.23E). It is not possible to identify the other host cells, although their features are consistent with neurons. There are variations in the thickness of the cyst wall between tissue cysts, with some showing deep invaginations of the limiting

membrane forming a complex of interconnecting channels all embedded in homogeneous granular material. There is also some vesicle formation on the inner aspect (Figure 2.23E) (Ferguson and Hutchison, 1987a). In the mature cysts, the bradyzoites appear more elongated than the tachyzoites, with a posteriorly located nucleus (Figure 2.23B). There are numerous micronemes and few dense granules, although this is variable. The rhoptries have more bulbous ends and are uniformly electron-dense. However, the major difference is the presence of numerous polysaccharide granules (Figures 2.23B, 2.23D).

The majority of tissue cysts appear as single structures, but it is possible to find small groups of tissues cysts of different sizes. It has been suggested, from the very early studies (Lainson, 1958), that this could represent daughter cyst formation resulting from the escape of individual bradyzoites to form new cysts. However, extensive immunocytochemical and ultrastructural studies have failed to find supporting evidence. It is possible to observe the formation of groups of cysts from as early as 14 days p.i. (Figure 2.22A). In addition, when examined by electron microscopy, the cysts, although of different sizes, appeared to be of similar maturity. The bradyzoites in all the cysts appear as mature organisms with no evidence of the feature described above for immature cysts. In addition, given the immunological response of the host to exposed parasite antigens, it would be expected that any escaped bradyzoite would invoke an inflammatory response (see section 2.4.4).

2.4.4 Inflammatory changes in the brains of infected mice

Toxoplasma infection of the brain is associated with inflammatory changes in and around the meninges and certain blood vessels within the brain (Ferguson *et al.*, 1991). During the acute phase



FIGURE 2.24

- (A) Small blood vessel from the brain of a chronically infected mouse showing a number of monocytes (M) and plasma cells (P) cuffing the vessel and also a plasma cell in the neuropil. Bar = $10 \mu m$.
- (B) Light micrograph of the brain of a chronically infected mouse showing numerous inflammatory cells within the meninges and cuffing the blood vessels (arrows). Note the tissue cyst (Cy) invokes no reaction. Bar = $20 \mu m$.
- (C) Electron micrograph of a large vessel close to the meninges showing the large number of cuffing monocytes (M) and plasma cells (P). Bar = $10 \mu m$.

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many of the inflammatory cells are lymphocytes and monocytes, with a few polymorphic leukocytes. In chronically infected mice, with numerous tissue cysts in the brain, there is the continuous presence of inflammatory cells, which cuff the small blood vessels within the neuropil and the vessels of the meninges (Figures 2.24A, 2.24B, 2.24C). However, it should be noted that these inflammatory cells show no tropism toward the tissue cysts (Figure 2.24B). In the chronic infections (3–24 months p.i.), the majority of inflammatory cells are plasma cells or monocytes/macrophages (Figure 2.24C). In addition, it is possible to observe numerous plasma cells around the small vessels and free in the neuropil (Figure 2.24A).

2.4.5 Cyst rupture in immunocompetent hosts

One of the clinical problems in immunocompromised hosts is recrudescence of the infection resulting in stage conversion back to actively proliferating and tissue-destructive tachyzoites. To examine the situation in the immunocompetent host, the brains of immunocompetent chronically infected mice have been examined. It is found that, indeed, a very small percentage of tissue cysts are rupturing at any given time during chronic infections (Ferguson et al., 1989). The initial change appears to be the death of the host cell. With the exposure of the parasite antigen in the cyst wall, there is evidence of a rapid and massive cell-mediated immune response involving numerous inflammatory cells (monocytes and even neutrophils). These are observed around the still apparently intact cyst (Figure 2.25A). With the rupture of the cyst wall, there is a further influx of macrophages into the cyst (Figure 2.25C). The macrophages phagocytose the bradyzoites, where there is fusion with lysosomes resulting in the formation of phagolysosomes and destruction of the parasites (Figure 2.25B). This immune response results in the formation of small inflammatory lesions (microglia nodules) with some evidence of host-cell apoptosis but limited hosttissue damage. The bradyzoites appear to be destroyed before they can undergo replication or



FIGURE 2.25

- (A) Low-power image of a tissue cyst with an intact cyst wall (CW) but with loss of the host cell. Note the number of inflammatory cells, monocytes (M), surrounding the cyst. Bar = $10 \,\mu m$.
- (B) Detail from the tissue cyst in Figure 2.25C showing a macrophage with a phagocytic vacuole containing degenerating bradyzoites (Br). Bar = $1 \mu m$.
- (C) Section through a ruptured tissue cyst in an immunocompetent host, showing the fractured cyst wall (CW) partially enclosing the bradyzoites (Br). Note the numerous macrophages (M) surrounding and invading into the tissue cyst and phagocytosing the bradyzoites. Bar = $5 \mu m$.

stage conversion to tachyzoites. These observations have been confirmed by immunocytochemical examination of the lesions.

2.4.6 Development in vitro

It is often stated that *T. gondii* is very easy to culture, and this has made it very popular as a

molecular model. However, it needs to be emphasized that normally only the tachyzoite and tachyzoite development occur in cell cultures. More recently it has been possible to trigger tissue cyst formation, but to date there has not been a single successful attempt to reproduce the development undergone by the coccidian stages.

2.4.6.1 Tachyzoite development in vitro

In vitro, tachyzoites undergo similar development to that described above (sections 2.2.4, 2.2.5), irrespective of the type of host cell used. The host–parasite relationship and the proliferation by endodyogeny and repeated endodyogeny is identical to that described above.

2.4.6.2 Bradyzoite development in vitro

This was first described in astrocytes in the1980s (Jones et al., 1986), and techniques for inducing this stage conversion were identified in the 1990s. It was observed that factors (pH changes, oxygen tension) that induce stress in the culture system appear to stimulate conversion to cyst formation. Unlike the in vivo situation, where there is marked selection of the type of host cell (neurons, muscle cells), in vitro almost any cell type can act as a host cell for cyst formation. In certain in vitro studies (Ferguson, unpublished), the process of cyst formation from the time the parasite entered the host cell is similar to that observed in vivo. However, other studies suggest that there may be some tachyzoite-like development before the vacuole takes on the features of the tissue cyst (Soete et al., 1994).

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Population Structure and Epidemiology of *Toxoplasma gondii*

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3.1 Introduction

- 3.2 Markers for genetic studies
- 3.3 Parasite population genetics
- 3.4 Factors affecting transmission and genetic exchange
- 3.5 Molecular epidemiological studies
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- 3.7 *Toxoplasma* genotype and human disease3.8 Conclusions
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3.1 INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite infecting all warm-blooded animals, with a world-wide distribution. It causes a large range of clinical manifestations in humans, from abortion and congenital infection to eye disease and fatal encephalitis. There are marked biological differences between *Toxoplasma* isolates in terms of their pathogenicity to mice. Most are avirulent, producing chronic asymptomatic infections; however, a few are highly virulent and result in fatal acute toxoplasmosis (Howe *et al.*, 1996). Given the biological and epidemiological diversity of the parasite, high levels of genetic variation may also be predicted, particularly given the potential for meiotic recombination in this protozoan with a well-described sexual cycle (Frenkel *et al.*, 1970; Hutchison *et al.*, 1970).

Multiple genetic markers have been developed to differentiate *Toxoplasma* strains and analyze the parasite population structure, and the consensus of these studies is that the vast majority of isolates belong to two or three clonal lineages (Howe and Sibley, 1995). However, these isolates were predominantly obtained from humans and domesticated species in Europe and North America, and extension of epidemiological screening across a wider geographical and host range reveals higher levels of variation and recombination among some parasite populations (Ajzenberg *et al.*, 2004; Lehmann *et al.*, 2004; Ferreira *et al.*, 2006).
3.2 MARKERS FOR GENETIC STUDIES

Numerous markers have been described for typing of Toxoplasma isolates (Table 3.1). Initially, prior to sequencing of the Toxoplasma genome, methods such as isoenzyme analysis and restriction fragment length polymorphism (RFLP) were used. However, as sequence information became available specific polymorphic markers were identified, and the discovery of these has been greatly facilitated by the development of the Toxoplasma genome project (Ajioka et al., 1998; Manger et al., 1998; http://toxodb.org; http://ToxoMap.wustl. edu/). A wide range of genetic markers is now available, but it should be emphasized that full appreciation of parasite population genetics requires the use of multiple markers in multilocus analysis.

3.2.1 Markers for population genetic studies

3.2.1.1 Isoenzymes

The first studies of strain diversity relied on isoenzymes. Six polymorphic enzymatic systems were characterized in Toxoplasma (aspartate aminotransferase, glutathione reductase, amylase, glucose phosphate isomerase, acid phosphatase, and propionyl esterase), and these were found to exhibit only 2-3 isoforms in a collection of 83 Toxoplasma stocks (Dardé et al., 1988, 1992; Dardé, 1996; Ajzenberg et al., 2002a). This allowed the description of 12 zymodemes within this collection. The majority of stocks clustered into three main zymodemes Z1, Z2, and Z3. Zymodeme 4 was closely related to zymodeme 2, differing by only one allozyme. The designation of zymodemes Z1, the Z2-4 cluster, and Z3 were later demonstrated to be consistent with the three main lineages, type I, type II, and type III respectively, defined by multilocus analysis of coding and non-coding DNA (Howe and Sibley, 1995; Dardé, 1996). Other zymodemes (Z5-12) found were represented by single isolates, each of which were later defined with additional markers to be atypical or recombinant strains.

The value of multilocus enzyme electrophoresis as a tool for genetic analysis has been demonstrated for many parasites (Gardener and Howells, 1972; Sargeaunt and Williams, 1979; Miles *et al.*, 1980). The technique gives a good level of resolution, but its main handicap for *Toxoplasma* is that it requires large numbers of purified parasites (approximately 7×10^6 tachyzoites per enzyme). This is particularly difficult for slow-growing isolates, and it may take up to 2 months of repeated passage to obtain sufficient quantities of tachyzoites for analysis (Dardé *et al.*, 1992; Dardé, 1996).

3.2.1.2 Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) have been detected by sequencing of single copy genes, and now constitute the most abundant group of genetic markers. They have been found in genes coding for major antigens located on the surface of the parasite, in dense granules or rhoptries, or within the matrix of the cyst (Sibley and Boothroyd, 1992; Parmley et al., 1994; Rinder et al., 1995; Meisel et al., 1996; Windeck and Gross, 1996; Fazaeli et al., 2000a; Grigg et al., 2001a; Tinti et al., 2003; Khan et al., 2005a). SNPs have also been detected in genes encoding structural proteins, such as α - and β -tubulin or actin (Lehmann et al., 2000); in those encoding enzymes, such as dihydrofolate reductase, dihydropteroate synthase, thymidilate synthase, nucleoside triphosphatase and DNA polymerase alpha (Asai et al., 1995; Biñas and Johnson, 1998; Aspinall et al., 2002); and in genes of unknown function, such as 850, 950, L328, 62B, 226, C19, and B1 (Sibley and Boothroyd, 1992; Grigg and Boothroyd, 2001).

More recently, Khan *et al.* (2005a) have designed 200 new SNP-RFLP markers which, together with 50 existing markers, map to approximate 300-kb intervals across the 14 chromosomes of *T. gondii* genome. These were used to look at genetic variation and recombination in the progeny of type I, II, and III strains. In common with earlier work by Grigg *et al.* (2001a), they found that in the three predominant

MARKERS FOR GENETIC STUDIES

P	henotypic or genetic markers	References
•	Isoenzymes: aspartate aminotransferase, amylase, propionyl esterase, glucose phosphate isomerase, glutathione reductase, acid phosphatase	Dardé <i>et al.</i> , 1992; Dardé, 1996
•	Genes coding for major antigens: surface antigens: <i>SAG1, SAG2, SAG3, SAG4, SAG5,</i> <i>MAG1, BSR4, SRS1, SRS2, SRS3, SRS4</i>	Sibley and Boothroyd, 1992; Rinder <i>et al.</i> , 1995; Lehmann <i>et al.</i> , 2000; Grigg <i>et al.</i> , 2001a; Tinti <i>et al.</i> , 2003
	organite antigens: <i>GRA1, GRA2, GRA3, GRA4, GRA6, ROP1</i>	Sibley and Boothroyd, 1992; Parmley <i>et al.</i> , 1994; Meisel <i>et al.</i> , 1996; Fazaeli <i>et al.</i> , 2000a; Grigg <i>et al.</i> , 2001a
	nucleoside triphosphatase: NTPase	Asai <i>et al.</i> , 1995
•	Other genes with known function: actin (<i>ACT1</i>), α-tubulin (<i>TUB1</i>), β-tubulin (<i>TUB2</i>), <i>B10</i> , dihydrofolate reductase (<i>FOL1</i>), <i>DPHS</i>	Lehmann <i>et al.</i> , 2000; Aspinall <i>et al.</i> , 2002
	DNA polymerase alpha (POL1)	Biñas and Johnson, 1998
	Intergenic spacer (IGS)	Fazaeli <i>et al.</i> , 2000b
•	Genes with unknown function: <i>850</i> , <i>950</i> , <i>L328</i> , <i>62B</i> , <i>226</i> , <i>C19</i> , <i>B1</i>	Sibley and Boothroyd, 1992; Grigg and Boothroyd, 2001
•	Microsatellites: low polymorphism: β-tubulin (<i>TUB2</i>), myosin-A (<i>TgM-A</i>), W35, B17, B18 high polymorphism:	Costa <i>et al.</i> , 1997; Ajzenberg <i>et al.</i> , 2004
	 ESTs (<i>N60608</i>, <i>N82375</i>, <i>N83021</i>, <i>N61191</i>, <i>AA519150</i>) M6, M33, M48, M95, M102, M163 <i>cB21-4</i> 	Ajzenberg <i>et al.</i> , 2002a Blackston <i>et al.</i> , 2001
•	Sequences repeated in the genome: <i>BS</i> TGR <i>REP</i> (mobile genetic elements)	Sibley and Boothroyd, 1992 Cristina <i>et al.</i> , 1991; Høgdall <i>et al.</i> , 2000 Terry <i>et al.</i> , 2001

TABLE 3.1 Genotypic and phenotypic markers used in studies of genetic polymorphism of

 Toxoplasma gondii; numerous other polymorphisms were recently detected by Khan et al., 2005a

strains of *Toxoplasma* each locus contained only two alleles (Grigg *et al.*, 2001a; Su *et al.*, 2003). These markers clearly have great potential for mapping genes associated with phenotypic traits such as virulence in experimental and epidemiological studies.

SNPs are detected by DNA sequencing. This can be used directly as a typing method. Multilocus sequencing, based on a large number of loci, is one of the best approaches to detect polymorphism and to analyze the structure of a population. However, as sequencing is expensive and timeconsuming, PCR-RFLP methods are generally developed following detection of polymorphic restriction sites for endonucleases. PCR-RFLP is less informative than sequencing, as it supposed that all isolates will fall into the pattern of restriction sites primarily described for a few strains. For example, a high level of polymorphism was detected by *GRA6* sequence analysis (9 allelic sequences among 30 stocks), but the *GRA6* PCR-RFLP method was only capable of differentiating 3 groups (Fazaeli *et al.*, 2000a). Despite this fact, the PCR-RFLP of single-copy genes remains the most commonly used method for typing *T. gondii* isolates (Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Howe *et al.*, 1997; Biñas and Johnson, 1998). Although the method is rapid and easy to use, technical problems – such as incomplete amplification of *SAG2* gene (Fuentes *et al.*, 2001) or incomplete digestion of the DNA by restriction enzymes (Lehmann *et al.*, 2000) – may limit its reproducibility.

The most commonly used specific marker is the *SAG2* gene polymorphism analyzed by PCR-RFLP using two restriction sites (Howe and Sibley, 1995). This was the first method described to differentiate the three main genotypes of *T. gondii*. Strain-typing strategies relying entirely on the *SAG2* locus were adopted in many studies (Howe *et al.*, 1997; Owen and Trees, 1999; Fuentes *et al.*, 2001; Dubey *et al.*, 2002), leading to the misidentification of atypical or recombinant strains.

3.2.1.3 Microsatellites

Microsatellite sequences are short tandem repeats of two to six nucleotides. They are known to be hypervariable due to the accumulation of length mutations by intra-allelic polymerase slippage on microsatellite sequence during replication. This makes them very informative neutral markers, well suited for individual identification of isolates. They are considered to be rapidly evolving sequences. In T. gondii some microsatellite markers appear to evolve rather slowly, as demonstrated by the relatively low number of alleles detected and their stability in a large number of isolates of the three main Toxoplasma strain types (Costa et al., 1997; Ajzenberg et al., 2004, 2005). The data obtained with these microsatellites correlate well with the patterns observed via multilocus PCR-RFLP on single copy genes (Ajzenberg et al., 2002a, 2004). However, microsatellites are more polymorphic than current SNP markers, and therefore give greater resolution in genetic population studies. To date, 13 microsatellites and 1 minisatellite have been described for genotyping *Toxoplasma* isolates (Costa *et al.*, 1997; Blackston *et al.*, 2001; Ajzenberg *et al.*, 2002a, 2002b, 2004, 2005; Lehmann *et al.*, 2003, 2004; Sreekumar *et al.*, 2003). They are located either in the introns of known genes (*TUB2* coding for β -tubulin, *TgM-A* coding for myosin-A) or in Expressed Sequence Tags.

Microsatellite polymorphism can be evaluated by PCR (which requires only a small amount of DNA), and allele sizing can be achieved with fluorescent primers and an automatic sequencer, giving highly reliable results. Sequencing of the microsatellite and its flanking regions can confirm that the length variation is due to variation in the number of nucleotide repeats, and can also lead to detection of SNPs either in the microsatellite itself or in its flanking region (Ajzenberg *et al.*, 2004). A multiplex PCR for five microsatellites allowing multilocus analysis of isolates following a single PCR amplification was recently described (Ajzenberg *et al.*, 2005).

3.2.1.4 RAPDs

A random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) uses arbitrary primers to generate DNA fragments that can discriminate T. gondii strains. This analysis has been used to discriminate mouse-virulent strains and nonvirulent strains (Guo and Johnson, 1995; Guo et al., 1997; Ferreira et al., 2004), but the low stringency conditions used can lead to difficulties in interpretation. Simple sequence repeat anchored-PCR (SSR-PCR), using a single microsatellite primer, is an alternative approach which has better reproducibility due to the higher stringency conditions used (Ferreira et al., 2004). Neither of these methods can be used for direct typing of clinical specimens as host DNA may also be amplified, obscuring the profile produced by the parasite.

3.2.1.5 Serotyping

A rather different approach for strain typing and for population genetic study is the use of synthetic peptides derived from polymorphic sites of the genes coding for *Toxoplasma* antigens (mainly *GRA6* and *GRA7*) (Kong *et al.*, 2003). The detection of antibodies against these peptides would allow identification of the parasite strain via serotyping, with no need for strain isolation or DNA extraction. The method has not yet been applied to epidemiological studies.

3.2.2 Markers for individual characterization of stocks and isolates

Several strategies have been proposed for fingerprinting of isolates, to allow epidemiological tracking, or identification of laboratory lines. These use either multiple-copy genes or an association of several rapidly evolving markers in order to give sufficient resolution to identify isolates beyond the lineage level.

3.2.2.1 Multiple-copy loci

Several techniques have used multiple-copy loci to detect a high polymorphism among Toxoplasma isolates. For example, probing a total genomic DNA digest with a ³²P-labelled BS probe generates a stock-specific pattern for a laboratory strain such as RH (Sibley and Boothroyd, 1992; Howe and Sibley, 1994; Messina et al., 1996). The BS probe detects a family of elements containing a series of 90 GAA minisatellite repeats flanked by moderately related, but non-identical, sequences (Messina et al., 1996). Similarly, TGR sequences (Cristina et al., 1991, 1995), a family of repeated DNA sequences analyzed by PCR-RFLP, revealed 10 different patterns in a population of 22 Toxoplasma isolates (Literák et al., 1998), and sequencing of the amplified products showed that each isolate had its own unique TGR sequence (Høgdall et al., 2000).

The *REP* elements are segments of repetitive DNA that transpose with relatively high frequency in the *Toxoplasma* genome and that are flanked by two distinct repeats, found in both direct and inverted formation (Terry *et al.*, 2001). This peculiar structure allows them to be amplified by a single primer PCR. Banding patterns observed after PCR of *REP* elements are identical for all mouse-virulent isolates, but all mouseavirulent isolates had different banding patterns. The fingerprinting of isolates allowed by these markers can facilitate epidemiological studies on human and animal outbreaks.

3.2.2.2 Multilocus analysis with more polymorphic microsatellite markers

Another approach for the individual characterization of isolates is multilocus typing using several polymorphic markers. For instance, the discriminatory power of an association of 8 microsatellite markers was 0.997 in a population of 83 Toxoplasma stocks, as calculated by Simpson's index (maximum = 1.0), indicating that, with the typing method used, nearly all stocks have a different genotype (Ajzenberg et al., 2002a). The high discriminatory power of these 8 microsatellite markers makes them highly appropriate for epidemiological studies of T. gondii, and for ratification of laboratory strain genotype. One added advantage of these markers is that they can be used for detection of mixed infections, even if they are caused by isolates belonging to the same lineage. Here, microsatellite typing is superior to analysis of multicopy loci (Ferdig and Su, 2000). As T. gondii is haploid, only one peak (i.e. only one length of the considered microsatellite after electrophoresis of the amplified product) is expected for a given locus corresponding to one allele. More than one peak will be detected if mixed infections with different alleles are present in the sample (Ajzenberg et al., 2002a). A pyrosequencing method (based on SAG2 polymorphism alone) was recently proposed to detect these mixed infections (Sreekumar et al., 2005).

3.3 PARASITE POPULATION GENETICS

3.3.1 Classical biases in population genetics

3.3.1.1 Number of loci and chromosome location

Clearly, only restricted inferences can be made from analysis of individual polymorphic markers. Detection of recombination events and interpretation of parasite population structure requires multilocus genotyping. The sensitivity of this analysis increases with the number of markers used, but it is important to remember that the inheritance of alleles from two parental strains may be disproportionate in recombinants. For example, strain CL19, the progeny of a type II \times type III cross, bore only 7 type II alleles in a total of 71 loci analyzed (Sibley et al., 1992). CL19 could easily be misidentified as type III strain rather that a II/III recombinant if only a small number of markers were used. Similarly, genotyping of a number of recombinant I/III strains with five microsatellites and six isoenzymes shows that they have inherited 10 type I alleles but only one type III allele (microsatellite TgM-A) (Ajzenberg et al., 2002a, 2004). These strains had previously been placed in zymodeme 1 (= type I) by isoenzyme analysis alone (Dardé, 1996).

Two mechanisms - independent assortment and crossing-over - lead to genetic recombination during meiosis. The independent assortment, which is the random separation of paternal and maternal homologous chromosomes, can only be detected by markers located on different chromosomes. The crossing-over, which is the exchange of genetic material by non-sister chromatids of paternal and maternal homologous chromosomes, is detected by markers located on the same chromosome. However, the amount of crossing-over between any two loci on a chromosome is proportional to the distance between them (if they are close then crossing-over is rare; if they are farther away there is more crossing-over). Thus, only markers far apart on the same chromosome must be used. Altogether, in population genetics, a choice of markers should include markers on different chromosomes together with markers far apart on the same chromosome. The mapping of a large numbers of polymorphic sites in T. gondii facilitates the choice of appropriate markers (Khan et al., 2005a).

3.3.1.2 Sampling of isolates

One of the most important prerequisites for understanding population structure is appropriate unbiased sampling of isolates (Dardé, 2004). In a parasite like Toxoplasma, which has such extensive distribution over a wide range of hosts and habitats, this criterion is difficult to meet. The majority of studies are based on the analysis of small numbers of isolates originating from specific host groups over a restricted geographical range. These studies may appear to give insights into local interactions between parasites and their hosts, but there is the risk that associations are simply a reflection of sample bias. Until recently, the majority of Toxoplasma isolates originated from Europe (mostly France) and North America (Howe and Sibley, 1995; Dardé, 1996; Ajzenberg et al., 2002a). Although isolates from a focal area may be of interest in estimating the extent of recombination, without bias due to geographical distance (Tibayrenc et al., 1991), wider biogeographical sampling is needed to appreciate the extent of strain variation and genetic exchange.

In addition to the geographical bias, these isolates were mostly collected from symptomatic toxoplasmosis in humans (congenital toxoplasmosis, or reactivation in immunocompromised patients) and domestic or peridomestic animals (mostly sheep, pigs, chickens, cats). Again, bias in the host origin of isolates prevents any overview of parasite population structure.

Over time this bias is being redressed, and studies have extended over a wider geographical and host range. A large number of studies based on a monolocus typing (SAG2) concern isolates obtained from chickens in Brazil (Dubey et al., 2002, 2003a, 2003b), Argentina, Colombia, Venezuela, Guatemala, Peru, Mexico, the West Indies, Israel, Egypt, Africa, and Sri-Lanka (Dubey et al., 2003c, 2003d, 2004a, 2004b, 2004c, 2005a, 2005b, 2005c, 2005d, 2005e, 2005f, 2005g), from Brazilian cats, pigs, dogs, and humans (Dubey et al., 2004d; Da Silva et al., 2005; de A Dos Santos et al., 2005; Vallochi et al., 2005), and from wildlife species in North America (Dubey et al., 2004e, 2004f) (see Table 3.2). These isolates constitute an important resource for better understanding of the genetic structure of Toxoplasma. The question arises as to whether this expanded collection of isolates exhibits the same restricted genetic variation and a clonal population structure

Text continued on page 59

	Geographical origin	Number of isolates	Host origin	Typing method	Type I	Type II	Type III	Atypical or recombinant	Reference
	Europe								
Multilocus typing	France	86	Human	5 microsatellites	8%	85%	2%	5%	Ajzenberg <i>et al.</i> , 2002b
	Portugal	15	Pigs	<i>SAG2</i> PCR-RFLP; 5 microsatellites		73.3%	26.7%		de Sousa <i>et al.,</i> 2006
Monolocus typing	Austria	67	Chicken	SAG2 PCR-RFLP	0	100%	0	NA ^a	Dubey <i>et al.,</i> 2005b
	France	90	Human	SAG2 PCR-RFLP	15%	77%	8%	NA	Honoré <i>et al.,</i> 2000
	France	37	Human	1 microsatellite		100%		NA	Costa <i>et al.,</i> 1997
	France	68	Human	SAG2 PCR-RFLP	10%	81%	9%	NA	Howe <i>et al.,</i> 1997
	Great Britain ^b	32	Human	SAG2 PCR-RFLP + sequencing	31%	34%	3%	NA	Aspinall <i>et al</i> ., 2002
	Great Britain	13	Sheep	SAG2 PCR-RFLP		100%		NA	Owen and Trees, 1999
	Portugal		Chicken	SAG2 PCR-RFLP	33.3%	66.7%			Dubey <i>et al.</i> , 2006
	Spain	25	Human	SAG2 PCR-RFLP	40%	40%	20%	NA	Fuentes <i>et al.,</i> 2001
	Spain	34	Sheep	SAG2 PCR-RFLP	8.8%	85.3%	5.9%	NA	Fuentes <i>et al.,</i> 2004a
	Spain	55	Human	SAG2 PCR-RFLP	25%	62%	7%	NA	Fuentes <i>et al.,</i> 2004b
	Spain	26	Cats	SAG2 PCR-RFLP	15%	85%		NA	Montoya <i>et al.,</i> 2004
	USA								
Multilocus typing	USA (California	a) 13	Sea otters	<i>SAG2, SAG1</i> PCR-RFLP		46%		54% (type X)	Cole <i>et al.</i> , 2000
	USA (California	a) 35	Sea otters	SAG1, SAG3, GRA6, B1 PCR-RLFP + sequencing		40%		60% (type X)	Miller <i>et al.</i> , 2004

 TABLE 3.2 Geographical and host species distribution of Toxoplasma genotypes

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PARASITE POPULATION GENETICS

	Geographical origin	Number of isolates	Host origin	Typing method	Type I	Type II	Type III	Atypical or recombinant	Reference
	USA	25	Pigs	SAG2 PCR- RFLP + 6 microsatellites		20%	80%		Lehmann <i>et al.,</i> 2003
	USA (Iowa)	43	Pigs	<i>SAG2, SAG1</i> PCR-RFLP		81.4%	9.3%	9.3%	Mondragon <i>et al.</i> , 1998
Monolocus typing	USA (Ohio, Massachusetts)	19	Chicken	SAG2 PCR-RFLP		26.3%	73.6%	NA	Dubey <i>et al.</i> , 2003e
	USA	36	Wild animals	SAG2 PCR-RFLP	5.5%	86%	8%	NA	Dubey <i>et al.</i> , 2004e
	USA (Mississippi, South Carolina)	11	Wild animals	SAG2 PCR-RFLP		18.8%	81.8%	NA	Dubey <i>et al.</i> , 2004f
	South America								
Multilocus typing	Brazil (Minas Gerais)	20	Dogs, chickens, humans	8 loci, PCR-RFLP				100% (R) ^c	Ferreira <i>et al.</i> , 2006
	French Guiana	12	Human	5 microsatellites				100%	Ajzenberg <i>et al</i> ., 2004
Monolocus typing	Argentina	9	Chicken	SAG2 PCR-RFLP	11%	11%	78%	NA	Dubey <i>et al</i> ., 2003d
	Argentina	17	Chicken	SAG2 PCR-RFLP	23.5%	17.6%	58.8%	NA	Dubey <i>et al</i> ., 2005f
	Brazil (Parana)	13	Chicken	SAG2 PCR-RFLP	54%		46%	NA	Dubey <i>et al</i> ., 2003b
	Brazil (Parana)	37	Cats	SAG2 PCR-RFLP	40.5%		59.4%	NA	Dubey <i>et al</i> ., 2004d
	Brazil (Rio de Janeiro)	48	Chicken	SAG2 PCR-RFLP	70%		27%	NA	Dubey <i>et al</i> ., 2003a
	Brazil (São Paulo)	25	Chicken	SAG2 PCR-RFLP	68%		32%	NA	Dubey <i>et al</i> ., 2002

 TABLE 3.2 Geographical and host species distribution of Toxoplasma genotypes—cont'd

	Brazil (Sao Paulo)	7	Pigs	SAG2 PCR-RFLP	71.4%		28.5%	NA	de A dos Santos <i>et al.</i> , 2005
	Brazil	9	Dogs	SAG2 PCR-RFLP	44.4%		55.5%	NA	da Silva <i>et al.,</i> 2005
	Colombia	24	Chicken	SAG2 PCR-RFLP	29.1%		70.8%	NA	Dubey <i>et al.,</i> 2005c
	Colombia	33	Humans, cats, birds	SAG2 PCR-RFLP	93.9%		3%	3%	Gallego <i>et al.</i> , 2006
	Guatemala	8	Chicken	SAG2 PCR-RFLP	37.5%		62.5%	NA	Dubey <i>et al.,</i> 2005e
	Mexico	6	Chicken	SAG2 PCR-RFLP	16%		83%	NA	Dubey <i>et al.</i> , 2004b
	Peru	10	Chicken	SAG2 PCR-RFLP	70%		30%	NA	Dubey <i>et al.,</i> 2004a
	Venezuela	13	Chicken	SAG2 PCR-RFLP		23%	77%	NA	Dubey <i>et al.,</i> 2005f
	Africa								
Multilocus typing	Cameroun, RCA, Senegal	8	Human	5 microsatellites		12.5%		87.5%	BRC ToxoBS group, personal data
Monolocus typing	Mali, Burkina Faso, DRCongo, Kenya	16	Chicken	SAG2 PCR-RFLP	6.25%	75%	18.75%	NA	Dubey <i>et al.</i> , 2005b
	Egypt	21	Chicken, ducks	SAG2 PCR-RFLP		14%	86%	NA	Dubey <i>et al.,</i> 2003c
	Other								
Multilocus typing	Caribbean islands	2	Human	5 microsatellites				3/3	BRC ToxoBS group, personal data
	New Caledonia	2	Human	5 microsatellites			2/2		BRC ToxoBS group, personal data

Continued

 TABLE 3.2 Geographical and host species distribution of Toxoplasma genotypes—cont'd

	Geographical origin	Number of isolates	Host origin	Typing method	Type I	Type II	Type III	Atypical or recombinant	Reference
	Reunion Island	1	Human	5 microsatellites				1/1	BRC ToxoBS group, personal data
Monolocus typing	India	7	Chicken	SAG2 PCR-RFLP		28%	72%	NA	Sreekumar <i>et al.</i> , 2003
	Sri Lanka	12	Chicken	SAG2 PCR-RFLP		50%	50%	NA	Dubey <i>et al.</i> , 2005g
	Israel	19	Chicken	SAG2 PCR-RFLP		89%	11%	NA	Dubey <i>et al</i> ., 2004c
	Grenada, West Indies	36	Chicken	SAG2 PCR-RFLP	13.9%	2.77%	80.5%	NA	Dubey <i>et al</i> ., 2005a

^aNA = not available (monolocus typing). ^bmixed infections (*SAG2* type I and type II): 31%. ^cR = recombinant.

as the original panel of isolates. Unfortunately the preliminary conclusions of all these studies are not valid in term of population genetics, as they use monolocus typing (*SAG2*), which cannot assess genetic diversity and recombination events. Only multilocus studies may answer this question.

To date, 182 isolates of unusual host or geographical origin (i.e. other than European or North American domestic isolates) have been analyzed by multilocus typing (Table 3.2). Most of these were from Brazil (n = 75), but also French Guiana (n = 9) and India (n = 7). The isolates from other parts of the world are very rare: Africa (n = 3), Japan (n = 3), Uruguay (n = 3), Panama (n = 3), Guadeloupe Island (n = 1), Reunion Island (n = 1), Barbados (n = 1), Argentina (n = 1), and Australia (n = 1). Unusual hosts were mostly sea otters (n = 51), but also rodents (n = 7), bears (n = 7), deer (n = 2), monkeys (n = 2), cougar (n = 1), wallaby (n = 1), turkey (n = 1), dove (n = 1), starling (n = 1), Pacific harbor seal (n = 1), and California sea lion (n = 1)(see Table 3.3 for references).

3.3.2 Population structure

3.3.2.1 Clonal population structure

The first studies considering a relatively large population of stocks with several independent genetic markers were conducted with 6 enzymatic systems on 86 stocks (Dardé et al., 1992; Dardé, 1996; Ajzenberg et al., 2002a), and with 6 independent single-copy loci analyzed with PCR-RFLP on 106 stocks (Howe and Sibley, 1995). They were performed on collections comprising wellestablished laboratory strains, such as RH, M7741, C56, Me49, Prugniaud, and more recent isolates originating mainly from human toxoplasmosis cases in France and the USA. Tibayrenc et al. (1991), by meta-analysis of published isoenzyme data on a limited collection of Toxoplasma stocks (Dardé et al., 1988, 1992), proposed that T. gondii has a clonal population structure. This working hypothesis received support from Sibley and Boothroyd (1992), who proposed that T. gondii was subdivided into two major clonal lineages, of which one is virulent in mice. Later, Howe and Sibley (1995)

postulated the existence, confirmed by phylogenetic studies on 106 stocks with 6 PCR-RFLP markers, of three lineages (designated types I, II, and III) instead of two (Figure 3.1). It is now well accepted that *T. gondii*, in common with many other parasitic protozoa, exhibits a clonal population structure. This clonal structure was further confirmed in multilocus studies using 8 microsatellite markers



FIGURE 3.1 Dendrogram of 106 *T. gondii* strains derived from restriction fragment length polymorphism analysis of six single-copy loci amplified by polymerase chain reaction. Strains are grouped into three main lineages: I, II, III. Horizontal scale and values at branch points are estimates of percent nucleotide sequence divergence. From Howe and Sibley (1995); reproduced with permission.

TABLE 3.3 Multilocus s	studies wit	h unusual	isolates (i	.e. unusual	host or ge	eographic	cal origin)		
Reference]	Loci (chroi	nosome lo	cation)			Typing method	Unusual isolates
Howe and Sibley, 1995	SAG2 (VIII)	SAG1 (VIII)	850 (V)	ROP1 (XI)	L328 (VII)	62 (IX)		PCR-RFLP	23 ^b
Dardé, 1996	ASAT (ND ^a)	GSR (ND)	AMY (ND)	GPI (ND)	PE (ND)	ACP (ND)		Isoenzymes	8 ^c
Lehmann <i>et al.</i> , 2000	SAG2 (VIII)	TUB1 (IX)	TUB2 (IX)	FOL1 (ND)	MAG1 (VIII)	<i>B10</i> (XII)	ACT1 (Ib)	Sequencing	3 ^d
Grigg <i>et al.</i> , 2001a	SAG2 (VIII)	SAG1 (VIII)	SAG3 (XII)	SAG4 (VIIa)	BSR4 (IV)			Sequencing	4 ^e
Lehmann <i>et al.</i> , 2003	SAG2 (VIII)	<i>M163</i> (XII)	M33 (IV)	<i>M48</i> (Ia)	<i>M102</i> (VIIa)	M6 (XII)	M95 (VIIa)	PCR-RFLP + microsatellite	3^{f}
Sreekumar <i>et al</i> ., 2003	SAG2 (VIII)	<i>M163</i> (XII)	M33 (IV)	M48 (Ia)	<i>M102</i> (VII)	M6 (XII)	M95 (VIIa)	PCR-RFLP + microsatellite	7 ^g
Miller <i>et al</i> ., 2004; Conrad <i>et al</i> ., 2005	SAG2 (VIII)	SAG1 (VIII)	SAG3 (XII)	<i>B1</i> (IX)	GRA6 (X)			PCR-RFLP + sequencing	52 ^h

Lehmann <i>et al.</i> , 2004	SAG2 (VIII)	M163 (XII)	M33 (IV)	M48 (Ia)	<i>M102</i> (VII)	M6 (XII)	<i>M</i> 95 (VIIa)		PCR-RFLP + microsatellite	53 ⁱ
Ajzenberg <i>et al.</i> , 2004	<i>TUB2</i> (IX)	W35 (II)	<i>B18</i> (VII)	<i>B17</i> (XII)	<i>ТgM-А</i> (Х)				Microsatellite	22 ^j
Ferreira <i>et al.</i> , 2006	SAG2 (VIII)	SAG1 (VIII)	SAG3 (XII)	<i>L363</i> (VIIb)	GRA6 (X)	<i>B1</i> (IX)	cB21-4 (III)	cS10-A6 (VIIa)	PCR-RFLP + microsatellite	20 ^k

^aND: not done.

^bBrazil (2: OH3, S11), Australia (1: Tg96), Japan (3: Tg132, FUK, Tg17), Panama (2 cats: K117c, K125; 1 dove:G622-M), Bears (7: B41, B51, B62, B70, B73, B74, B75), Deers (2: WTD-1, WTD-3), Turkey (1: T61), Rodents (4: R18, R943, R961, R977).

^cUruguay (2: OPA, CASTELLS), French Guiana (1: RUB), Japan (1: Tg132), Argentina (1: PIG3), Australia (1: Tg96), Monkey (1: SQM), Guinea pig (1: 76K).

^dCougar (1: COUGAR2), Sea otter (1: OTTER3), Wallaby (1: WALLABY1)

^eFrench Guiana (2: RUB, VAND), Uruguay (1: CASTELLS), Cougar (1: COUGAR)

fStarling (1), Rodents (2)

^gIndia (7: TgCInd-1-TgCind-7)

^hSea otters (50), Pacific harbor seal (1), California sea lion (1)

¹Brazil (53: CK194-218, CK300, CK304-305, CK 307, CK309, CK312-316, CK319, CK323, CK328, CK332, CK335, CK337, CK340-345, CK347, CK351, CK353, CK357-358, CK364).

^jFrench Guiana (9: RUB, VAND, GUY-2001-DOS, IPP-2002-BAT, GUY-2002-MAT, GUY-2002-KOE, GUY-2002-BAS, GUY-2003-MEL, GUY-2003-ADA), Africa (3: WIK, GANGI, PSP-2003-KOM), Uruguay (3: ATIH, OPA, CASTELLS), Guadeloupe island (1: PSP-2003-ERO), Reunion island (1: TOU-2002-ALI), Monkey in Barbados (1: ENVL-2002-MAC), Bear (2: B41, B73), Deer (1: WTD-1), Turkey (1: T61)

^kBrazil (20: D1-D8, CH1-CH3, AS28, BV, N, EGS, RAR, SAF, EFP, C4, P)

on 83 stocks (Ajzenberg *et al.*, 2002a), sequencing of 7 independent single-copy genes on 16 stocks (Lehmann *et al.*, 2000), and sequencing of 15 unlinked loci on 18 stocks (Grigg *et al.*, 2001a).

The main criteria for a clonal population structure (Tibayrenc, 1998) in *T. gondii* are:

- 1. The isolation of identical multilocus genotypes over large geographic areas and at an interval of several years. For instance, the RH strain isolated in 1939 in USA has the same multilocus pattern as other strains isolated 40–60 years later in Europe. Type II strains have been isolated 40 years apart in France and in the USA.
- 2. The small number of different multilocus genotypes and the over-representation of the observed genotypes by comparison with panmixic expectations, providing evidence of a high linkage disequilibrium. In Howe and Sibley (1995), the PCR-RFLP analysis of 6 independent loci on a collection of 106 stocks detected only 15 different genotypes from a possible 1728 different combinations of alleles. This high linkage disequilibrium was observed even when linkage disequilibrium tests were performed on geographical subdivisions of the *Toxoplasma* sample to avoid the bias due to geographical distance (Ajzenberg et al., 2002a).
- 3. *The correlation between independent set of markers*, such as isoenzymes, PCR-RFLP on independent single-copy genes, or microsatellite analysis.

Sequencing of introns or housekeeping genes in a number of natural stocks concluded that withinlineage allelic diversity is virtually absent (Biñas and Johnson, 1998; Lehmann *et al.*, 2000). Moreover, comparative sequence analysis of individual genes estimates only 1 percent divergence at the DNA sequence level between lineages. The relatively lack of intratypic variation associated with a low divergence between lineages strongly suggests that these three clonal lineages have emerged as the dominant strains relatively recently. It was proposed that these three clonal lineages shared a common ancestor 10 000 years ago (Su *et al.*, 2003). The recent expansion of these three strains and their huge predominance in diverse natural populations of *Toxoplasma* suggests a high level of adaptation to transmission or colonization within a particular ecological niche (Su *et al.*, 2003), and it is tempting to speculate that this may reflect the origins of agriculture and the spread of domesticated species.

3.3.2.2 Recombination and genetic diversity

The clonal population structure with a low genetic diversity is surprising, considering the broad geographic and host range of this parasite and its capacity for sexual reproduction. One of the main consequences of genetic exchange is recombination of genotypes among different loci (Tibayrenc, 1993). The null hypothesis of panmixia (random mating and genetic exchange) has been rejected in Toxoplasma, as there is very little evidence of recombination in the population studied. For instance, in the survey of 106 stocks by Howe and Sibley (1995), only 4 were encountered with extensively mixed genotypes. A clonal structure does not totally exclude recombination events, but rather indicates that such recombination is not important enough to alter significantly the prevailing clonal population structure (Tibayrenc et al., 1990, 1991). In fact, the studies which genotyped the largest collection of isolates (Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Dardé, 1996; Ajzenberg et al., 2002a) concluded that recombination was exceedingly rare in natural populations. However, although recombination may be infrequent, it should not be ignored.

Archetype strains The clonal lineages themselves have emerged from recombination events. Detailed sequencing of 18 mainly unlinked polymorphic genes in 3 archetype strains identified a total of just 2 allelic classes at all loci examined, and these 2 alleles segregated randomly between the three lineages (Grigg *et al.*, 2001a). This limited, essentially dimorphic, gene pool indicates that the major lineages within the species share a common ancestry and have emerged as a result of meiotic recombination (Grigg and Suzuki, 2003). To emphasize the fact that the sexual recombination between

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two distinct founders is responsible for this emergence, the two allelic types are referred to as A (Adam) and E (Eve) (Grigg *et al.*, 2001a) (Table 3.4). More recently, Khan *et al.* (2005a), mapping the genome with 250 SNP markers to approximately 300-kb intervals across the 14 chromosomes of *T. gondii* genome, confirmed that in the 3 predominant strains each locus contained only 2 alleles. Further, asymmetric distribution of strain-specific SNPs implies that very little recombination has occurred between strains over the intervening period.

For example, among the three lineages the recombinant nature of type III is clear, as it possesses several specific markers such as *BSR4* and *SAG4* (Grigg *et al.*, 2001a) or *TgM-A* (Ajzenberg *et al.*, 2004) but it also carries markers characteristic of

type II (e.g. *GRA4*, *ROP1*, *SAG1*, *ACT1*, *TUB1*, *FOL1*, and *B10*) and type I strains (e.g. *MAG1*, *SAG2*, *SAG3*, *GRA1*, *GRA3*) (Lehmann *et al.*, 2000; Grigg *et al.*, 2001a).

Recombinant strains Among *Toxoplasma* strains, a small percentage of them appear to exhibit higher levels of recombination. They possess A and E allelic classes identical to those found in the three major lineages, but these have segregated differently among the loci analyzed (Table 3.4) (Howe and Sibley, 1995; Grigg *et al.*, 2001a; Ajzenberg *et al.*, 2004; Ferreira *et al.*, 2006). The recombinant genotypes are related to the three main lineages but the inclusion of these mixed genotypes in phylogenetic analysis decreases the robustness of association between the type I,

Locus chromosome	SAG1 VIII	SAG2A VIII	SAG3 II	SAG4A VII	BSR4 IV	Genotype
Type I (7)	•	0	0	0	0	Ι
Type II (8)	0	•	•	0	0	II
Type III (7)	0	\otimes	\otimes	•	•	III
Type IV (4)	•	\otimes	\otimes	•	nd	IV
ELG	0	•	•	0	0	II (drifted)
TONT	•	0	\otimes	•	•	Sibling?
SSI 119	•	\otimes	\otimes	•	•	Sibling?
P89	•	\otimes	\otimes	•	•	Sibling?
P80	•	•	\otimes	0	0	Sibling?
2035	•	0	\otimes	0	nd	Sibling?
RUB	•	0	•	•	♦	New
VAND	•	0	•	•	♦	New
MAS	•	♦	\otimes	♦	♦	New
CASTELLS	•	•	\otimes	•	•	New
COUGAR	•	•	•	•	•	New

TABLE 3.4 Genotype of non-lineage strains by DNA sequence analysis (adapted from Grigg and Suzuki, 2003)

Multilocus sequence analysis of seven type I, eight type II, seven type III, and four type IV strains revealed no withinlineage polymorphism and different combinations of the two allelic classes over the five loci investigated. *P80, P89, SSI, TONT, 2035 represent novel recombinants, possibly sibling progeny, bearing new combinations of the alleles found in the three archetypal lineages of *Toxoplasma*.

• E allele; \bigcirc A allele; \bigcirc type III lineage A allele defined by one (*SAG2A*) or three (*SAG3*) nucleotide polymorphisms from the consensus sequence; \blacklozenge represents a unique allele with > 0.4% polymorphism from either A or E.

II, and III strains (Ajzenberg *et al.*, 2004) (Figure 3.2). The strains may reflect ongoing rare recombination events, or could be the less successful sibling progeny of the three major lineages (Grigg and Suzuki, 2003) or even extant versions of parental lineages of one of the three archetypal lineages (Boothroyd *et al.*, 2005). Isolates with mixed genotypes have been sampled mainly from tropical areas such as Brazil (Ferreira *et al.*, 2006), Africa, the Caribbean, and Reunion Island (Ajzenberg *et al.*, 2004), but a few have been described from wildlife (bears and deer) in North America (Howe and Sibley, 1995; Ajzenberg *et al.*, 2004). The isolation of recombinant strains



FIGURE 3.2 Phylogram of 43 *Toxoplasma gondii* strains as determined by analysis of the sequences of five microsatellite markers (*TUB2*, *W35*, *TgM-A*, *B18*, *B17*). *Hammondia hammondii* was used as the outgroup. The tree was built by Wagner analysis after bootstrapping with 100 repetitions. FG indicates a French Guianan strain. Adapted from Ajzenberg et al. (2004).

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from the 'domestic' population of Toxoplasma in Europe or North America is very uncommon; they include four isolates from North American pigs, P62, P80, P89, and P105 (Mondragon et al., 1998), five DNA samples (including four identical described as type IV) from human ocular toxoplasmosis in North America (Grigg et al., 2001b), two isolates from AIDS patients in the USA (Howe and Sibley, 1995), and four isolates from human congenital toxoplasmosis in France (Ajzenberg et al., 2002b, 2004). The number of recombinant strains in the literature will probably dramatically increase when chicken isolates sampled all over the world by Dubey's laboratory (references already cited) will be characterized by multilocus analyses.

Atypical genotypes More rarely, 'atypical' or 'exotic' strains are discovered (Grigg et al., 2001a; Su et al., 2003; Ajzenberg et al., 2004). These have many unique polymorphisms and 'novel' alleles (Table 3.4). Phylogenetic analysis shows that the atypical genotypes are distributed throughout the tree without any obvious structuration, and cannot be related to the three main lineages (Ajzenberg et al., 2004) (Figure 3.2). However, global observation of the genetic diversity indices showed that although allelic diversity was high, the level of genetic polymorphism of sequences remained very low in agreement with previous observations of diversity in Toxoplasma strains (Grigg et al., 2001a; Su et al., 2003; Ajzenberg et al., 2004). At some loci, atypical strains show evidence of the dimorphic allele patterns that typify the clonal lineages (Su et al., 2003). This strongly suggests that they have introgressed with the clonal lineages through subsequent crosses (Sibley, 2003).

Only 14 strains with atypical genotypes are described in the literature. The first, MAS, isolated from a case of human congenital toxoplasmosis in France, and the second, CASTELLS, taken from an aborted sheep in Uruguay, have been well characterized with many markers in many studies (Dardé, 1996; Grigg *et al.*, 2001a; Su *et al.*, 2003; Ajzenberg *et al.*, 2004). Additional atypical strains include a cougar isolate from Canada (Lehmann *et al.*, 2000; Grigg *et al.*, 2001a; Su *et al.*, 2003), an

atypical genotype (named type X) from marine mammals (Miller *et al.*, 2004; Conrad *et al.*, 2005), a second isolate (IPP-2002-URB) from a human congenital case in France (Ajzenberg *et al.*, 2004), and nine strains from French Guiana (Carme *et al.*, 2002; Ajzenberg *et al.*, 2004).

The French Guianan (FG) strains were isolated from human patients with severe disseminated toxoplasmosis, except for one strain (IPP-2002-BAT) taken from a case of asymptomatic congenital toxoplasmosis. Since infections occurred in individuals living in the rainforest, eating undercooked game and drinking untreated river water (Carme et al., 2002), the strains are probably transmitted from wildlife species. Variation in the FG strains (RUB, VAND) was first detected by isoenzyme analysis (Bossi et al., 1998; Dardé et al., 1998), then multiple polymorphisms were reported via sequencing of antigen genes SAG1, SAG2A, SAG3, SAG4, BSR4 (Grigg et al., 2001a) and GRA6 (Fazaeli et al., 2000a). The nine FG strains have also been genetically characterized by multilocus microsatellite sequencing together with 34 strains from diverse host and geographical origins (Ajzenberg et al., 2004). Each French Guianan strain had a unique multilocus genotype, the genotypic diversity was maximal (number of different genotypes on the total number of genotypes, G = 1), and the majority of atypical alleles were found in FG strains and, for some alleles, only in FG strains (Table 3.5). The level of heterogeneity between the FG strains contrasts markedly with 'domestic' isolates from human congenital toxoplasmosis in France, where, in 57 consecutive cases, 96.5 percent of isolates had only one genotype, type II (Ajzenberg et al., 2002b).

3.3.3.3 New data on population structure

Genetic exchange can have many effects: it may lower linkage disequilibria, shuffle genotypes and reduce phylogenetic divergence (Tibayrenc and Ayala, 2002). There is evidence that, as with many parasites, the use of sexual recombination may vary in *Toxoplasma*. Microsatellite analysis found lower linkage disequilibrium in a Brazilian chicken sample than in a North American domestic animal

Isolate	Host	Origin	type	TUB2	W35	TgM-A	B18	B17
BK	Human	Netherlands	Ι	1	1	1	1.3	1
BEV	Human	France	II	2.3	2	2	2	2.3
NED	Human	France	III	2.3	3	3	1.3	2.3
IPP-2002-BAT	Human	Fr. Guiana	R III/I	1	3	3	4	2.3
RUB	Human	Fr. Guiana	Atypical	2.3	3	3	7	9
GUY-2003-BAS	Human	Fr. Guiana	Atypical	2.3	2	4	5	5
GUY-2003-KOE	Human	Fr. Guiana	Atypical	2.3	4	4	5	4
GUY-2003-MEL	Human	Fr. Guiana	Atypical	2.3	2	4	2	5
GUY-2001-DOS	Human	Fr. Guiana	Atypical	2.3	4	4	1.3	5
GUY-2002-MAT	Human	Fr. Guiana	Atypical	1	7	4	1.3	11
VAND	Human	Fr. Guiana	Atypical	1	2	4	4	5
GUY-2003-ADA	Human	Fr. Guiana	Atypical	1	4	3	6	4

TABLE 3.5 Genotypic diversity of nine French Guianan strains in comparison with three archetypalstrains (BK, BEV, and NED)

Alleles are defined by sequence polymorphisms in microsatellite regions of five markers (*TUB2*, *W35*, *TgM-A*, *B18*, *B17*). Alleles 1, 2, 3 are reserved for clonal lineages I, II, and III. Allele 2.3 means that types II and III share the allele. Allele 1.3 means that types I and III share the allele. Allele 4 and above are used for atypical alleles (grey background).

sample, suggesting a higher rate of outcrossing in Brazil (Lehmann *et al.*, 2004). The central role of sexual recombination in Brazilian isolates has been confirmed by Ferreira *et al.* (2006), who found recombinant genotypes in 20 isolates via PCR-RFLP at 8 independent loci.

Similarly, Ajzenberg et al. (2004) found that molecular and phylogenetic analysis of 21 'domestic' and 22 unusual isolates (i.e. unusual owing to their different host or geographical origin) were not in agreement with a strictly clonal model. The molecular data showed many shuffled genotypes, which is inconsistent with a theoretical clonal structure characterized by a global association between alleles due to linkage disequilibrium (Tibayrenc et al., 1990). Phylogenetic reconstruction also showed weak relationships between these isolates (Figure 3.2), which did not present the typical structure of a clonal organism such as Trypanosoma cruzi, Entamoeba histolytica or Leishmania (Tibayrenc et al., 1990; Bañuls et al., 1999). This implies that phylogenetic divergence of T. gondii is obscured by recombination. From these genetic and phylogenetic analyses, the most parsimonious hypothesis is that *T. gondii* presents a complex population structure with a mix of clonal and sexual propagation as already described for other parasites belonging to the Apicomplexa phylum, such as *Cryptosporidium* (Mallon *et al.*, 2003) or *Plasmodium falciparum* (Anderson *et al.*, 2000).

The above evidence suggests that sexual recombination may be more common between Toxoplasma isolates in the wild life cycle (e.g. French Guiana), and in areas where breeding is recent or not intensive and cat domestication recently introduced (e.g. Brazil, West or Central Africa, the Caribbean). Thus, under-representation of the wildlife isolates might explain the predominance of archetypal I, II, and III strains and the description of a global clonal structure in the previous studies. Comparison of microsatellite data from North American and Brazilian isolates suggests that either clonal or sexual propagation may be selected under different environmental conditions (Lehmann et al., 2004). At present, there is clear evidence that strains with clonal propagation and peridomestic transmission

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dominate in North America and Europe. However, it is less clear whether this pattern extends to cover sympatric wildlife species in these regions, due to the patchiness of epidemiological sampling. Beyond these regions of the world, it is very difficult to interpret the patterns of parasite transmission and genetic exchange.

Considering the distribution of atypical and recombinant strains, it might be suggested that they are found in environments that are less dominated by domesticated species (e.g. Brazil, Africa, the Caribbean islands). It is tempting to speculate that the atypical strains reflect a more panmictic population structure with a more diverse range of parasite genotypes sustained by recombination of strains in this species-rich environment. However, care should be taken not to over-interpret this evidence of apparent recombination. Although atypical genotypes, such the cougar (Lehmann et al., 2000) and French Guianan strains (Ajzenberg et al., 2004), are phylogenetically unrelated to the three main lineages, they may represent 'unknown' clones which are adapted to wild hosts. The key to resolving this question lies in more intensive epidemiological sampling of this environment. If new clonal types do exist, they must be distinguished from ephemeral clones according to the characteristics of a clonal structure - namely, that a new strain type should be widespread over an extensive geographical and host range, and should persist over a long period of time (Tibayrenc et al., 1990).

New clonal types have already been proposed for example, type IV, which is associated with ocular toxoplasmosis (Grigg et al., 2001b); type X, from sea otters (Miller et al., 2004); and an identical mixed I/III genotype which has been described in humans from Africa (Ajzenberg et al., 2004). It is possible to discuss the validity of type IV or of the 'African type', as these rest on a very limited set of isolates; however, there are good arguments that type X should be considered a new, emerging type. Type X has been genetically defined by the association of two type II alleles at SAG2A and SAG3 with three atypical alleles at B1, SAG1, and GRA6 (Miller et al., 2004). Isolates conforming to this type have been obtained from a variety of marine mammals, including 38 isolates from post mortems of sea

otters, 2 from a Pacific harbour seal, and 1 from a California sea lion (Miller et al., 2004; Conrad et al., 2005). An additional C8 RAPD marker revealed genetic heterogeneity among these type X isolates, and further demonstrated that the profile of type X was distinct from the three major lineages (Conrad et al., 2005). Clearly, the genotype occurs in a range of hosts across the Californian coastline, and the only remaining question is whether it is stable over time and space. There is some evidence in favor of this, as the atypical type X allele at SAG1 has been previously described in wild animals and humans (allele 3 in Howe and Sibley, 1995). Very recently, the atypical type X allele at GRA6 has also been identified in a free-living jaguar in French Guiana (D. Ajzenberg, personal communication).

3.4 FACTORS AFFECTING TRANSMISSION AND GENETIC EXCHANGE

3.4.1 Biological factors

Although *Toxoplasma* has a sexual cycle and its potential for recombination has been experimentally proven (Sibley *et al.*, 1992; Khan *et al.*, 2005a), evidence from the field suggests restricted use of this cycle. As suggested by Tibayrenc and Ayala (2002), the clonal population structure is probably a consequence of upstream inhibition of recombination rather than a downstream elimination of recombinant genotypes by natural selection. Several of the biological properties of *Toxoplasma* may account for the predominant clonal structure:

- 1. The sexual stage infection in the cat is relatively transient, and it is thus likely that the majority of infections involve only a single *Toxoplasma* isolate derived from a single prey source. This means that autofertilization or 'selfing' would be common and would limit the flow of genes between strains (Howe and Sibley, 1995).
- 2. Any macrogametes of the parasite remain unfertilized but are capable of forming oocysts in the small intestine of cats by parthenogenesis (Ferguson, 2002).

- 3. The parasite does not have an obligatory sexual cycle, and can be transmitted asexually through carnivory (Howe and Sibley, 1995; Su *et al.*, 2003). Su *et al.* (2003) have proposed that archetype clonal strains may be adapted to this transmission route, and this would endow them with the ability to transmit more efficiently among intermediate hosts, bypassing sexual recombination in the definitive host.
- 4. There is evidence that, in some host species, *Toxoplasma* may be serially vertically transmitted (Morley *et al.*, 2005). This would lead to clonal expansion of the parasite in that species (Duncanson *et al.*, 2001).
- 5. Toxoplasma is known to induce a strong immune response both in intermediate and in definitive hosts. In intermediate hosts, although superinfection can occur leading to a mixed infection (Dao et al., 2001), it is likely that this is rare in nature. There are very few occasions where genetically different isolates have been retrieved from the same individual (Ajzenberg et al., 2002a; Dubey et al., 2003a, 2005a). This factor has the effect of partitioning the parasite within hosts, and is key to the transmission dynamics of the parasite. In definitive hosts, it has been shown that occasionally a cat experimentally infected with a given strain can be infected with a different strain leading to a low oocyst production, but it was not possible to determine if the two strains mated in these experiments (Dubey, 1995).

3.4.2 Environmental factors

Though it is believed that *T. gondii* has no geographical and host boundaries (Howe and Sibley, 1995), it may well be that particular genotypes are associated with specific host groups or with different geographic locations. To date, isolates have been obtained from many different species (see Tables 3.2 and 3.3). Variation has been seen in the relative abundance of strains. At this stage it is difficult to read too much into these studies, in part because most of them rest on measuring variation at a single locus and in part

because the strains often come from different geographical regions. One feature emerging from these ongoing studies is that the genotype of an isolate may reflect its geographical origin to a greater extent than its host species – for example, chickens in Brazil were infected with *SAG2* type I and III isolates (Dubey *et al.*, 2002, 2003a, 2003b) while the same species in the USA carried mainly *SAG2* type II parasites (Dubey *et al.*, 2003e). Attempts to understand the transmission of the parasite between hosts must clearly take this geographical variation into account.

If genotypes are selected by host species, this could be driven either by opportunities for transmission between sympatric hosts or by variation in susceptibility due to local adaptation. If this is true, then there may be an intrinsic co-evolutionary link between host and parasite genotypes. Only a limited number of host species, such as cats, a few meat-producing animals, and peri-domestic mammals and birds, are involved in T. gondii's domestic cycle, which may limit the complexity of the parasite genetic pool in this cycle. Among many genotypes, the three clonal lineages seem to be most successfully adapted to these domestic hosts (Lehmann et al., 2003). They would have diverged about 10000 years ago, which coincides with the domestication of companion and agricultural animals (Su et al., 2003). In Europe or North America, intensive breeding of a narrow range of domestic meat-producing animals together with cat domestication offered a major niche to these three lineages. Nowadays, these three major clonal lineages likely prevail due to increases in human travel and in the trade of food animal products between countries. Furthermore, farms are reservoirs of infection (small peri-domestic rodents and birds, and cats) from which transmission of clonal types can radiate to the surrounding wild environment (Lehmann et al., 2003), leading to an impoverishment of genetic diversity. Human activities in North America and Europe, which diminished recombinations and thus gene flow in Toxoplasma, may favor genetic drift for Toxoplasma evolution. This would reduce the adaptative potential of this parasite in a domestic environment, and its biodiversity.

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If Toxoplasma strains have co-evolved with particular host species, then it may also be true that the density and diversity of host species is linked to parasite diversity. In this case, speciesrich habitats like the rainforest, which harbors many species of mammals and birds, could sustain a greater diversity of parasite genotypes in order to colonize the maximum of ecological niches. At the moment, there is some evidence that strains circulating in the wild environment in certain parts of the world (notably South America) are different from the archetype strains and more diverse. It is still difficult to judge whether they are ancient relict populations or reflect biogeographical separation. As further sampling and genotyping helps us to understand gene flow in these populations, we will discover whether they do make greater use of the sexual cycle or resolve into a further complex of clonal lineages with occasional recombinants. The frequency with which recombinant strains arise depends on two factors: the degree of genetic diversity and the rate of transmission. If both are high, then the likelihood of mixed infections and recombination in the felid host increases. In France, for instance, type II is so predominant that even if a cat ingests two infected prey in a short period of time, recombination would most probably occur between two type II isolates, leading to production of a type II oocyst. In a wild environment, where the genotypic diversity is much higher, recombination between different genotypes would lead to oocysts with a mixed genotype. Furthermore, experimental re-infection of an intermediate host has been shown to occur when the genotype of the second infecting isolate is different from the genotype of that of the first (Dao et al., 2001). Thus, due to the higher genetic diversity, re-infection could be more frequent in wild intermediate hosts, leading to a higher frequency of mixed infections in wild nature.

In this hypothesis, in a wild environment, the two forces affecting genetic variation (genetic drift and recombination) are strong. This permits *Toxoplasma* to conserve an optimal capacity for adaptation. However, as the increasing urbanization results in a greater interaction between the domestic and wild environments, it will be interesting to

study the *Toxoplasma* population at the intersection of these two worlds.

3.5 MOLECULAR EPIDEMIOLOGICAL STUDIES

To determine the patterns of transmission of Toxoplasma requires intensive studies which employ polymorphic markers to track strains in contiguous space and time. Such studies are rare, but perhaps the best examples are the careful studies of marine mammals, in which close typing of isolates shows infection by both type II strains and by isolates clustered into a new strain, type X (Miller et al., 2004). The risk of infection in sea otters was related to the level of freshwater runoff (Miller et al., 2002), and a hotspot of type X strain transmission was mapped to a single site (Morro Bay) on the California coast (Miller et al., 2004). It is proposed that oocysts in runoff from water fall into marine sediments and may be concentrated in mollusks - a common food source for the otters. Although Toxoplasma can survive in bivalves (Lindsay et al., 2004), there is as yet no evidence that this occurs in nature and the terrestrial source of this unusual genotype has still to be discovered. However, extension of this approach to the region inland from Morro Bay should throw light on these questions and perhaps provide the first genetically defined transmission network for Toxoplasma.

A second system which has been subject to molecular epidemiological study is the transmission of *Toxoplasma* in sheep. A longitudinal study of UK sheep revealed consistently high levels of congenital transmission, with over 40 percent of live-born lambs affected (Duncanson *et al.*, 2001; Williams *et al.*, 2005). Analysis of 10-year pedigree records proved supportive of vertical transmission, finding that abortion and *Toxoplasma* infection are more common in some maternal lineages (Morley *et al.*, 2005). Mobile genetic element markers (Terry *et al.*, 2001) have been used to characterize the strains circulating in the flock, and these found evidence of a clonal genotype in longitudinal studies (Figure 3.3). Occasionally,



FIGURE 3.3 PCR amplification using the single primer extension with repA and repB MGE primer of *T. gondii* from ovine chord samples. Samples were taken from Charollais sheep on a Worcester farm in March 1999 (1–4), January 2000 (5–8), and March 2000 (9), and from a Lancaster farm in March 2000 (10–12). The majority of isolates within a flock are clonal, but a few variant strains are found.

new genotypes were seen, perhaps reflecting infection from oocysts. Evidence for vertical transmission has also been reported in other species, including mice and rats (Beverley, 1959; Dubey *et al.*, 1997; Owen and Trees, 1999; Marshall *et al.*, 2004). It is characteristic that vertical transmission may be overlooked, as parasites adapted to this route are predicted to have low pathogenicity and therefore to be cryptic (Dunn and Smith, 2001).

The cat is believed to play a central role in the transmission of *Toxoplasma*, yet, ironically, there are no genetic data that demonstrate oocyst-induced infection. The most frequently cited evidence relates to the serious outbreak of toxoplasmosis in Vancouver Island (Bowie *et al.*, 1997). This epidemic lasted for 6 months between 1994 and 1995, and caused 100 cases of clinical human disease. Mapping the sites of infection led to the

conclusion that the outbreak was caused by oocyst contamination of the local water reservoir. Infected feral cats and cougars were both found in the watershed area (Aramini *et al.*, 1998), but attempts to extract oocysts from the water supply were unsuccessful. The only isolate found in the neighborhood was isolated from cougar feces and was found to have an atypical genotype (Lehmann *et al.*, 2000; Grigg *et al.*, 2001a). This isolate was supposed to be at the origin of the outbreak. Similarly, other reported oocyst-associated infections also rely on circumstantial evidence (Benenson *et al.*, 1982; Bahia-Oliveira *et al.*, 2003).

In summary, although population-based studies are revealing large-scale patterns of genetic variation in *Toxoplasma*, more intensive molecular epidemiological studies are required to elucidate transmission networks, and this is clearly a priority if we are to understand and control the transmission of the parasite.

3.6 TOXOPLASMA GENOTYPE AND BIOLOGICAL CHARACTERISTICS

The practical implication of a clonal population structure is that biological characteristics can be attributed to a genetically well-defined subset of the parasitic population. In the case of *Toxoplasma*, the relationships between the three main lineages and some biological characteristics are now well established.

Virulence in mice is the most recognized phenotypic marker: type I strain led to a widespread parasite dissemination and death of mice less than 10 days after inoculation of <10 tachyzoites; in contrast, mice survived to infection with a type II strain (50 percent lethal dose (LD50) >10³) and tachyzoite dissemination was much less extensive. Type III is also generally considered as avirulent in mice, although progressive deterioration and death of mice, notably with neurological symptoms, can occur a few weeks or months after inoculation. The genetic differences between strains elicit a different immune response in the host that could in part explain the different patterns of virulence (Gavrilescu and Denkers, 2001; Fux *et al.*, 2003; Nguyen *et al.*, 2003; Diana *et al.*, 2004; Robben *et al.*, 2004; Saeij *et al.*, 2005a).

The higher virulence of type I in mice compared with types II or III has been correlated with in vitro biological properties: type I displays enhanced migration in vitro, as well as enhanced transmigration across polarized MDCK or across extracellular matrix. It also shows a higher rate of ex vivo penetration of lamina propria and submucosa (Barragan and Sibley, 2002). This ability to cross epithelial barriers rapidly and reach the bloodstream within hours post-infection might be an important predeterminant of parasite dissemination in vivo in susceptible host species. In cell culture, type I grows faster than type II or III and has a lower rate of interconversion from tachyzoite to bradyzoite than type II strains (Soete et al., 1993). The higher growth rate of type I parasites has been suggested to be due to a higher reinvasion rate rather than to a shorter doubling time (Saeij et al., 2005a).

Although these *in vitro* studies demonstrate different intrinsic properties of the different strains, the host response is essential for expression of virulence: strain virulence is not the same across host species – for example, type I strains, which are highly virulent in mice, are not pathogenic in rats (Zenner *et al.*, 1999).

Atypical and naturally recombinant strains are usually more virulent in mice than are types II or III; however, owing to their genomic diversity they cannot be directly compared. They exhibit differences in mouse virulence and in other biological properties which probably reflect the differences in the combination of genes they have inherited (Grigg and Suzuki, 2003). Experimental crosses should prove useful in mapping the relationships between genotype and phenotype, and identifying 'virulence genes'. Interestingly, a cross between two avirulent strains, ME49 (type II) and CEP (type III), gave rise either to avirulent progeny like their parents or to progeny with enhanced virulence (Grigg et al., 2001a). Remarkable differences in the dissemination patterns in mice were observed between a virulent progenitor from a type II \times III cross (LD50 was at least three logs

lower than that of either parents) and its nonvirulent siblings strain (Saeij *et al.*, 2005b).

3.7 TOXOPLASMA GENOTYPE AND HUMAN DISEASE

3.7.1 Circumstances of isolation and typing

The circumstances during which T. gondii can be isolated from human cases of toxoplasmosis are relatively rare. They necessitate the retrieval of tachyzoites from pathological samples collected for diagnosis purposes (blood, amniotic fluid, broncho-alveolar lavage, etc.), or of cysts in tissues collected via biopsy or necropsy. Isolates from human toxoplasmosis come predominantly from cases of congenital toxoplasmosis or from immunodeficient patients, and much less frequently from symptomatic acquired toxoplasmosis in immunocompetent patients. Even in clinical cases, strain isolation via mouse inoculation is not easy to perform. Besides, it has also been suggested that isolation in mice imposes a selective pressure that may retrieve one strain from a mixed infection (Villena et al., 2004). This hypothesis needs more investigation.

The sensitivity of the PCR-based methods theoretically allows direct analysis of the parasite genotype from primary clinical sample. This would reduce the eventual risk of bias in strain selection imposed by isolation techniques and enable genotyping to be performed on small quantities of pathological products, such as ocular fluids, or on formaldehyde fixed tissues (Costa *et al.*, 1997; Grigg *et al.*, 2001b). In reality, the low number of parasites present in some samples and the frequent presence of PCR inhibitors often led to negative results even with nested PCR (Howe *et al.*, 1997; Fuentes *et al.*, 2001; Terry *et al.*, 2001). Besides, nested PCRs amplify the risk of DNA contamination.

Strain isolation or direct typing on pathological products can only be performed during symptomatic toxoplasmosis. To fully interpret the influence of *Toxoplasma* genotype on the different clinical aspects of human toxoplasmosis, we must also genotype the vast majority of human infections that are asymptomatic. Serological typing, a non-invasive method dependent on recognition of variant antigenic determinants (Kong *et al.*, 2003), may be useful in generating data on all the strains circulating in the human population.

From the first published studies (Dardé *et al.*, 1992; Howe *et al.*, 1997; Ajzenberg *et al.*, 2002b) it was evident that type II isolates were largely predominant (about 80 percent) in human symptomatic toxoplasmosis. However, it should be borne in mind that these studies were mainly performed on human patients from Europe and the USA, and that the situation might well be different in other continents.

3.7.2 Congenital toxoplasmosis

Congenital toxoplasmosis is the main source of *Toxoplasma* isolation in humans (amniotic fluid, placenta, cord blood, tissues of aborted fetuses). Amniotic fluids or even placentas are available mainly in countries (such as France) where a systematic prenatal screening of congenital toxoplasmosis is performed. In this case, nearly all the isolates responsible for congenital toxoplasmoses, including the majority of asymptomatic cases at birth, can be submitted for genotyping. Otherwise, strain isolation comes mainly from the more severe cases of symptomatic congenital toxoplasmosis, introducing a clinical bias.

Typing performed on all the isolates consecutively isolated in congenital cases in French laboratories revealed that they almost all belonged to type II (Table 3.6) (Ajzenberg et al., 2002b). Type II isolates were found in all the different aspects of congenital disease, including lethal infection, severe neuro-ocular involvement, isolated chorioretinitis, or latent toxoplasmosis. The main factor determining the severity of congenital infection remains the stage of pregnancy at which the disease is acquired (Table 3.7). Thus type II strains, which are non-virulent in mice, can sometimes prove highly pathogenic to the human fetus, but are also the only ones (together with a very few type III strains in Ajzenberg et al., 2002b) found in benign or latent congenital toxoplasmoses.

Very few type I, atypical, or recombinant strains were isolated from congenital toxoplasmosis, but these were usually observed in severe cases. The higher growth rate of type I isolates in mice and their remarkable transepithelial migratory ability (Barragan and Sibley, 2002) would imply a high risk of transplacental transmission and severe infection of the developing fetus. However, in some cases type I strains have been isolated from placenta without any development of congenital infection in children. This raises the question of whether an immunocompetent pregnant woman can more easily control a type I infection and prevent congenital transmission (Ajzenberg *et al.*, 2002b).

The conclusion that type II strains are most frequently responsible for congenital toxoplasmosis was drawn mainly from French data. In Spain, a first study from Fuentes et al. (2001) reported SAG2 type I strains in six out of eight cases of congenital toxoplasmosis. The clinical selection of mainly severe cases may explain this difference, particularly as, in a further report from Spain (Fuentes et al., 2004b), the proportion of SAG2 type II strain was higher (67 percent). However, it is likely that there will be geographical variations in strains associated with congenital toxoplasmosis. In countries of South America, such as Brazil, Colombia, or French Guiana, the majority of strains sampled to date in the environment are type I, atypical or recombinant strains (Ajzenberg et al., 2004; Ferreira et al., 2006). In the few reports from these countries, recombinant I/III strains, type I or at least SAG2 type I strains were implicated in severe cases of congenital toxoplasmosis (Ferreira et al., 2006; Gallego et al., 2006). Further, among the isolates consecutively collected in France from congenital toxoplasmosis cases, genotypes other than type II were found essentially in cases where the mother had acquired the infection in other countries (New Caledonia, Reunion Island, Brazil, French Guiana) (ToxoBS BRC France, unpublished data).

Thus, before it can be concluded that one genotype is more adapted than another to congenital transmission, we need data from different parts of the world, as the higher proportion of one type in

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	Number of isolates	Type I	Type II	Type III	Atypical or recombinant	Mixed infection	Reference
France	86	8%	85%	8%	5%		Ajzenberg <i>et al.,</i> 2002b
France	37		100%		NA ^a		Costa <i>et al.</i> , 1997
France	13		100%		NA		Howe <i>et al.</i> , 1997
Great Britain	19	31%	37%		NA	31%	Aspinall et al., 2003
Spain	24	25%	67%	8%	NA		Fuentes et al., 2004b
Spain	8	75%	12.5%	12.5%	NA		Fuentes et al., 2001
Brazil	4				$100\% \ R^b$		Ferreira <i>et al</i> ., 2006
USA	17	6%	70%	18%	6%		Howe and Sibley, 1995
Colombia	6	83.3%		16.6%			Gallego <i>et al.</i> , 2006

 TABLE 3.6 Distribution of genotypes in human congenital toxoplasmosis

^aNA = not available (monolocus typing).

^bR = recombinant genotypes.

human disease may simply reflect a disproportionately high infection with this genotype in meat-producing animals and environment of the same geographic areas.

3.7.3 Immunodeficient patients

Typing of isolates originating from immunodeficient patients was reported by Howe *et al.* (1997) and Honoré *et al.* (2000) (Table 3.8). In these two studies, typing was based on only a single locus (*SAG2* PCR-RFLP) and some atypical or recombinant genotypes could have been misidentified. *SAG2* type II strains were equally predominant (76 percent) in cerebral and in disseminated toxoplasmoses, in AIDS patients, and in other immunocompromised patients (lymphoma, organ transplant). In mice, type II strains produce high numbers of cysts which are more prone to reactivate in experimentally immunocompromised mice (Howe and Sibley, 1995). If they were to have the same behavior in humans, this would explain their predominance in cases of toxoplasmic encephalitis. However, about 15 percent of isolates observed in these patients

TABLE 3.7 Distribution of genotypes in congenital toxoplasmosis according to clinical severity(adapted from Ajzenberg et al., 2002b)

Clinical presentation	Number of isolates	Period of maternal infection (WA ^a)	Type I	Type II	Type III	Atypical
Fetal death	6	2–11		6		
Neonatal death	3	Unknown	1	2		
Severe disease	21	7–17	2	16		3
Benign or asymptomatic at birth	45	15–38		43	2	
Infected placenta, non- infected child	4	14–20	4			

WA = weeks of amenorrhea.

	Number of isolates	Type of Immune deficiency	Type I	Type II	Type III	Atypical or recombinant	Mixed infection	Reference
France	45	AIDS	13%	76%	11%	NA ^a		Howe <i>et al.</i> , 1997
France	55	AIDS	12.7%	76.4%	10.9%	NA		Honoré <i>et al.</i> , 2000
Great Britain	8	AIDS	50%	13%	13%	NA	25%	Aspinall <i>et al.,</i> 2003
Spain	31	AIDS	26%	58%	16%	NA		Fuentes <i>et al.</i> , 2004b
Spain	13	AIDS	23%	53.8%	30.7%	NA		Fuentes <i>et al.</i> , 2001
France	10	Non-AIDS	10%	80%	10%	NA		Howe <i>et al.,</i> 1997
France	16	Non-AIDS	19%	75%	6%	NA		Honoré <i>et al.</i> , 2000
USA	8	AIDS	62.5%		12.5%	25%		Khan <i>et al</i> ., 2005b

 TABLE 3.8
 Distribution of Toxoplasma SAG2 genotypes in immunocompromised patients

^aNA = not available (SAG2 monolocus typing).

belong to *SAG2* type I, suggesting that *SAG2* type I strains can also give rise to cysts and reactivate in human tissues. Furthermore, in a recent study reporting direct genotyping performed on cerebral spinal fluid from eight human immunodeficiency virus-positive patients, Khan *et al.* (2005b) found that a majority of these patients had infections with type I strains or strains containing type I alleles.

These first studies were performed in France and in the USA, but the likely origin of the patient infection was not recorded. In a more recent French collection of isolates, only recombinant I/III isolates were detected in immunocompromised patients (AIDS and non-AIDS) infected in West Africa. Again, this indicates the importance of taking the geographical origin into account (ToxoBS BRC France, unpublished data).

3.7.4 Immunocompetent patients

Toxoplasma gondii is rarely isolated in immunocompetent symptomatic patients. The few isolates originating from toxoplasmic lymphadenopathies in Europe belong mainly to type II (Dardé, 1996).

In ocular toxoplasmosis (Table 3.9), genetic typing has been performed directly from parasite DNA extracted from vitreous fluid (Grigg et al., 2001b). This investigation revealed an unusual abundance of type I (3/12) or recombinant genotypes I/III (5/12). In this study, the only type II isolates were observed in cases of chorioretinitis due to cyst reactivation in immunocompromised patients. It can also be noted that, in France, chorioretinitis following congenital toxoplasmosis is observed with type II strains (Ajzenberg et al., 2002b). These data suggested that cases of acquired ocular toxoplasmosis are more likely to be due to type I or recombinant genotypes. This was recently confirmed in Brazil, where a SAG2 type I was found in 10 cases of ocular toxoplasmosis (Vallochi et al., 2005), and in Korea (Lin et al., 2005). The higher prevalence of acquired ocular toxoplasmosis in Brazil may be due to the higher frequency of type I, atypical, or recombinant genotypes circulating in this country. This may also explain the high frequency (20 percent of 97 cases) of ocular involvement in the Victoria outbreak in British Columbia, where an atypical cougar isolate

Geographical origin	Number	Туре І	Type II	Type III	Recombinants	Reference
Brazil	11	11			NA ^a	Vallochi <i>et al.</i> , 2005
Korea	1	1				Lin <i>et al.</i> , 2005
USA	5				5	Boothroyd and Grigg, 2002
USA	6	1			5	Grigg <i>et al</i> ., 2001b

TABLE 3.9 Distribution of genotypes in ocular toxoplasmosis in immunocompetent patients(presumably acquired ocular toxoplasmosis)

^aNA = not available (SAG2 monolocus typing).

was suspected (Bowie *et al.*, 1997; Burnett *et al.*, 1998; Lehmann *et al.*, 2000), and the 100-fold higher incidence of ocular toxoplasmosis in patients born in Africa compared to patients born in Britain (Gilbert *et al.*, 1999).

The virulence of atypical strains is also apparent in the rare cases of severe toxoplasmosis observed in immunocompetent patients with multi-organ failure (Debord et al., 1996; Bossi et al., 1998; Dardé et al., 1998; Carme et al., 2002; Ajzenberg et al., 2004). These patients presented high and prolonged fever associated with pneumopathy, hepatic involvement, and sometimes myositis or cardiac failure due to myocarditis or pericarditis. Several deaths due to toxoplasmosis were observed in these immunocompetent patients. These infections, all due to atypical strains, were acquired after wild-game consumption, or after drinking water in the wild forests of French Guiana. Such severe cases are more occasionally observed elsewhere. The high virulence in humans of these atypical strains may be the consequence of intrinsic properties, but also of an inappropriate immune response to an unusual organism.

3.7.5 Preliminary conclusions on *Toxoplasma* genotype and human disease

Relationships between human genotype and human disease certainly exist, but are still difficult to assess because of the host immune status and genetic background (Suzuki *et al.*, 1996; Mack *et al.*, 1999), and because of other factors of virulence such as the infecting dose or parasite stage. Type II strains, which are described as the most prevalent in human disease, are 'virulent' for immature

fetuses and for immunocompromised patients, but are also responsible for many asymptomatic or benign toxoplasmosis in more mature fetuses and probably for most of the asymptomatic infections in immunocompetent patients in Europe. This may also be the case for type III strains, although we have very few reports of human disease associated with this genotype. Type I strains have certainly been associated with higher virulence in some patients (acquired ocular disease, cases of disseminated congenital toxoplasmosis), but their detection in cases of reactivation of chronic infection in immunocompromised patients or in placentas with no congenital infection suggests that they can also be responsible for asymptomatic infections in immunocompetent patients. The situation for atypical or recombinant strains in humans is even more complex, as it may depend on the combination of genes of the different types. It has been suggested that in recombinant genotypes, virulence is associated with the presence of a majority of type I alleles. Such human infections with natural recombinant strains, together with experiments in mice with natural or experimental recombinants, will in the future reveal the genetic bases of traits that lead to higher virulence in humans.

3.8 CONCLUSIONS

The population structure of *Toxoplasma* has been analyzed with a variety of molecular probes. They reveal a clonal population structure in isolates in North America and Europe, with three dominant strain types. These strains are sustained by a peridomestic transmission, and appear to have arisen from one or a few crosses between ancestral isolates and then spread together with domesticated species. Outside this geographical region the greater complexity of genotypes and strains isolated from South America appears to show greater evidence of recombination. Large areas of the inhabited world, such as Africa or Asia, remain totally unexplored. It is likely that Toxoplasma, which has multiple forms of transmission, can adapt its phenotype to different environments, and that either clonal or sexual reproduction might prevail in different habitats. There is need for more extensive sampling and multilocus analysis before drawing a definitive conclusion. The priority for the future is to bring together population genetic and phenotypic analysis in order to predict the distribution, transmission cycle in the environment, and pathogenesis (virulence) of strains in humans.

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Clinical Disease and Diagnostics

E. Petersen and O. Liesenfeld

4.1 Introduction

- 4.2 Clinical disease
- 4.3 Diagnosis of infection with *Toxoplasma* gondii in the human host

4.4 Treatment of toxoplasmosis *References*

4.1 INTRODUCTION

The first human case ascribed to infection with *T. gondii* was a child with hydrocephalus reported by Jankû in 1923 (Jankû, 1923). Systemic infection with *T. gondii* was first reported in 1940 (Pinkerton and Weinman, 1940), and Sabin reported the first case of encephalitis due to *T. gondii* (Sabin 1941). Lymphadenopathy was recognized as a key symptom by Siim (1951) and Gard and Magnusson (1951). Encephalitis due to *T. gondii* in immuno-compromised patients was first reported from patients with Hodgkin's disease during immunosuppressive treatment (Flament-Durand *et al.*, 1967).

The term '*T. gondii* infection' describes the asymptomatic course of infection observed in the majority of infected immunocompetent individuals. In contrast, 'toxoplasmosis' describes the symptomatic course of infection with *T. gondii. T. gondii* infection and toxoplasmosis are discussed here in the setting of immunocompetent and immunocompromised patients

4.2 CLINICAL DISEASE

4.2.1 Immunocompetent patients

4.2.1.1 Adults with acute acquired T. gondii *infection, including pregnant women*

Acute acquired infection in immunocompetent individuals most often manifests as asymptomatic diseases (*T. gondii* infection). Only a minority of individuals acutely infected with *T. gondii* present with signs or symptoms of the infection (Remington, 1974). The symptoms vary from a short, selflimiting, unspecific illness, to severe symptoms with prolonged fever, fatigue, and retinochoroiditis (Masur *et al.*, 1978; Teutsch *et al.*, 1979; Benenson *et al.*, 1982; Luft and Remington 1984; Bowie *et al.*, 1997). Cervical lymphadenopathy is the most common clinical presentation; lymph nodes are discrete and non-tender, and measure only a few centimeters in diameter (McCabe *et al.*, 1987). Rare manifestations of toxoplasmosis in apparently immunocompetent individuals include pneumonitis, hepatitis, myocarditis, polymyositis, and fever of unknown origin.

Toxoplasmosis may account for 5 percent of clinically significant lymphadenopathy cases (McCabe et al., 1987). Fever, malaise, night sweats, myalgias, sore throat, and hepatosplenomegaly may be observed, and small numbers of circulating atypical lymphocytes seen. Adenopathy and symptoms usually resolve within a few months to, at most, a year. These nodes are firm, are not fixed to the tissue, and do not suppurate. Biopsy demonstrates follicular hyperplasia, epithelioid histiocytes blurring the margins of the germinal centers, focal distention of sinuses with monocytoid cells, and occasionally cysts or tachyzoites of T. gondii. Occasionally, acquired infection may be associated with myositis or a sepsis-like syndrome.

Toxoplasma retinochoroiditis may be the result of postnatally or congenitally acquired infection. Involvement of the eye occurs during the acute stage of infection, and reactivation is observed during the latent stage of the infection. Patients presenting with acute eye lesions caused by T. gondii more frequently show postnatally acquired rather than congenital infection (M. Holland, Congress of the European Association for Vision and Eye Research, Vilamoura, Portugal, 2005). Most commonly, blurred vision, scotoma, pain, photophobia, and epiphora are present, and may result in complete or partial loss of vision or in glaucoma (Holland et al., 1996; Remington et al., 2001). Involvement of the macula results in impairment or loss of vision. Retinochoroiditis, due to T. gondii, is a relapsing disease, owing to reactivation of latent infection. During reactivation, bradyzoites (in cysts) transform to tachyzoites, which proliferate and cause acute chorioretinitis, and this is associated with symptoms of blurred vision, scotoma, pain, photophobia, and epiphora. It is not clear whether impaired vision in adult patients is mainly due to congenital infection with T. gondii or to acquired infection. One study in the United Kingdom found a lifetime risk of symptomatic T. gondii eve disease of approximately 2 per 10 000, and a 100-fold higher risk in persons born in West Africa but living in the UK (Gilbert *et al.*, 1999). Eye disease due to *T. gondii* is more widespread in Brazil, and a recent study found a prevalence of retinochoroiditis in adults of 1.2 percent (de Amorim Garcia *et al.*, 2004).

Acute *T. gondii* infection in pregnant women does not differ from *T. gondii* infection in other immunocompetent individuals. The infection is most commonly asymptomatic, although cervical lymphadenopathy may occur. Due to the asymptomatic course, acute infection often goes unnoticed and therefore may be transmitted to the fetus.

4.2.1.2 Congenitally infected children

During the 1940s there was improved understanding that maternal infection resulted in congenital toxoplasmosis in newborns. In 1953, Feldman reported a series of 103 children of whom 99 percent had eye lesions, 63 percent had intracranial calcifications, and 56 percent had psychomotor retardation (Feldman, 1953). These observations initiated interest in congenital infection among scientists in Europe (Couvreur, 1955). In Gothenburg, Sweden, 50 percent of mothers had had previous infection with T. gondii and 2 out of 23260 children had clinical toxoplasmosis during a 1948-1951 study period (Holmdahl and Holmdahl, 1955). A study from Austria reported frequent symptoms in children with congenital toxoplasmosis (Eichenwald, 1957). A French study concluded that treatment prevented transmission from mother to child and reduced the clinical symptoms in children (Couvreur and Desmonts, 1962), and another study from France showed that the seroprevalence in pregnant women in Paris was 85 percent, with a high risk of Toxoplasma infection in seronegative women (Desmonts et al., 1965). This was followed by a larger study from France of 374 pregnancies (Desmonts and Couvreur, 1974).

Congenital infection results when acquired *T. gondii* infection occurs in seronegative pregnant women. Women who are seropositive before conception do not, as a rule, transmit infection to the fetus. There have, however, been a few rare

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cases reported of congenital infection where the mother acquired infection 1–2 months before conception (Vogel *et al.*, 1996). In addition, immuno-compromised women (HIV-infected women, or women treated with corticosteroids) may transmit latent infections during pregnancy, resulting in congenital infection (Mitchell *et al.*, 1990).

The clinical manifestations of congenital toxoplasmosis vary depending on the trimester in which the infection was acquired. There is an inverse relationship between the rate of transmission and the severity of the infection (Dunn et al., 1999). Infections acquired shortly before or around the time of conception in almost all cases do not result in transmission to the fetus. Infections acquired in the first trimester will result in congenital infection in 10-25 percent of fetuses (Dunn et al., 1999). The rate of transmission rises to 30-50 percent in those women infected in the second trimester, and 60-70 percent for those infected in the third trimester. Treatment of the mother with antiparasitic drugs may reduce the incidence of congenital infection (Gilbert et al., 2003).

The clinical manifestations of congenital toxoplasmosis are most severe if infection is acquired before week 26 of gestation. In these cases, the central nervous system is commonly affected; non-specific signs include retinochoroiditis, strabismus, blindness, epilepsy, psychomotor or mental retardation, encephalitis, microcephaly, intracranial calcification, hydrocephalus anemia, jaundice, rash, and petechiae due to thrombocytopenia (Remington *et al.*, 2001). Newborns infected in the third trimester may be asymptomatic at birth, but sequelae (such as retinochoroiditis) often develop later in life.

A recent European multicenter study found that out of 244 newborns with congenital toxoplasmosis, 19 had cerebral calcifications; treatment before 4 weeks of diagnosis reduced the risk of this finding (Adjusted Odds Ratio 0.28; CI 0.08–0.75) (Gras *et al.*, 2005). The same study found retinochoroiditis in 12 percent (30/255) of newborns with congenital toxoplasmasis; however, treatment did not reduce the risk of retinochoroiditis (Gras *et al.*, 2005).

4.2.2 Immunocompromised patients

4.2.2.1 HIV-infected patients

The increased frequency of Toxoplasma encephalitis in patients with AIDS was reported soon after the start of the HIV epidemic (Roue et al., 1984; Enzensberger et al., 1985; Suzuki et al., 1988), and Toxoplasma encephalitis was an important endstage cause of death in HIV patients before the introduction of HAART (Highly Active Anti-Retroviral Therapy). Toxoplasmosis in patients with AIDS is most often the result of reactivation of latent disease following rupture of cysts in the central nervous system. The frequency of reactivated toxoplasmosis depends on the rate of seroprevalence and, apparently, the concentration of IgG antibodies (Derouin et al., 1996; Hellerbrand et al., 1996). Patients with reactivated toxoplasmosis often present with signs and symptoms of disease in the brain and/or the eye (Liesenfeld et al., 1999). Acute acquired infection in AIDS patients has been reported and may involve multiple organs. Toxoplasma encephalitis is the most common presentation of reactivated toxoplasmosis in AIDS patients (Luft and Remington, 1992). The disease most often presents with subacute focal deficits, including hemiparesis (39-52 percent). Altered mental state (30-42 percent), seizures (15-29 percent), cranial nerve disturbances (7-28 percent), abnormalities of speech (6-26 percent), cerebellar signs (9-30 percent), meningismus (10-16 percent), and behavioral/psychomotor manifestations (30-42 percent) including psychosis, dementia, and anxiety have been observed.

Pulmonary toxoplasmosis presenting as febrile illness with cough and dyspnea has been reported (Rabaud *et al.*, 1996).

4.2.2.2 Cardiac and kidney transplants

Patients with organ transplants or malignancy may also get central nervous system or pulmonary involvement, or myocarditis, due to reactivation of disease. The majority of *T. gondii* infections in immunocompromised hosts are reactivations of previous infections (Mele *et al.*, 2002). The seroprevalence is low in northern Europe, less than 2 percent in northern Sweden (Forsgren *et al.*, 1991; Evengård *et al.*, 2001), and higher further south – up to 60 percent in Poland (Paul *et al.*, 2000) – as well as being strongly age-dependent. Clinical signs of infection are similar to those in AIDS patients, and involve the brain, heart, and lungs. The organisms may also be present in eyes, liver, pancreas, adrenal glands, and kidneys. The initial presentation is often fever. *Toxoplasma* infection has also been described after heart, kidney, and liver transplantations (Aubert *et al.*, 1996; Giordano *et al.*, 2002; Renoult *et al.*, 2004; Wulf *et al.*, 2005). In most cases, infection manifests within 3 months following transplantation.

Seronegative patients may be infected with T. gondii through transplanted organs of seropositive donors. More rarely, seropositive transplant recipients may reactivate their latent infection due to the transplant-related immunosuppression. Thus, the frequency of transplant-related infection depends on the seroprevalence of infection with T. gondii in the population. Gallino et al. (1996) reported that 78 percent (14/16) of infection in T. gondii-naïve seronegative recipients receiving a cardiac transplant from a T. gondii-positive donor seroconverted; in contrast, only 10 percent (6/59) donor-negative-recipient-positive cases developed serological evidence of Toxoplasma infection. The use of antiparasitic prophylaxis also impacts the rate of infection. A review of 257 heart transplants between 1985 and 1993, and 33 heartlung transplants, found that 4.5 percent (13) were donor Toxoplasma-positive, recipient-negative, of which 9 were followed up and only 1 patient seroconverted. All patients received trimethoprim/ sulfamethoxazole prophylaxis for pneumocystis (Orr et al., 1994). In patients receiving a cardiac transplant, six weeks of pyrimethamine prophylaxis reduced infection from 57 percent (4/7) to 14 percent (5/37) (Wreghitt et al., 1992).

4.2.2.3 Bone marrow and hematopoietic stem cell transplants

The prevalence of *T. gondii* in BMT patients also varies with the seroprevalence in the population

(ranging from 0.5 percent in the USA to 5 percent in France). Toxoplasmosis in patients with allogeneic stem-cell or bone-marrow transplantation was previously considered a rare event. Most patients are seropositive for T. gondii before transplantation, and reactivate the latent infection. Symptoms of T. gondii infections in bone-marrow transplant patients include fever (43 percent), seizures (14 percent), headaches (13 percent), confusion (13 percent), and pulmonary symptoms (12 percent); 92 percent had more than one symptom, and the average onset was 62 days post-BMT (range 1-689) (Mele et al., 2002). Mortality rates are high (Chandrasekar et al., 1997). The European Group for Blood and Bone Marrow Transplantation reported on 106 allogenic stem-cell transplants, of which 55 percent of the donors were Toxoplasma IgGpositive. All received prophylaxis with trimethoprim and sulfamethoxazole for 6 months, and 15 percent (16/106; 95 percent CI: 8-21 percent) had at least one T. gondii PCR-positive blood sample and 6 percent (6/106; 95 percent CI: 1-10 percent) experienced clinical disease due to T. gondii (Martino et al., 2005). The median time to diagnosis from onset of symptoms was 42 days (range 1-178 days). The presenting symptoms were localized encephalitis in four patients and pulmonary toxoplasmosis in one patient, and one patient presented with acute disseminated disease (Martino et al., 2005).

4.3 DIAGNOSIS OF INFECTION WITH TOXOPLASMA GONDII IN THE HUMAN HOST

Congenital infection of the fetus in women infected just before conception is extremely rare, and even during the first few weeks of pregnancy the maternal–fetal transmission rate is only a few percent (Dunn *et al.*, 1999). Strategies for control and prevention of congenital toxoplasmosis vary between countries, and the diagnostic challenges are different in pre- and neonatal screening programs.

Systematic prenatal screening is performed in Austria, France, and Slovenia, and widespread on-demand screening takes place in Belgium, Germany, Italy, and Spain. Samples are obtained during pregnancy and analyzed for *Toxoplasma*specific IgM and IgG antibodies. When seroconversion is detected, the mother is infected and treatment is usually started. The biggest diagnostic challenge is the situation when *Toxoplasma*specific IgG and IgM antibodies are found in the first sample after conception, where the time of infection is the key to estimating whether the fetus is at risk or not (Ades, 1991).

Neonatal screening for congenital toxoplasmosis is performed in New England, Denmark, and parts of Brazil by analyzing the blood samples obtained on filter paper (Guthrie cards) on the fifth day postpartum (Guerina et al., 1994; Lebech et al., 1999; Neto et al., 2004). Detection of Toxoplasma-specific IgM antibodies eluted from the PKU filter paper is followed by a request for a blood sample from both the mother and infant for confirmatory testing (Sørensen et al., 2002). Of these sera, 15-20 percent are found to be negative for Toxoplasma-specific IgM (Naot et al., 1981; Decoster et al., 1992; Lebech et al., 1999). Low levels of Toxoplasma-specific IgM antibodies may be found for up to several years after acute infection, and the mere demonstration of low levels of Toxoplasma-specific IgM antibodies is therefore not regarded, by itself, as a sign of acute infection with T. gondii (Liesenfeld et al., 1997, 2001a, 2001b).

The measurement of the avidity of IgG antibodies was first demonstrated for T. gondii infections in 1989 (Hedman et al., 1989, 1993; Lecolier and Pucheu, 1993), and has since then been further developed (Dannemann et al., 1990; Marcolino et al., 2000; Beghetto et al., 2003; Petersen et al., 2005); however, it does not work at the beginning of the infections, with low levels of IgG antibodies (Press et al., 2005). Since previous studies have shown that some individuals have low-avidity IgG antibodies many months after infection (Petersen et al., 2005), the hypothesis whether treatment influences the maturation of IgG-antibodies was also tested. Based on just 12 untreated patients, it seems that treatment and/or pregnancy may delay the IgG maturation (Petersen et al., 2005).

A study of the diagnostic value of different diagnostic tests for acute infection with *T. gondii*, including *Toxoplasma*-specific IgG, IgM, and IgA antibodies, and the IgG-avidity index, demonstrated that the combination of a sensitive test for *Toxoplasma*-specific IgM antibodies and a *Toxoplasma*-specific IgG-avidity assay had the highest predictive value of the time of infection (Robert *et al.*, 2001).

4.3.1 *Toxoplasma* antigens and diagnostic assays

T. gondii is a complex protozoan parasite with three distinct life-stages, each with stagespecific expression of antigens (Kasper and Ware, 1989; Singh et al., 2002). The most immunodominant antigen is the tachyzoite-specific surface antigen 1, SAG1 (previously known as P30), which comprises up to 5 percent of the protein of the tachyzoite (Burg et al., 1988). The antigen expressed in E. coli has been shown to be recognized by natural SAG1-antibodies (Harning et al., 1996), and SAG1 is considered a prime candidate antigen in diagnostic tests because of its immunodominance and lack of known cross-reactivity to antigens from other microorganisms. Other surface antigens (SAG2, SAG3, and SAG4) have been identified, SAG2 and SAG3 being tachyzoite-specific, and SAG4 bradyzoite-specific (Cesbron-Delauw et al., 1994; Cesbron-Delauw, 1995; Howe and Sibley, 1994; Odberg-Ferragut et al., 1996). Two other groups of T. gondii antigens have been studied for use in diagnostic assays: the dense granule antigens, GRAs, in particular GRA1 and GRA6 (Lecordier et al., 2000) and GRA7 (Fischer et al., 1998); and the microneme antigens, MICs (Garcia-Règuet et al., 2000; Lourenco et al., 2001; Cerede et al., 2002). The MIC antigens have also been shown to be important in the induction of protective immunity (Beghetto et al., 2005). Bradyzoite-specific antigens like the bradyzoite antigen 1, BAG1 (Bohne et al., 1993), and matrix antigen 1, MAG1 (Parmley et al., 1994), should in theory be important in the antibody repertoire in infections past the acute stage, but it remains to find their place in future diagnostic assays. The diagnostic value of oocyst-specific
antigens has been studied in a single study of *T. gondii* oocyst-infected cats, but has not yet been tested in humans (Dubey *et al.*, 1995).

A summary of laboratory findings in the diagnosis of toxoplasmosis is found in Table 4.1.

4.3.2 The historical development of diagnostic assays

The complement fixation assay, CFA, was the first diagnostic test for Toxoplasma-specific antibodies (Warren and Sabin, 1942; Steen and Kåss, 1951). The dye test described by Sabin and Feldman (1948) is based on antibody-mediated killing of live T. gondii parasites in the presence of complement. If antibodies are present in the sample, the parasites are made penetrable for methylene blue and are colored in the presence of complement; if antibodies are not present, the parasites remain unstained against the blue background of the methylene dye. The dye test, DT, has proved a very sensitive assay, but the requirement for live T. gondii parasites makes it difficult and expensive to perform, and the test is now only performed in a few reference laboratories (Reiter-Owona et al., 1999). The DT is not included in reference panels circulated as part of external quality control programs, and multicenter studies show a considerable variability (Petithory *et al.*, 1996; Reiter-Owona *et al.*, 1999; Rigsby *et al.*, 2004). Immunofluorescence assays, IFA, were introduced in the 1960s (Ambroise-Thomas *et al.*, 1966) and proved specific, but with a lower sensitivity compared to the DT. The IFA for *Toxoplasma*-specific IgM antibodies is still used by some centers because it is highly specific, but it has a low sensitivity (Robert *et al.*, 2001).

The Enzyme Immuno Assay technique, EIA, became available in 1972 (Engvall and Perlmann, 1972). The first *Toxoplasma*-specific IgM assay was developed by Remington and Miller (1966), and the first EIA-based assay by Naot and Remington (1980). By the end of the 1980s the direct EIA measuring *Toxoplasma*-specific IgG antibodies and the μ -capture EIA measuring *Toxoplasma*specific IgM antibodies were well established in reference centers, and the first commercial test produced by industry had been introduced (Schaefer *et al.*, 1989). The μ -capture IgM assays were an improvement over the direct EIA IgM

TABLE 4.1 Summary of laboratory findings in the diagnosis of toxoplasmosis

- Acute infection (e.g. lymphadenitis): IgM serology is positive; serial specimens will demonstrate an increasing IgG titer.
- Acute toxoplasmosis in pregnancy: the serologic response is identical to that seen in other patients with acute *T. gondii* infection; however, the issue for the transmission of disease is whether infection occurred during the pregnancy, as IgM can persist for months after an acute infection and the risk for transmission is only present if infection occurred during the pregnancy. The use of IgG-avidity (low in acute infection) and differential agglutination, as well as the presence of IgA and/or IgE antibodies (which disappear sooner than IgM), can help to define the time of infection and the need for an evaluation of congenital transmission *in utero*.
- Chronic infection: IgM is negative, and IgG is present and does not change with serial specimens.
- Reactivation of disease in immune suppression (e.g. *Toxoplasma* encephalitis in HIV/AIDS): IgM is negative and IgG is present. In rare cases no detectable serologic response to *T. gondii* may be seen; however, the absence of serology is an indication to obtain a tissue sample for diagnosis. While the sensitivity of PCR has been variable in this setting, if PCR is positive it is useful for diagnosis.
- Congenital toxoplasmosis: for diagnosis *in utero*, the procedure of choice is PCR of amniotic fluid combined with ultrasound imaging of the fetus. Newborns will be IgG positive, due to maternal antibodies. The presence of IgM or IgA in a newborn confirms the diagnosis of congenital infection. Serial serology with a stable or rising IgG titer also confirms the diagnosis of congenital disease.

assays, but continued to have problems with falsepositive results (Liesenfeld *et al.*, 1997). The development of the Immunosorbent Agglutination Assays, ISAGA, solved this by using whole-cell formalin-fixed *T. gondii* (Pouletty *et al.*, 1984), and tests based on this technique are regarded as highly sensitive and specific for *Toxoplasma*specific IgM and IgA antibodies (LeFichoux *et al.*, 1984; Pouletty *et al.*, 1985). Immunoblot using single antigens has also been tested as a means of improving diagnostic sensitivity (Gross *et al.*, 1992).

A method to measure the maturation of Toxoplasma-specific IgG antibodies to determine the time of infection was described by Hedman et al. (1989). The test explores the increasing avidity (sum of all affinities) of the specific IgG antibodies with the maturation of the immune response, and in the original study it was shown that the time of infection could be determined within a 3-month window after infection. The test has been adapted to automated systems (Petersen et al., 2005). Newer IgG-avidity tests allow exclusion of an acute infection within the last 3-4 months in patients with high-avidity antibodies. In contrast, the presence of low- or intermediate-avidity IgG antibodies does not necessarily allow diagnosis of an acute infection since the maturation of IgG antibodies may show marked differences between individuals. The same principle is used in the differential agglutination test (Thulliez et al., 1989).

4.3.3 Diagnosis of *Toxoplasma* gondii infection in pregnant women

In countries where prenatal screening programs are in place, a test of the first blood sample from the pregnant women for *Toxoplasma*-specific IgM and IgG antibodies is performed. Approximately 5 percent of seropositive women in the first trimester have *Toxoplasma*-specific IgM antibodies, but only approximately 4 percent of these give birth to a child with congenital *Toxoplasma* infection. It is therefore a considerable problem to determine whether women with specific IgM antibodies are infected before or after conception. This is particularly the case in countries where testing of pregnant women at the beginning of pregnancy is common. This problem has been partly solved by obtaining two samples from pregnant women to see whether there is any development of the immune response. It is generally agreed that there is development of the *Toxoplasma*-specific IgG antibody response within the first 8 weeks after infection, after which the IgG levels are maintained at a high level, with or without declining IgM antibodies (Jenum *et al.*, 1997, 1998).

The problem of too many low-level *Toxoplasma*specific IgM-positive patients and the question of whether the diagnostic performance could be improved by repeating the same tests 2 weeks apart were investigated in a European multicenter study (Robert *et al.*, 2001). All highly sensitive assays were found to have a low specificity, and single tests were unable reliably to distinguish between acute and latent infections. Only the sequential analysis of sera by a highly sensitive IgM assay in combination with IgG-avidity testing gave excellent diagnostic performances. In contrast, IgA or IgM assays were less useful in diagnosing acute infections by confirming positive IgM results.

4.3.3.1 IgG-avidity index

In a European multicenter study, many laboratories contributed samples from patients for whom the time of infection was known. This panel was used to determine the proportion of sera showing specific IgM and IgA antibodies to *T. gondii* (Pouletty *et al.*, 1984), as well as the IgG-avidity index within 1–3 months, 3–12 months, or more than 12 months after seroconversion. These data were used to propose a two-level strategy for diagnosis (Robert *et al.*, 2001).

4.3.3.2 Combined two-test strategies

Robert *et al.* (2001) demonstrated that the best strategy for diagnosing acute and recent infection with *T. gondii* was a two-test strategy, with a sensitive IgM test first, followed by an IgG-avidity test. Thus the study confirmed the need for the *Toxoplasma*-specific IgG-avidity index assay in the diagnosis of acute and recent infection.

The increased use of the *T. gondii* IgG-avidity test has highlighted an inherent problem with the test, in that many pregnant women have long-lasting low IgG-avidity antibodies; the IgG-avidity assay therefore needs further development, which could be by the use of recombinant antigens (Beghetto *et al.*, 2003).

4.3.4 Improvement of EIA tests for *Toxoplasma*-specific IgG and IgM antibodies

The problems with IgM-based diagnostic tests in T. gondii infections have resulted in attempts to improve them. The accepted reference test is the ISAGA, but most analyses are performed with an EIA capture test. The assays use whole-cell, lysed T. gondii as antigen, and attempts have been made to improve the test by using recombinant antigens (Ferrandiz et al., 2004). The ISAGA IgM and EIA IgM, and IgM immunofluorescence, were evaluated in a prospective European cohort study, EMSCOT, of women diagnosed with primary T. gondii infection during pregnancy and newborns identified through neonatal screening. The EMSCOT study provided data on sensitivities for diagnosing congenital infection in the newborn of four Toxoplasma-specific IgM-antibody assays and three Toxoplasma-specific IgA-antibody assays. The study also provided data on the sensitivity of neonatal testing related to the estimated gestational age of infection with T. gondii, showing that the IgM seropositivity at birth only detects infections in the second half of pregnancy.

The study included 5223 samples from 996 children, of which 3742 were tested with an EIA system, 2011 with an ISAGA IgM, and 316 with the IgM-immunofluorescence assay. The children were followed for 1 year to ascertain the diagnosis by demonstrating the presence of *Toxoplasma*-specific IgG antibodies at 12 months of age, which is the gold standard for confirming congenital infection with *T. gondii*. The sensitivity for EIA tests was low – 29.3 percent – clearly demonstrating the need for better tests. The study was the first to provide data on the sensitivity of *Toxoplasma*-specific IgM antibodies related to

gestational age at infection. It was clearly demonstrated that a sensitivity of 50 percent is not reached until after the thirtieth gestational week (Petersen *et al.*, 2006a).

4.3.5 Recombinant IgG assays, adults

Conventional assays have so far used whole-cell, lysed *T. gondii* antigens, which have batch variations. With increasing emphasis on the need for reproducibility, the use of recombinant antigens in diagnostics assays provides a theoretical advantage.

Previous studies have shown that the GRA1, GRA7, and SAG1 molecules are immunodominant (Johnson et al., 1992; Harning et al., 1996; Jacobs et al., 1999; Aubert et al., 2000; Li et al., 2000). Pietkiewicz et al. (2004) demonstrated that recombinant antigens including a mixture of GRA1, GRA7, and SAG1 were not as sensitive as wholecell, lysed antigen if sera had an IgG titer of less than 1:1600 using an EIA test and less than 1:512 in an IgG-immunofluorescence test. The test did, however, have a 100 percent sensitivity in a panel of sera from individuals who had Toxoplasmaspecific IgM and/or IgA-antibodies (i.e. those in whom the infection was recent) (Pietkiewicz et al., 2004). Future assays for Toxoplasma-specific IgG antibodies relying on recombinant antigens need to include a panel of antigens, and the test with recombinant antigens has not yet been optimized to the same sensitivity as the whole-cell, lysed antigen assay.

4.3.6 Recombinant IgM and IgG assays, newborns

Diagnostic assays based on recombinant antigens for measuring the *Toxoplasma*-specific IgM antibodies were evaluated in infants with or without congenital toxoplasmosis born to mothers with toxoplasmosis acquired during pregnancy (Petersen *et al.*, 2006a). Antigen fragments from the MIC2, MIC3, M2AP, and SAG1 proteins were tested in an EIA test (RecEIA) on 104 serum samples from newborns born to mothers infected with *T. gondii* during pregnancy. Of these, 35 were

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congenitally infected, and 34 out of 35 (97 percent) serum samples from the congenitally infected patients reacted with at least one of the recombinant antigens (Buffolano *et al.*, 2006).

Remarkably, all sera from 22 *Toxoplasma*infected newborns who were clinically and serologically undiagnosed at birth were reactive using the IgM RecEIA analysis, allowing the confirmation of congenital toxoplasmosis as soon as 2 months after birth. The presence of *T. gondii*-specific IgM antibodies against recombinant MIC2, MIC3, M2AP, and SAG1 antigens may be used for the early postnatal diagnosis of congenital toxoplasmosis.

It was also found that the newborn *Toxoplasma*infected child primarily produces IgG_2 and IgG_3 against recombinant *Toxoplasma* antigens, whereas the maternally transferred antibodies were primarily IgG_1 (Buffolano *et al.*, 2006). Thus subclass analysis of serum samples from mother and child against defined recombinant antigens may further improve diagnosis of congenital *Toxoplasma* infection in newborns.

4.3.7 The *Toxoplasma*-specific IgG-avidity index

The maturation of the IgG response varies considerably between individuals. In the study of Lappalainen et al. (1993), two seroconverting mothers already had an IgG-avidity index above 20 percent at the time of diagnosis; however, most patients had developed an IgG-avidity index above 15 percent after 180 days (Lappalainen et al., 1993). A study from France found an average IgGavidity index of 0.2 in pregnant women infected within 5 months (Lecolier and Pucheu, 1993). The original method developed by Hedman et al. (1989) used serial dilutions tested in EIA with and without 6M urea, but automated assays today calculate the IgG-avidity index from two single measurements of the sample with and without urea. This introduces an uncertainty, although experiments with only two serum sample dilutions showed an excellent agreement with IgG-avidity measurements using four serial serum sample dilutions (Korhonen et al., 1999). Prince and Wilson (2001) evaluated the IgG-avidity assay using single-dilution assays with and without urea and showed that, because the signal obtained in an EIA system is not linear, it makes a difference whether the *Toxoplasma* IgG-avidity index is calculated from the OD values or the activity measured in international units of *Toxoplasma*-specific IgG antibodies per ml (Prince and Wilson, 2001).

The IgG-avidity results found in one study (Petersen et al., 2005) demonstrated that a persistent low IgG-avidity index poses a diagnostic problem, at least in pregnant women receiving treatment during pregnancy (Petersen et al., 2005). Up to half of the patients with acute infections may show a low or borderline IgG-avidity index 6 months after the infection (Rossi, 1998; Montoya et al., 2002), which is in concordance with the results reported in our study (Petersen et al., 2005). The LIAISON® avidity results were compared with the semiautomated VIDAS system for measuring the Toxoplasma-specific IgG-avidity index, and there was a good correlation between the results from the two systems, showing that persistence of low-level Toxoplasma-specific IgG-avidity antibodies is an inherent problem of measuring the Toxoplasmaspecific IgG-avidity index unrelated to the assay system.

The cut-off value defining a low IgG-avidity index differs markedly between different studies, and one study found that patients infected within the past 3 months had an IgG-avidity index of below 0.45 (Holliman *et al.*, 1994). A comparison between the VIDAS and the Labsystems IgG-avidity index showed a correlation coefficient of 0.6 in pregnant women but 0.88 in other patients (Alvarado-Esquivel *et al.*, 2002), but the difference was not further discussed. Improvement of the IgG-avidity assay using Western blot technique has been attempted, and revealed differences in the maturation of the specific IgG to different antigens (Villavedra *et al.*, 1999).

The IgG response matures rapidly in some individuals, and this has been reported in several studies. For instance, the cut-off of the *Toxoplasma*-specific IgG-avidity index using the VIDAS system (bioMérieux) was defined as 0.3 to ensure that all sera from acute infections had a low-avidity index (Pelloux *et al.*, 1998). The same study showed that,

at least in pregnant women, a low IgG-avidity index persisted up to 9 months post-infection, and all women were treated. In a study of T. gondiiinfected pregnant women identified prospectively through prenatal screening, Jenum et al. (1997) found that 2 out of 73 women had an IgG-avidity index above 0.2 before 20 weeks of gestation, but many continued to have a low IgG-avidity index even a year after infection. It is assumed that all women were treated during pregnancy. The IgG-avidity results found in one study (Petersen et al., 2005) demonstrated that long-lasting low IgG-avidity was a common finding in pregnant women. The study compared the IgG-avidity maturation in treated pregnant women with samples from patients with acute infection with T. gondii who were not pregnant and were not treated, and found a significantly more rapid IgG maturation during the first 4 months after infection in subjects who were not treated and not pregnant (Petersen et al., 2005).

The observation that the *Toxoplasma gondii*specific IgG maturation is delayed in treated pregnant women compared to non-treated nonpregnant individuals has been reported in one previous study, which found significantly delayed IgG maturation in treated individuals (Sensini *et al.*, 1996). Our finding that treatment may influence the IgG-avidity maturation underlines the need for further studies to better clarify the avidity maturation process in pregnant women under therapy in comparison with untreated individuals. If confirmed, different cut-off values will have to be defined for treated and untreated and/or pregnant and non-pregnant individuals.

4.3.8 Molecular and other diagnostic techniques

The diagnosis of acute toxoplasmosis may be established by the detection of anti-*T. gondii* antibodies by serological tests, or of tachyzoites or *T. gondii*-specific DNA in body fluids or tissue samples. In most cases of toxoplasmosis in immunocompetent individuals, diagnosis is established by serological tests; however, molecular (i.e. PCR) diagnostic tests have proven useful in

the diagnosis of infection *in utero* as well as in immunocompromised hosts.

The detection of T. gondii tachyzoite DNA in body fluids and tissues by PCR amplification is effective to diagnose congenital (Grover et al., 1990), ocular (Montoya et al., 1999), and cerebral toxoplasmosis (Holliman et al., 1990). PCR should be considered the gold standard for diagnosis of in utero infection. Sensitivity in initial reports was 100 percent, but subsequent studies have indicated this is very dependent on the gestational age of the infection (Switaj et al., 2005; Thalib et al., 2005). Sensitivity also varies with gene target (for example, the B1 gene is present at 35 copies, and AF146527 is present at 300 copies). In a French study of 2000 consecutive amniotic fluid samples, it has been confirmed that a positive PCR correlates with disease and that the PCR is more sensitive than any other available test (Thulliez, 2001). Isolation of T. gondii from blood or body fluids (e.g. CSF, or amniotic or BAL fluids) establishes diagnosis of the acute infection. Isolation can be performed by inoculation of the samples in mice or in tissue cultures. The demonstration of tachyzoites in histological sections or smears of body fluids by immunoperoxidase staining with anti-T. gondii antibodies also establishes the diagnosis (Conley et al., 1981). This technique has been very useful in the diagnosis of CNS mass lesions in the setting of HIV/AIDS.

4.3.9 Diagnosis of *Toxoplasma* gondii infection in live-born neonates

Diagnosis of congenital infection with *T. gondii* is difficult at birth if *Toxoplasma*-specific IgM and/or IgA antibodies are not present, because present diagnostic methods can only distinguish between maternal and fetal IgG with difficulty. The traditional method of diagnosing congenital toxoplasmosis in IgM- and IgA-negative newborns is to wait up to 12 months and observe whether the maternal *Toxoplasma*-specific IgG antibodies disappear. If the child has been treated continuously with sulfadiazine and pyrimethamine the synthesis of *Toxoplasma*-specific IgG antibodies can be suppressed, and the serological confirmation of the infection can sometimes not be made with certainty before the second year of life (Wallon et al., 2001). This situation is found when T. gondii infection is suspected but Toxoplasma-specific IgM and/or IgA antibodies cannot be demonstrated in the child, and parasitological investigations like PCR analysis for Toxoplasma-specific nucleic acid is negative or not appropriate. IgM and IgA antibodies do not cross the placenta, and neonatal screening programs for congenital toxoplasmosis are based on the detection of Toxoplasma-specific IgM antibodies eluted from blood spots from PKU-filter papers (Guthrie cards) (Guerina et al., 1994; Lebech et al., 1999; Sørensen et al., 2002; Neto et al., 2004). Different cutoffs for maternal and newborn Toxoplasma-specific IgM antibodies have been proposed (Candolfi et al., 1993). It has been hypothesized that treatment of acute toxoplasmosis during pregnancy reduces the duration of the Toxoplasma-specific IgM response, but two studies have not found such an effect (Gras et al., 2004; Petersen et al., 2006a).

Demonstration of Toxoplasma-specific IgG antibodies with different specificities in sera from the mother and child shows that the child synthesizes his or her own IgG antibodies, confirming that the child is infected with T. gondii. Previous studies have shown that transferred maternal and neosynthesized T. gondii-specific IgG antibodies can be differentiated by immunoblot or immunocomplexing (Chumpitazi et al., 1995; Robert-Gangneaux et al., 1999; Gross et al., 2000; Remington et al., 2004). Differentiation of the specificities of IgG antibodies in the mother and child can also be carried out by comparing T. gondii antigen precipitated with maternal or child sera before performing an electrophoresis of the antigen-antibody complex (Pinon et al., 1996, 2001; Robert-Gangneux et al., 1999). The immunoblot technique and immunocomplexing were compared in a doubleblind study and found to be equally sensitive (Pinon et al., 2001). The immunoblot technique identifies newborns with congenital toxoplasmosis with a sensitivity of approximately 70 percent (Rilling et al., 2004; Tissot-Dupont et al., 2003), increasing to 85 percent within the first 3 months

of life (Gross *et al.*, 2000; Rilling *et al.*, 2004; Tissot-Dupont *et al.*, 2003). These results still leave 15–30 percent of congenitally infected newborns without a confirmed diagnosis.

To improve the diagnosis of congenital toxoplasmosis, a two-dimensional immunoblot (2DIB) assay was developed that is capable of distinguishing between maternal and neonate *Toxoplasma*specific IgG with a better sensitivity than previous assays (Nielsen *et al.*, 2005). The 2DIB methodology has greatly increased the resolution of the antibody response by allowing identification of up to 1000 spots, whereas the most sensitive immunoblots do not allow distinction of less than 50 bands – often considerably less (Nielsen *et al.*, 2005).

4.3.10 Immunocompromised patients

Because reactivation of latent Toxoplasma infection is the most common cause of toxoplasmosis in immunocompromised patients, detection of T. gondii IgG antibodies is indicated. Patients with a positive result are at risk of reactivation of the infection; patients with a negative result should be instructed on how they can prevent becoming infected. The most important factor in the management of the seropositive immunosuppressed patient is to consider T. gondii as a potential causative agent in patients presenting with nonspecific symptoms including focal symptoms from CNS, heart, lungs, and liver. Since immunocompromised patients do not reliably produce antibodies, serology has been replaced by direct detection methods such as PCR analysis of T. gondiispecific nucleic acid. The definitive diagnosis of toxoplasmosis relies on detection of T. gondii DNA by PCR or on histologic demonstration of the parasite. Whereas tachyzoites are diagnostic of the active infection, T. gondii tissue cysts may indicate latent infection. In patients with toxoplasmic encephalitis, blood, CSF, and brain tissue may be used to detect T. gondii-specific DNA. Sensitivities of PCR range between 25 and 80 percent for blood, and 35 and 100 percent for CSF samples (Parmley et al., 1992; Vidal et al., 2004; Colombo et al., 2005).

Pulmonary toxoplasmosis is well known in HIVinfected patients with low CD4+ T-cell counts (Rabaud et al., 1996). A study of bronchoalveolar lavage (BAL) samples from 332 Danish HIV-infected patients found 2.1 percent (7/332) positive samples using a new, sensitive, real-time PCR method (Petersen et al., 2006b). The patients were in an advanced stage of immunosuppression, with a mean CD4+ T-cell count of 39×10^6 per liter (range $0-161 \times 10^6$ per liter; normal values > 650 × 10⁶ per liter). Monitoring bone-marrow transplant patients by PCR on peripheral blood, BAL fluid, and CSF (according to local symptoms), and treatment with pyrimethamine for positive PCR results, reduced the mortality to the same levels as for T. gondiinegative BMT patients. The same strategy could be applied to other immunosuppressed patients at the risk of developing T. gondii infection, including those patients with HIV infection.

4.4 TREATMENT OF TOXOPLASMOSIS

With regard to therapy, it is useful to separate toxoplasmosis into several categories (Table 4.2). The decision to treat is based on the location of the infection, the immune status of the patient, and whether or not a woman with acute toxoplasmosis is pregnant. There are virtually no large, wellcontrolled clinical trials to establish the ideal therapeutic strategy; however, there have been several studies of prophylaxis and treatment for toxoplasmosis in the setting of AIDS or congenital disease. The recommended therapies are based on extrapolations from in vitro studies and animal models (mostly murine), and on the clinical experience and practice of physicians experienced in the treatment of T. gondii infection. The standard therapeutic agent for the treatment of toxoplasmosis is

 TABLE 4.2 Toxoplasma infections and treatment indications

Syndrome		Treatment ^a
1.	Asymptomatic infection, latent infection detected by serologic test being positive	Not required
2.	Adenopathy, fever or malaise in the immunocompetent host	Not required ^b
3.	Disseminated disease (i.e. CNS, heart, or hepatitis) in the normal host,	PYR/SULFA
	or laboratory infection with tachyzoites	
4.	Infection during pregnancy	SPR – PYR/SULFA ^c
5.	Congenital toxoplasmosis	PYR/SULFA ^d
6.	Ocular toxoplasmosis	PYR/SULFA and steroids
7.	Infection in immunocompromised hosts:	
	• AIDS	PYR/SULFA
	transplantation	PYR/SULFA
_	acute disease	PYR/SULFA

^aRecommended primary treatments as described in the text. PYR, pyrimethamine; SULFA, sulfonamides; SPR, spiramycin; steroids, corticosteroids.

^bPainful adenopathy may respond to indomethacin, prolonged adenopathy may respond to PYR/SULFA. ^cInfection during pregnancy as determined by seroconversion of the mother is treated with spiramycin. If the fetus is confirmed to have toxoplasmosis, by ultrasound, amniocentesis or cordocentesis, then PYR/SULFA is given, alternating with SPR.

^dCongenital toxoplasmosis is treated until the infant is 6–12 months old. Most experts recommend a year of therapy.

the combination of pyrimethamine and a sulfonamide (such as sulfadiazine) or, in the case of sulfonamide allergy, clindamycin. In general, the drugs to treat toxoplasmosis are active against the rapidly replicating tachyzoite stage and have limited efficacy against tissue cysts; therefore, patients treated for toxoplasmosis will have a latent infection at the conclusion of treatment.

Pyrimethamine is a substituted phenylpyrimidine - that is, an inhibitor of dihydrofolate reductase. The serum half-life of pyrimethamine is 35-175 hours, and serum levels on a dose of 25-75 mg per day range from 1 to 4.5 mg/l (Weiss et al., 1988). Serum levels for an individual are not predictable, due to the wide variation in absorption and serum half-life. CSF levels of pyrimethamine are 10-25 percent of the corresponding serum levels. Dose-related bone-marrow suppression with thrombocytopenia, neutropenia, and anemia may develop. Folinic acid (leucovorin) is routinely given at a dose of 5-10 mg/d orally to prevent these effects. Folinic acid does not inhibit the action of pyrimethamine on T. gondii, as this organism cannot take up folinic acid.

Sulfonamides inhibit dihydrofolic acid synthetase, which is another enzyme involved in folic acid metabolism; thus they are synergistic with pyrimethamine. These drugs are well absorbed, with good penetration into cerebrospinal fluid. Adverse reactions to sulfonamides are common, particularly in AIDS patients. Bone-marrow suppression is seen, and this responds to folinic acid. Hypersensitivity reactions with a rash and Stevens–Johnson syndrome have been reported.

4.4.1 Asymptomatic infection or latent infection

Immunocompetent individuals with latent toxoplasmosis as evidenced by positive serology do not require treatment. In AIDS patients who are seropositive for *T. gondii*, the risk of developing encephalitis has been estimated at 10–20 percent. Trimethomprim-sulfamethoxazole, or dapsone plus pyrimethamine, is effective in preventing encephalitis (Torres *et al.*, 1993; Bozzette *et al.*, 1995). In cardiac transplantation, prophylaxis with pyrimethamine for 6 weeks is used for *T. gondii* seronegative recipients receiving hearts from seropositive donors (Wreghitt *et al.*, 1992).

4.4.2 Acquired toxoplasmosis

In immunocompetent individuals treatment is rarely needed; however, in a rare patient whose symptoms are persistent, treatment should be as described for disseminated disease. Myocarditis, encephalitis, a sepsis syndrome with shock, and hepatitis are occasionally seen. In these patients, treatment should be given with pyrimethamine (100-mg loading dose and 25–50 mg/d) and sulfadiazine or trisulfapyrimidines (4–8 g/d) for 4–6 weeks. Folinic acid (5–10 mg/d) should also be given. Infections acquired through a laboratory accident or blood transfusion should also be treated as described above.

4.4.2.1 Acquired toxoplasmosis during pregnancy

Acutely infected pregnant women should be given 3 g/d of spiramycin in divided doses once maternal infection is suspected or diagnosed, to decrease transmission (McAuley et al., 1994). Spiramycin is a macrolide antibiotic that has activity against T. gondii due to its ability to affect apicoplast function. Amniocentesis, fetal blood monitoring, and fetal ultrasonography should be used to assess infection in the fetus. Ultrasonography should be done every 2-4 weeks, as ventricular dilatation may develop in as little as 10 days. If fetal toxoplasmosis is diagnosed by demonstration of fetal IgM, culture from amniotic fluid or fetal blood, PCR of amniotic fluid or fetal blood, or ultrasonographic evidence of ventricular dilatation, then specific therapy with pyrimethamine (25-50 mg/d), sulfadiazine (3-4 g/d), and folinic acid (5-15 mg/d) should be administered to the mother (McAuley et al., 1994); this can be alternated every 3 weeks with spiramycin (3 g/d) until delivery. The majority of infants born to women treated with this regimen had subclinical disease at birth. Pyrimethamine should not be used in the first 14-16 weeks of pregnancy due to concerns about teratogenicity.

4.4.3 Congenital toxoplasmosis

At present, a clinical trial is in progress to address the optimal therapy and duration of therapy for children with congenital toxoplasmosis (R. McLeod, personal communication). Published results from this study suggest that treatment has benefit for congenital infection compared to historical controls (McAuley et al., 1994; McLeod et al., 2006). It has been demonstrated that neonates who appear normal at birth (subclinical disease) may later demonstrate serious sequelae (primarily retinochoroiditis). Congenital toxoplasmosis can be managed by treatment with a loading dose of pyrimethamine (PYR) of 2 mg/kg per day for 2 days, followed by PYR 1 mg/kg per day for 2 months, and then PYR 1 mg/kg every other day for the next 10 months. In addition, sulfadiazine or trisulfapyrimidine at 100 mg/kg per day in two divided doses and folinic acid 5 mg every other day is administered. Corticosteroids (1 mg/kg per day) should be added in patients with active macular disease or active CSF profiles (CSF protein >1 g/dl).

4.4.4 Ocular toxoplasmosis

There have been limited trials of the different antitoxoplasmal drugs in this disorder (Holland *et al.*, 1996). Since the retinochoroiditis is self-limiting, comparative trials are essential. Pyrimethamine and sulfadiazine appear to be effective in decreasing inflammation, but do not appear to shorten the time course of the retinitis. Corticosteroids (prednisone 1–2 mg/kg per day) are indicated if the macula, optic nerve head, or papillomacular bundle are involved. When prednisone is given, it is tapered when pigmentation (healing) begins. Clindamycin (1200 mg/d) has been used as an alternative drug, but was found to be inferior to pyrimethamine/ sulfadiaine (Tabbara and O'Connor, 1980).

4.4.5 *Toxoplasma* infection in immunocompromised hosts

The treatment for encephalitis due to *T. gondii* is pyrimethamine 200 mg (loading dose) followed by

50-75 mg/d, with sulfadiazine 1-1.5 g every 6 hours and folinic acid 10-25 mg/d (Liesenfeld et al., 1999). In patients intolerant of sulfadiazine, clindamycin 600-1200 mg every 6 hours can be used with pyrimethamine (Remington et al., 1991). Alternative combinations with reported efficacy in case reports include pyrimethamine with one of the following: clarithromycin 1 g every 12 hours; atovaquone 1250 mg every 12 hours; azithromycin 1200 mg every day; or dapsone 100 mg every day. A recent meta-analysis of published studies on the use of trimethoprim-sulfamethoxazole for the treatment of Toxoplasma encephalitis concluded that, especially in resource poor environments, the outcome of therapy with this agent is similar to that of pyrimethamine and sulfadiazine (Dedicoat and Livesley, 2006).

Therapy is often started empirically, and response is expected within 14 days. If no response is seen, then brain biopsy is required for diagnosis. Corticosteroids are often used to control intracranial hypertension due to mass effect. Desensitization to sulfadiazine has been reported to be successful. In about 30 percent of patients relapse of encephalitis occurs when treatment is stopped, although relapse may take several weeks. Patients are therefore maintained on pyrimethamine 25 mg/d, sulfadiazine 4 g/d, and folinic acid 10 mg/d, after they have completed a 6–8-week course of primary treatment. With the use of antiretroviral therapy, if the CD4+ lymphocyte count is restored to over 200, secondary prophylaxis can be discontinued.

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Ocular Disease Due to *Toxoplasma gondii*

P. Latkany

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5.1 INTRODUCTION

Even though ocular toxoplasmosis is the most common etiology of posterior uveitis in the United States and the world, it remains a poorly understood disease. For instance, there is limited understanding as to why macular lesions are common in congenitally infected individuals, limited understanding regarding the details of ocular recurrences, and there is no agreement on best treatment. There is no regimen that can eliminate the bradyzoite stage of infection; therefore, once an individual is infected by *Toxoplasma gondii* the retina is randomly undetectably 'seeded', resulting in a chance for local recurrences in the future. There is irreversible damage to the involved retina where a recurrence occurs. If a recurrence occurs within the central macula, the consequence is severe visual morbidity.

Toxoplasma gondii is one of the most common parasitic infections in the world, with as many as one-third of all humans being infected (Jackson and Hutchison, 1989; Holliman, 1997). Seroprevalence appears to be diminishing in some regions, and is less common in colder climates; the seropositivity is 22.5 percent in the United States and 74 percent in El Salvador (Montoya and Liesenfeld, 2004). Acquired toxoplasmosis is usually an asymptomatic systemic infection (Bowie et al., 1997); however, severe acquired disease has been reported (Carme et al., 2002). Several features, including size of inoculum (Liesenfeld, 1999), sex (Roberts et al., 1995), immune status (Suzuki et al., 1996), and virulence of organism (Su et al., 2002), influence the outcome of infection. Lymphadenopathy is the

most common manifestation of acute systemic infection.

Contrary to previous belief, it appears that most people with ocular toxoplasmosis are not congenitally infected but have been infected postnatally. The rate of ocular involvement in acquired toxoplasmosis was previously estimated to be no more than 3 percent (Perkins, 1973), but more recent data suggest that it is as high as 20 percent (Burnett *et al.*, 1998). Congenital infection with *Toxoplasma* can cause a range of effects, from fetal demise or fetal abnormalities to minimal symptomatic sequelae. The classic triad of signs suggestive of congenital toxoplasmosis are chorioretinitis, intracranial calcifications, and hydrocephalus (Jones *et al.*, 2001a).

Of the myriad signs and symptoms associated with congenital Toxoplasma infection, Toxoplasma chorioretinitis is the most common, being present in 70-90 percent of patients (Stagno et al., 1977; Couvreur et al., 1984; Koppe et al., 1986; McAuley et al., 1994). Indeed, chorioretinitis may be the sole symptomatic manifestation of congenital infection (Fair, 1958; Alford et al., 1974). Congenital infection is serious and should be managed by experts; estimates of the rate of occurrence range from 400 to 4000 births in the United States each year (Lopez et al., 2000). Almost one-fourth of eyes affected by ocular toxoplasmosis in congenital disease are legally blind, and congenital toxoplasmosis can cause central and even complete vision loss (Bosch-Driessen et al., 2000). No well-designed recent randomized controlled trials have been conducted of the treatment of Toxoplasma retinochoroiditis, and there is no widespread agreement regarding optimal therapeutic agents or the duration of treatment (St Georgiev, 1993).

Currently, there is considerable controversy concerning the treatment of ocular toxoplasmosis. Some clinicians reserve treatment for active disease unless there is imminent threat to a patient's vision – for example, the presence of lesions within the macula. There has even been controversy regarding the efficacy of medicines during acute illness (Gilbert *et al.*, 2002). One meta-analysis of three randomized controlled treatment trials found little evidence supporting the use of routine antibiotic treatment for acute *Toxoplasma* chorioretinitis (Stanford *et al.*, 2003). In addition, long-term treatment of patients with chronic recurrent *Toxoplasma* chorioretinitis remains controversial (Kopec *et al.*, 2003). There are confounding issues of chronic suppressive treatment that remain unexplored. For instance, it is not known whether the outcome in the treatment of chronic ocular disease is dependent on the strain of *T. gondii* – i.e. type I virulent strains or type II avirulent strains – causing the infection.

5.2 HISTORICAL FEATURES OF OCULAR TOXOPLASMOSIS

The first description of ocular disease as a result of *T. gondii* infection was in 1923 by Jankû. Levadit subsequently identified the parasite observed by Jankû as *T. gondii* (Levadit, 1928). At the time, ocular involvement was noted as a manifestation of congenital disease in newborns, but it was not until 1939 that this became widely appreciated (Wolf *et al.*, 1939). Rieger is credited with the origin of the concept of postnatally acquired *T. gondii*, as well as the theory that recurrence may be related to immunocompromised states (Rieger, 1951). These ideas were revolutionary in 1951, and remain topics of inquiry today.

In 1952, the link between *T. gondii* and eyes with chorioretinal lesions was confirmed by Helenor Campbell Wilder Foerster (Holland *et al.*, 2002). In the years between the Sabin–Feldman dye test's introduction and this discovery, the potential role of *T. gondii* as an unrecognized cause of ocular disease in adults was under suspicion. Vail (Vail and Stephenson, 1943), Frenkel (1949), and Rieger (1951) all described series of adult patients with chorioretinal lesions and positive *T. gondii* antibody tests. Frenkel noted positive *Toxoplasma* skin tests on patients with chorioretinal lesions (Frenkel, 1949).

Clear histological evidence was not given until Wilder presented her landmark case series. This case series of 53 eyes that had been enucleated due to pain and blindness unequivocally established a strong relationship between *T. gondii* and the particular characteristics of its ocular manifestations. Through careful laboratory techniques

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and persistent investigation, she not only provided some answers to the enigma of Toxoplasma chorioretinitis but also put forth central questions that remain unanswered. Each eye in Wilder's cohort had lesions that were granulomatous with central necrosis, and T. gondii was consistently found in the necrotic areas. Serologic testing on these patients revealed all of them to test positive for T. gondii antibodies. As a result of Wilder's work, ocular toxoplasmosis resulting from congenital infection became accepted as the leading cause of posterior uveitis in otherwise healthy adults. This work solidified the hypothesis that toxoplasmosis, not tuberculosis, causes ocular disease characterized by retinochoroidal lesions. Prior to Wilder's report, tuberculosis was routinely erroneously ascribed as the source of what Wilder ultimately demonstrated was actually ocular toxoplasmosis.

Postnatally acquired infection with ocular involvement, as well as ocular manifestations of congenital disease, were fully characterized by Hogan in 1958; however, during the 1960s Hogan and associates made the incorrect assumption that ocular symptoms of toxoplasmosis occur largely in the presence of systemic symptoms and rarely alone (Hogan et al., 1964). They recognized that a large population of patients with postnatally acquired T. gondii infection are asymptomatic and deduced that ocular involvement in patients with acquired infection was uncommon. The classic teaching that most (if not all) Toxoplasma chorioretinitis is congenital was given further support in 1973 when Perkins concluded that nearly all cases of Toxoplasma ocular involvement in the UK resulted from congenital infection (Perkins, 1973). Based on the belief that ocular involvement only occurs immediately after infection, episodes of recurrent chorioretinitis in children and adults were attributed to congenital infection that went undetected at birth (Hogan, 1961). Hogan, Perkins, and others based their studies on a set of assumptions that are now known to be incorrect, and their conclusions are now being challenged.

Later studies have refuted many of the earlier assumptions about the nature and course of *Toxoplasma* retinochoroiditis. For example, it is now understood that ocular disease is often the only manifestation of recent, postnatally acquired infection (Ongkosuwito *et al.*, 1999). Also, retinal lesions have been known to develop long after initial infection (Silveira *et al.*, 2001). These new data have led to a shift in some basic beliefs about this disease and the consideration of the possibility that most ocular toxoplasmosis is acquired (Holland, 1999; Gilbert and Stanford, 2000).

Treatment of ocular toxoplasmosis with antimicrobial drugs began in the early 1950s. In 1953, Eyles and Coleman described the effects of pyrimethamine used in conjunction with sulfonamides. Hogan was the first to note that therapy seemed to elicit resolution in adult patients with Toxoplasma chorioretinitis (Hogan, 1958). A combination of antimalarial drugs and sulfonamides, specifically pyrimethamine and sulfadiazine, was used by Hogan in 1958, and remains widely used today (Holland and Lewis, 2002). Currently, drug therapy for ocular toxoplasmosis is usually administered only if there is reactivation of the infection. Some clinicians do not administer specific drug therapy when a peripheral Toxoplasma chorioretinitis recurrence occurs in an immunocompetent person; however, the current author believes that treatment should be administered to these patients.

A survey of the members of the American Uveitis Society highlights the lack of uniformity regarding therapy. The most common regimen used in the 1991 published survey was pyrimethamine, sulfadiazine, prednisone, and folinic acid in 32 percent of respondents, and an additional 27 percent added clindamycin to the most common regimen (Engstrom *et al.*, 1991). Other agents with activity against *Toxoplasma* include quinolones and macrolides. Adjunctive therapies such as laser treatment or cryotherapy (Jacklin, 1975) within and adjacent to chorioretinal scars are today rarely employed.

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Prior to the mid-twentieth century, *T. gondii* was not widely recognized as a cause of ocular disease in general or of chorioretinal lesions in specific. With the introduction of the Sabin–Feldman dye test (Sabin, 1948) it became apparent that *Toxoplasma* infection was not only widespread but

also largely asymptomatic. There is a wide variation of seroprevalence among different countries, geographic areas, and ethnic backgrounds, with as many as 22.5 percent of adults in the Unites States (Jones et al., 2001b) and 90 percent in Panama (Sousa et al., 1988) seropositive for antibodies against Toxoplasma. At least 0.6 percent of residents of Alabama (Maetz et al., 1987) and Maryland (Smith and Ganley, 1972) have chorioretinal scars consistent with prior Toxoplasma chorioretinitis. Without clusters of lesions, it is unknown whether a particular pigmentary lesion is a normal variant or secondary to Toxoplasma infection. Recent studies suggest that while T. gondii is still the leading cause of posterior uveitis, acquired disease occurs more frequently than congenital infection (Gilbert and Stanford, 2000).

Congenital toxoplasmosis appears to be the highest risk of a systemic infection for the development of ocular lesions. The risk of retinochoroiditis from intrauterine infection is 20 percent in the early childhood years, and can rise to as high as 80 percent in adolescence. Congenital infection of ocular toxoplasmosis has been estimated to affect 3000 infants born in the United States each year, with a resultant annual cost of between US\$400 million and US\$8.8 billion each year (Wilson and Remington, 1980; Roberts and Frenkel, 1990; Roberts et al., 1994). Toxoplasma chorioretinitis is present in 70-90 percent of patients with congenital Toxoplasma infection, and it is the most common manifestation of disease (Alford et al., 1974). Though 85 percent of congenitally infected infants appear normal at birth, studies indicate that if these patients are not treated then approximately 85 percent of them will go on to develop chorioretinal lesions, some resulting in vision loss, by adulthood (Koppe et al., 1974; Wilson et al., 1980).

Recently, a major paradigm shift has developed in the etiology of ocular toxoplasmosis. While the classical teaching is that most if not all ocular toxoplasmosis is secondary to intrauterine exposure, it is clear that acquired disease is more common than previously thought (Holland, 1999) – and indeed postnatal infection may account for the majority of *Toxoplasma* retinochoroiditis (Gilbert and Stanford, 2000). The origin of the classical teaching that ocular toxoplasmosis results from congenital infection was from studies showing that in South Pacific islands there is a high seroprevalence of exposure to toxoplasmosis prior to pregnancy and there is also a low rate of ocular disease (Darrell *et al.*, 1964). In addition, supporting the classical teaching is the rarity in earlier studies of multiple siblings having ocular toxoplasmosis. Moreover, there was no increase in ocular toxoplasmosis prevalence with age not paralleling the concomitant increase of seroprevalence of toxoplasmosis with age – both arguing against acquired disease as a source of ocular toxoplasmosis.

Recent studies have reported ocular toxoplasmosis in non-twin siblings (Glasner *et al.*, 1992) and ocular infection in acquired disease. Serologic testing at the Palo Alto Medical Foundation suggests that many patients displaying only ocular symptoms of toxoplasmosis may have recently acquired infection, as opposed to recurring congenital infection. It is plausible that previous lesions in these patients may be due to earlier, undetected episodes of acquired infection, as chorioretinal lesions acquired during childhood or in the periphery of the eye may go unreported and be incorrectly diagnosed as congenital.

Several serologic studies of specific populations suggest that ocular toxoplasmosis is mostly acquired. Seropositivity for T. gondii is relatively high in the region of Erechrim in southern Brazil. This is presumed to be due to the cultural practice of ingesting raw pork. One study found that the prevalence in Brazil of ocular disease increases with age (Glasner et al., 1992). Congenital toxoplasmosis is rare, since the majority of women are exposed to infection prior to their first pregnancy. Cases of non-twin siblings both having ocular toxoplasmosis (Silveira et al., 1988), along with the high rate of ocular disease there (17.7 percent) (Glasner et al., 1992), suggest that previous notions about the epidemiology of this disease are worth reconsideration. Higher rates of disease due to acquired infection in Brazil may be related to any number of factors. Various factors concerning the host, the parasite, and the environment have been hypothesized to affect the course of disease: differences in the genetics or age of the host upon

exposure; intensity, frequency, and duration of exposure; manner of transmission; and clonal type, life stage, and virulence of the parasite.

Acquired ocular toxoplasmosis has been reported in France (Couvreur and Thulliez, 1996), Canada (Burnett et al., 1998), Brazil (Glasner et al., 1992; Silveira et al., 2001), and the United States (McCannel et al., 1996; Montoya and Remington, 1996). A fascinating population-based epidemiologic study of a rural area of Brazil revealed that household correlated with severity of infection (Portela et al., 2004). No ocular disease was found in patients younger than 10 years of age, even though there was in 47 percent evidence of serologic infection. Of all age groups, 12.9 percent of seropositive patients had ocular lesions; patients older than 55 years of age had the highest prevalence of ocular lesions. Similar households had a slightly increased risk factor for ocular toxoplasmosis; however, age was the strongest risk for ocular lesions. High values for GIPL-specific IgA were associated with larger eye lesions. The IgA appeared to be directed against GIPL derived from tachyzoites.

Whereas in the United States it appears that type II strains of *Toxoplasma* are the most common strains associated with human infection, in southeast Brazil types I, III, or I/III hybrids with a relative absence of type II have been reported (Dubey *et al.*, 2002, 2003; Fux *et al.*, 2003).

Depending on the study, anywhere from 2 to 20 percent of infected individuals develop ocular toxoplasmosis (Glasner *et al.*, 1992; Couvreur and Thulliez, 1996; McCannel *et al.*, 1996; Montoya and Remington, 1996; Burnett *et al.*, 1998; Holland, 1999; Silveira *et al.*, 2001).

There are several modes of transmission of *Toxoplasma* to humans, and the exact hierarchy of importance of transmission is unknown and will likely vary depending on region. It is usually difficult to identify the source of exposure to *T. gondii* in a particular patient. Most transmission is assumed to occur by ingestion, whether it is from contaminated water, undercooked meat or contaminated produce. An example of an identified food source (deer) that appears to be associated with five cases of acquired toxoplasmosis

from handling or eating venison is compelling (Ross *et al.*, 2001). There are likely several as yet unidentified food sources within different cultures and regions that are a common source of transmission of toxoplasmosis. Unfiltered drinking water was identified as a major source for the high prevalence of infection in a rural region of northern Brazil (Bahia-Oliveira *et al.*, 2003). There was one report of aerosolization in a horse's stable as a source of transmission (Teutsch *et al.*, 1979).

Toxoplasmosis has been shown to occur as a result of contaminated municipal drinking water (Bowie et al., 1997), and even with a strict vegetarian diet (Hall et al., 1999). Contamination of a municipal reservoir was the source of a 1995 outbreak of toxoplasmosis in Victoria, British Columbia (Bowie et al., 1997), and this same problem has been associated with high risk for infection in Brazil (Bahia-Oliveira et al., 2003). Though the high rates of seropositivity in Brazil are traditionally attributed to the practice of ingesting raw or undercooked pork (Silveira et al., 1987), there is evidence that drinking contaminated water is linked with higher rates of ocular toxoplasmosis (Silveira, 2002). Infection from multiple sources could be part of the reason for the very high rates of postnatally acquired ocular toxoplasmosis. The details of the 1995 Victoria outbreak have been recounted by Bowie and associates (Bowie et al., 1997): 100 people were found to be acutely infected with T. gondii caused by the outbreak, 19 of whom had infectionrelated retinitis. The range of ages in the entire cohort was 6-83 years, but the eye disease cases were significantly older than other cases of symptomatic disease (such as lymphadenopathy).

5.4 THE MECHANISM OF TISSUE DAMAGE IN OCULAR TOXOPLASMOSIS

There are several theories as to why ocular toxoplasmosis results in its inflammatory process with resultant damage:

1. *Cyst rupture with subsequent infection*. Initially cysts generate little inflammatory response,

perhaps as a result of incorporation of host cell components into the cyst wall (Dutton et al., 1986a). Although there is no direct evidence of cyst rupture inducing inflammation, there is a related organism, Besnoitia jellisoni, in which a cyst can be observed ophthalmoscopically and can undergo a spontaneous rupture which induces an inflammatory response (Frenkel, 1955). However, there does appear to be cyst degeneration, by electron micrographic studies, in retinochoroidal lesions (Rao and Font, 1977). In addition, from animal models it appears that the number of organisms released during cyst rupture determines the presence of an inflammatory response, as 500 tachyzoites cause minimal reaction while 5000 tachyzoites induce more of an inflammatory reaction (O'Connor and Nozik, 1971; Culbertson et al., 1982).

- 2. *Toxic mediators released from parasite*. A dialysate from the peritoneal exudates of *Toxoplasma*-infected mice was injected intratvitreally into rabbit eyes and found to result in severe retina damage (Hogan *et al.*, 1971).
- 3. *Damage by inflammatory mediators*. Lysis of inflammatory cells within the retina results in surrounding tissue damage (Dutton *et al.*, 1986b).
- 4. Hypersensitivity to T. gondii antigens. Histopathologic demonstration of granulomas has been reported in Toxoplasma retinochoroiditis. Patients previously exposed to T. gondii develop a delayed-type hypersensitivity reaction to subcutaneously administered antigen (Frenkel, 1949). Cyst rupture results in exposure of sequestered antigen, which in turn results in an intraocular delayed-type hypersensitivity reaction. Injection of T. gondii antigen intraocularly did not result in an inflammatory response (Culbertson et al., 1982). From these experiments it is unlikely that T. gondii initiates the inflammatory cascade, though hypersensitivity may prolong inflammation once initiated.
- 5. *Auto-antigens*. Several assays on peripheral blood from patients indicate that humoral (Abrahams and Gregerson, 1982) and cell-mediated immunity (Nussenblatt *et al.*, 1989)

against retinal antigens are present. Animal studies demonstrate selective destruction of outer retina similar to that observed in experimental induced uveitis with S antigen. However, auto-antigens are unlikely to be the primary impetus behind visual morbidity as empirically patients who are immunocompromised do worse secondary to parasite replication. *In vitro* lymphocyte proliferation was found to S antigen in 16 of 40 patients with ocular toxoplasmosis (Nussenblatt *et al.*, 1989). In addition, 34 of 36 patients had photoreceptor activity as detected by indirect immunofluorescence.

5.5 HOST FACTORS IN OCULAR TOXOPLASMOSIS

The eye is an unusual immunologic environment designed to reduce inflammation, in that transforming growth factor β is constitutively expressed, as well as a high concentration of Fas ligand on ocular cells (Streilein and Stein-Streilein, 2000). In addition, Class I MHC molecules are downregulated (Streilein et al., 1997). The unusual immunologic microenvironment of the eye may decrease clearing of T. gondii infection. Transforming growth factor β has been shown to reduce interleukin-12 production of interferon γ (Hunter et al., 1995). Nitric oxide also plays an important role in interferon gamma-mediated host reaction against T. gondii in ocular toxoplasmosis (Langermans et al., 1992; Roberts et al., 2000). TNF-α, iNOS, IL-1, and IL-6 are all upregulated in ocular toxoplasmosis (Gazzinelli et al., 1994; Hayashi et al., 1996). Five genes, including one within the region of the H-2 gene, have been associated with an impact on survival after infection with T. gondii (McLeod et al., 1989). Class I MHC and CD8+ fraction of T cells determine cyst load after infection (Brown and McLeod, 1990).

Reactivation of *Toxoplasma* in the murine model of ocular toxoplasmosis has been demonstrated to be influenced by downregulation of interferon- γ and tumor necrosis factor- α (TNF α). Interleukin-6 (IL-6) enhanced intracellular reproduction of *T. gondii* (Beaman *et al.*, 1994), but this contradicts the finding of an IL-6 knockout model (Lyons *et al.*, 2001). Of note, the retinal pigmented epithelium produces a large amount of IL-6.

Vitreous fluid mitogenically-stimulated cell lines of ocular toxoplasmosis patients did not show any reactivity to retinal antigens, but demonstrated a strong response to *T. gondii* antigens (Feron *et al.*, 2001). In a human retinal pigment epithelial (HRPE) cell culture assay, interferon results in L-tryptophan starvation through induction of indoleamine 2,3-dioxygenase, an enzyme that converts tryptophan to N-formylkynurenine; however, the administration of exogenous tryptophan did not result in complete reversal of the inhibitory effect. Interferon- γ was the most potent cytokine in HRPE cells, and indeed by itself inhibited growth of parasite (Nagineni *et al.*, 1996).

Interleukin-10 in an intraocular inoculum route of infection in the mouse appears to play a role in limiting inflammation, and because knockout IL-10 mice have similar γ -interferon levels to controls, it appears to have only a partial role in regulation of γ -interferon production (Lu *et al.*, 2003).

A clue to the complexity of host factors in toxoplasmosis is that even though in animal models inhibition of TNF- α results in worse ocular disease, in humans medicines against TNF- α rarely result in disease recurrence. Infliximab, a chimeric antibody against TNF- α , is used to treat some forms of uveitis, as well as rheumatoid arthritis and Crohn's disease. It has been reported to be a cause of biopsy-confirmed CNS toxoplasmosis (Young and McGwire, 2005) but, despite the prevalence of *T. gondii*, by 2005 there were only 8 known cases of *T. gondii*-associated reactivation with over 600 000 patients having received the drug.

5.6 PARASITE FACTORS IN OCULAR INFECTION

Three clonal types of *T. gondii* predominate in nature (Howe and Sibley, 1995). Virulent strains of *T. gondii* appear to have their origin in a single, genetically homogeneous lineage. This is despite

the parasite's pervasive nature and ability to reproduce sexually (Sibley and Boothroyd, 1992). Recently, a composite genome map of the 14 chromosomes of T. gondii was reported (Khan et al., 2005). There is at most a 1 percent difference between the three strains, and reports examining only one locus are likely to misclassify Toxoplasma because of shared alleles between strains (Saeij et al., 2005a). The most surprising development regarding strains and ocular disease was the finding of several novel genotypes from clinical ocular samples (Grigg et al., 2001a). These novel strains had combinations of alleles from the three classical strains, and rather than having unique virulent genes it is likely that it is the combination of genes that results in increased virulence. It has been demonstrated experimentally that a combination of non-virulent strains can lead to more virulent progeny (Grigg et al., 2001b).

The identification of Toxoplasma genotype using peripheral blood samples of patients with ocular disease may have clinical and epidemiologic value (Bou et al., 1999). Most immunocompromised individuals do not have infection with the more virulent type I strain, but patients who are immunocompetent appear to have type I as a commonly identified strain associated with ocular disease (Fardeau et al., 2002). Type II strains appear to be the most common cause of human disease in the United States (Dardé, 2004). In a study limited by the sample size of 12 clinical isolates, patients with a higher proportion of atypical toxoplasmosis were also found to have clonal types associated with more virulence (8 out of 12) (Grigg et al., 2001a). In addition, all 6 patients who were immunocompetent with severe ocular toxoplasmosis had a more virulent SAG1 gene. When the samples were analyzed by PCR RFLP assay for SAG3, SAG2, and B1, 5 of 12 isolates were identified as having new recombinant strains. A larger cohort is required to validate whether type I-associated infection is in fact more likely associated with more severe ocular disease.

A recent validated multiplex PCR has been developed allowing rapid multilocus strain typing from five microsatellite marker locations (Ajzenberg *et al.*, 2005). Type II strain was present in 85 percent of isolates of a cohort of congenital toxoplasmosis from France (Bowie *et al.*, 1997; Ajzenberg *et al.*, 2005). Severe acquired ocular toxoplasmosis disease has been documented to occur from atypical strains (Burnett *et al.*, 1998; Grigg *et al.*, 2001a). In the domestic cycle of toxoplasmosis there are three clonal strains widely prevalent; however, in other areas where there are a wide variety of intermediate hosts (such as in French Guiana) atypical strains are more common.

While there is limited understanding as to why ocular disease remains one of the most frequent causes of recurrent morbidity, there is a suggestion from endothelial cell culture regarding why ocular disease is common in systemic infection (Smith et al., 2004). It appears that T. gondii grows better in retinal endothelial cells than in aortic, dermal, or umbilical endothelial cell lines, and even 2.8-fold higher than in human foreskin fibroblasts (Smith et al., 2004). There are two theories as to how T. gondii crosses the blood-brain barrier. One is that the tachyzoites cross the blood-brain barrier when circulating lymphocytes are arrested within the ocular vasculature and lyse, permitting free tachyzoites to infect adjacent retinal endothelium (Roberts and McLeod, 1999). An additional theory that has yet to be substantiated is that infected lymphocytes cross the blood-brain barrier present within the eye and transport T. gondii into the eye.

5.7 ANIMAL MODELS

5.7.1 Animal models of ocular toxoplasmosis

Several intraocular models of toxoplasmosis have been developed. The primate (Culbertson *et al.*, 1982), mouse, and rabbit (Nozik and O'Connor, 1968) models suffer from the lack of similarity to human toxoplasmosis, where ocular seeding occurs from systemic endogenous infection. Both marsupials and New World monkeys appear to be unusually susceptible to *Toxoplasma* infection, which is believed to be due to the absence of feline populations in their environment (Gustafsson *et al.*, 1997; Innes, 1997; Epiphanio *et al.*, 2003).

There are no ideal small-animal models of human ocular toxoplasmosis. The macula is the central anatomical segment of the retina within the visual axis that gives humans their most fine acuity. Only primates have maculas, and macular disease is an important feature of congenital toxoplasmosis. Since the mouse does not have a macula, it cannot demonstrate macular disease. In addition, the proportional volume of the lens is much greater and the proportional vitreous volume much less in the mouse than in the human eye. Despite these shortcomings, mice have been used to mimic human retinal disease. If mice can reliably be manipulated to develop lesions similar to human ocular toxoplasmosis then this is helpful, as such models provide a springboard to investigate human ocular toxoplasmosis.

5.7.2 Murine models of ocular toxoplasmosis

One example of a murine model of ocular toxoplasmosis is the injection of tachyzoites into the anterior chamber of the mouse (Hu et al., 1999). The technique is to remove some aqueous humor by paracentesis. (The eye is a closed environment, and removing fluid reduces the risk of raised intraocular pressure.) This is followed by an injection of 5 µL of parasite suspension in Dulbecco's modified Eagle medium. It should be noted, however, that the most significant route of transmission of ocular toxoplasmosis in humans is endogenous through systemic infection, and not directly through an exogenous route. The benefit of the model is that intraocular replicating parasites can be readily identified. The one difference from the human model is that the initial infection may permanently alter the normal blood-brain barrier of the eye, owing to the initial inflammatory response, and result in an atypical ocular environment.

This intraocular inoculation model has yielded interesting results. For instance, the model has demonstrated an extraordinary susceptibility of C57bl mice to mortality within 2 weeks, as compared to Balb/c mice which have almost no mortality from intraocular inoculation. A recent study

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reports that in murine models, genetic factors of the host mouse as well as the parasite strain are significant in determining susceptibility to experimental ocular toxoplasmosis (Lu *et al.*, 2005).

An alternative to avoid the damage from intraocular injection is the simple topical application of a parasite suspension of 10³ tachyzoites (RH strain) in RPMI (Gil et al., 2002). It is a surprisingly useful way to obtain a detectable intraocular parasite load with preservation of the intraocular architecture and no alteration of the lens architecture (Tedesco et al., 2005). Surprisingly, there appears to be no detectable difference in measured intraocular infection between the topical or the intravitreal route of infection in mice. Both routes had detectable parasites within intraocular vessels, glial reaction of the inner plexiform layer by day 7, and disruption of the retinal pigmented epithelium. Intravitreal injection of PBS alone also resulted in glial changes within the inner plexiform layer (Tedesco et al., 2005). Similar to rabbit models of ocular infection, a high inoculum dose given intraocularly can result in ocular disease even in previously primed mice (Hu et al., 1999; Dao et al., 2001). It has been demonstrated that even without the overwhelming of host defenses likely from intraocular inoculums, systemic re-infection of mice with different strains after previous seroconversion is possible (Dao et al., 2001).

A congenital model of ocular toxoplasmosis has been reported in which infected dams are inoculated during gestation (Hay et al., 1984). Unfortunately, a wide range of clinical disease occurred in this model. Murine congenital toxoplasmosis differs significantly from human disease. In the murine model there is significant opaque cataract formation, even with lens autolysis. This can be seen by examination using a 35-diopter lens, and documented with a Zeiss operating microscope (Dutton et al., 1986a). Even though ME49 strain infection of mice is a reliable model for Toxoplasma cysts in the brain, it is unusual to find evidence of intraocular toxoplasmosis even with PCR amplification. Antibodies in the same model against interferon- γ , TNFα, or CD4 and CD8 cells result in frequent demonstration of parasite in association with more severe ocular lesions (Gazzinelli et al., 1994).

L-NAME, through its inhibition of NO, can cause disease reactivation in chronically infected mice; however, the cyst load was not robust enough to identify on histopathology (Roberts *et al.*, 2000).

In vivo imaging of the mouse fundus is possible, and is greatly facilitated by the appropriate imaging set-up (Hawes et al., 1999). The digital Kowa Genesis Small Animal Fundus camera was an excellent imaging device for mice, and was available with a fluorescein angiographic mode. Digital capture helped instantly to confirm capture of an appropriate image. Kowa has plans to release an updated model. Regular clinical fundus cameras can also take images of the posterior pole of mice eyes, but this is not as easy and usually requires manipulation of the mouse eye to achieve the best image. Recent observations document systemic infection using in vivo imaging employing the IVIS system (Xenogen, Alameda, California) (Saeij et al., 2005b). This paper demonstrates some bioluminescence due to luciferase transgenic parasites with pixels correlating with ocular involvement; however, ex vivo imaging of eyes confirming this localization was not reported (Saeij et al., 2005b).

5.7.3 Hamster models of ocular toxoplasmosis

Frenkel first reported hamsters as an ocular model for toxoplasmosis (Frenkel, 1955). The RH strain he had used required therapy to prevent mortality, and the CJ strain did not consistently produce ocular lesions. Reliable hamster models of ocular disease with oral infection (Gormley et al., 1999) and intraperitoneal (Pavesio et al., 1995) injection have subsequently been reported, using 100 cysts of the ME49 T. gondii strain for infection. Although the ocular disease does not exactly mimic human disease, the models are attractive in that hamsters reliably develop ocular lesions with little systemic disease which resolve spontaneously without treatment. Unlike humans, ocular toxoplasmosis in the hamsters does not result in pigmentation, the overlying retina alone appears atrophic, and the disease is bilateral.

5.7.4 Rabbit models of ocular toxoplasmosis

Hogan was the first to create a published animal model of ocular toxoplasmosis by injecting tachyzoites into the carotid arteries (Hogan, 1951; Garweg et al., 1998); however, the RH strain used in this model frequently resulted in meningoencephalitis and rapid mortality. Beverley and others injected the inoculum into the anterior chamber of rabbits (Beverley et al., 1954). The BK strain in PBS inoculated intravitreally near the retinal wing of the posterior pole (the entry site was through the pars plana 4-5 mm behind the limbus at the superior rectus muscle insertion into the globe) into previously primed Burgundy rabbits resulted in an inflammatory response that mimics inflammation of human disease (Garweg et al., 1998). The caveats previously mentioned apply here, as any intraocular inoculum route results in disruption of the normal intraocular architecture. Inoculation of parasites into the suprachoroidal space differs from human infection in that there is a limited local infection followed quickly by systemic infection, and there is also a risk of retinal defects (Nozik and O'Connor, 1968). The suprachoroidal route does not induce an intraocular inflammatory response in primed rabbits, in contrast to the intravitreal route. In addition, high inoculum can result in ocular disease even in previously primed rabbits (Garweg et al., 1998). Cyclosporine A (Friedrich et al., 1992) and total body irradiation (O'Connor, 1983a) induce reactivation of ocular disease in previously infected rabbits, but local trauma does not induce reactivation in the rabbit (Nozik and O'Connor, 1970).

5.7.5 Feline models of ocular toxoplasmosis

A feline model of intracarotid inoculation of 5000 tachyzoites was successful in producing a reliable model of lesions of ocular toxoplasmosis (Davidson *et al.*, 1993). Because the feline model has primarily choroidal involvement, it differs from human ocular infection. Usually, similar to human infection, initial infection with *Toxoplasma* in cats is

subclinical (Dubey and Beattie, 1988). The organism has been found on histopathology throughout the eye (Dubey and Carpenter, 1993). Since over 50 percent of cats may be seropositive for T. gondii, antibody assays of ocular fluid have been developed to help diagnose Toxoplasma as an etiology of feline uveitis (Chavkin et al., 1994). The infrequent identification of organisms by histopathology (Peiffer and Wilcock, 1991) has led to theories hypothesizing an indirect T. gondii antigen etiology to feline Toxoplasma-related uveitis (Davidson and English, 1998). PCR assays of intraocular fluid have demonstrated, however, that direct infection due to T. gondii is the source of feline uveitis (Lappin et al., 1996; Burney et al., 1998). Less frequent ocular lesions occurred with other routes of infection. Oral administration of tissue cysts or oocysts resulted in infrequent ocular lesions (Dubey and Frenkel, 1972, 1974; Dubey, 1977). In a model of neonatal infection of ocular toxoplasmosis, it appeared that the Mozart strain (initially isolated from intraocular fluid) resulted in more frequent eye lesions than either Maggie or ME49 (Powell and Lappin, 2001). No areas of non-tapetal retina were infected in these cats, and neonatal infection can result in systemic infection with apparent involvement confined to the eye even without evidence of serologic seroconversion (Powell and Lappin, 2001). Necropsy has identified frequent intraocular lesions by transplacentally infected cats (Dubey et al., 1996).

5.7.6 Non-human primate models of ocular toxoplasmosis

As expected, non-human primates have the most similar ocular environment mimicking the human eye. In addition, the published models mimic current sensibility regarding human ocular toxoplasmosis. For example, the non-human primate models support the theory that recurrent ocular disease is from the direct presence of *T. gondii*, and not from indirect antigenic immunogenicity. The attempt to induce a necrotizing retinochoroiditis from either intravascular or intraocular injection of *T. gondii* antigens was unsuccessful in non-human primates (Newman *et al.*, 1982; Holland *et al.*, 1988a). In addition, no necrotizing retinitis developed in previously immunized primates after injection of live parasites; however, as expected, a local inflammatory response occurred when injected intraocularly in previously sensitized animals (Webb *et al.*, 1984). Despite total lymphoid irradiation of 2000 cGy, none of six Cynomolgus monkeys developed recurrent ocular disease even with intraocular injection of Beverley strain or the presence of preexisting chorioretinal lesions.

5.8 CLINICAL CHARACTERISTICS

5.8.1 The clinical features of ocular toxoplasmosis

The classic symptoms of ocular toxoplasmosis are similar to the classic symptoms of uveitis in general. When ocular toxoplasmosis is active, usually there is pain, redness, photophobia, and decreased vision. There are variations of the above classic four symptoms, with some patients, for instance, having only decreased vision. The pain and photophobia are minimal unless there is severe iridocyclitis. It can be difficult to detect early onset of any of these symptoms in children, as they cannot articulate their symptoms appropriately. As the congenital Toxoplasma study cohort (Mets et al., 1997) demonstrated, if children are instructed to promptly report any change in their vision to their caregivers, this can increase the detection of active disease. Ocular toxoplasmosis can present with unusual manifestations of retinal pathology. Instead of the classic severely involved focal chorioretinitis, it has also been reported to resemble unilateral acute idiopathic maculopathy (Lieb et al., 2004).

The most common clinical presentation of ocular infection due to *T. gondii* is a unilateral chorioretinitis associated with a pre-existing chorioretinal scar and an overlying vitritis. In addition, the clinical ophthalmic diagnosis of retinal vasculitis, of both arterioles and veins, is commonly made in active disease secondary to interaction between antibodies and antigens (O'Connor, 1974). *Toxoplasma gondii* accounts for

greater than one-fourth of all cases of posterior uveitis. Lesions can occur in any part of the fundus, but in patients with congenital infection, severe macular lesions appear more commonly than in acquired infection. One study (Mets et al., 1997) found that macular lesions were present in 58 percent of a cohort of 94 children with congenital toxoplasmosis, 76 of whom had 1 year of treatment with pyrimethamine and sulfadiazine. Peripheral scars were present in 64 percent. This could be due to the early vascularization of the posterior pole during fetal development, or to the unique vascularization of the fetal macula (which contains end arterioles), or to the higher concentration of cells. Evidence of bilateral infection without simultaneous bilateral active disease (lack of bilateral inflammation but the presence of bilateral scars) was found in 46 percent of eyes from one study (Hogan et al., 1964).

After resolution of an active lesion, patients will have decreased vision in the area of retinochoroiditis. If the lesion is small and peripheral, then the patient will probably be asymptomatic. If, however, the lesion is small but within the macula, then the patient will probably be symptomatic. It is useful to have patients check their vision one eye at a time on a daily basis. *Toxoplasma* lesions within one diskdiameter of the optic disk result in very significant 'downstream' visual field defects. This means that an entire region of retina away from the actual lesion, but whose communicating nerve fibers pass over the lesion, can have loss of input as measured by visual field testing (Stanford *et al.*, 2005).

Because of overlapping visual fields and the fact that ocular toxoplasmosis is usually active unilaterally not bilaterally – a change in vision may not be initially detected unless unilateral daily screening occurs. An Amsler Grid, a graph paper like grid of boxes, mounted to a flat surface may help compliance with self-vision screening. In addition to decreased vision in the area of reactivation, patients will likely have floaters or other media opacity related complaints that will vary depending on the degree of inflammation when the lesion was active and may persist after resolution of the underlying reactivation secondary to inflammatory debris being trapped within the vitreous.

Vision loss can be caused by many of the complications associated with ocular toxoplasmosis. Involvement of the macula or optic nerve can directly decrease central vision. Complications secondary to inflammation can indirectly affect vision. These include macular edema, vitreous opacity, epiretinal membrane, and retinal detachment (Friedmann and Knox, 1969; Mets et al., 1997; Bosch-Driessen et al., 2000). Under normal circumstances, peripheral scars can affect the visual field but do not impair central vision. However, in rare cases peripheral scarring may lead to central vision loss. An example of a rare manifestation of ocular toxoplasmosis is provided by one case wherein central vision loss occurred due to a giant macular hole (Blaise et al., 2005). This macular hole was the result of vitreous traction caused by peripheral ocular toxoplasmosis. Subretinal neovascularization in ocular toxoplasmosis has been reported as an unusual cause of vision loss (Cotliar and Friedman, 1982).

Ocular involvement has been shown to occur long after the time of infection, whether acquired or congenital infection. New lesions are likely to occur near the borders of existing lesions, and a larger lesion surrounded by smaller satellite lesions has been considered the hallmark of a recurrence of both congenital and postnatally acquired disease. Little is known about what influences the rate of recurrence of ocular toxoplasmosis. Though Bosch-Dreissen and associates report a cumulative increase in the prevalence of chorioretinal recurrence (Bosch-Driessen et al., 2002), it cannot be assumed that the risk of recurrence in an individual patient increases over time. Holland's impression was that the pattern of recurrence decreased over time (Holland, 2003). One possible explanation for this pattern is that tissue cysts in human hosts have a limited lifespan and lose their ability to reactivate. There is no evidence that short-term therapy at the time of infection has any effect on the pattern of recurrence (Bosch-Driessen et al., 2002).

5.8.2 Recurrence of ocular toxoplasmosis

The classic description of recurrent active *Toxoplasma* chorioretinitis is a focus of retinitis appearing at the border of a retinochoroidal scar;

however, there are several reports illustrating the variance in the clinical features of this disease. Active chorioretinitis does resolve without treatment, leaving a hyperpigmented scar, and recurrences develop as 'satellite' lesions. A recurrence is usually symptomatic, with the redness, pain, and light sensitivity and decreased vision that occur with any generalized panuveitis. Recurrence of chorioretinitis can lead to vision loss (Friedmann and Knox, 1969) and blindness.

There is limited understanding regarding the many factors that exist between infection and recurrence of disease. There is a strong pattern of recurrence during the teenage and adult years. Women with ocular toxoplasmosis are at a higher risk of recurrence during pregnancy (O'Connor, 1983b), though the fetus (except for rare reports – Silveira *et al.*, 2003) appears to be at risk only during a mother's initial infection.

Initially it was unclear what recurrence actually was, and it certainly remains unclear why recurrence occurs. Prior to the AIDS epidemic there was controversy as to whether recurrence represents an autoimmune process alone without the presence of active parasites. Histopathology on patients with ocular toxoplasmosis and HIV infection has demonstrated parasites in areas of involved retina. In addition, eyes that have received corticosteroid treatment alone have had very poor outcomes associated with parasites demonstrated on tissue biopsy (Sabates et al., 1981). Frenkel's theory (Frenkel, 1974) that recurrence represents a hypersensitivity reaction appears unlikely to be the central cause of recurrent ocular toxoplasmosis. Release of T. gondii antigen into the retina is not associated with a hypersensitivity reaction. Reactivation is thought to represent a shift of T. gondii from the dormant phase known as bradyzoites to the more active phase of tachyzoites. There is no clear understanding of how this shift from bradyzoite to tachyzoite occurs within the eye, or what factors influence or cause the shift.

Evidence of prior recurrence is the presence of inactive satellite lesions, which are local areas of chorioretinal scars. Recurrent lesions usually occur in close proximity to prior areas of chorioretinitis, as is evident in the usual clusters of scars that exist (Figure 5.1). Recurrent disease occurs CLINICAL CHARACTERISTICS



- FIGURE 5.1 Toxoplasma gondii chorioretinitis.
 (A) Left macula with old scar superior temporal to new active lesion.
 (B) Late fluorescein angiogram of (A) demonstrating incompetence of the blood-brain barrier of both the active and the old lesion.
 (C) Hypofluorescent lesion where active lesion is on (A) and vascular remodeling of superior temporal old lesion.
- (D) Active lesion in (A) within 2 mm of the fovea (red-free photograph).
- (E) Old chorioretinal lesion inferior to optic nerve with overlying vitreal condensation.



FIGURE 5.1, Cont'd *Toxoplasma gondii* chorioretinitis.(F) Nasal to optic nerve – there is an active lesion adjacent to old chorioretinal scar. This figure is reproduced in color in the color plate section.

when new areas of retina are involved in an infectious inflammatory process that results in permanent destruction of involved tissue. Resolution of the inflammatory process will usually occur spontaneously after several weeks. Although the general eye inflammation will resolve, the area of retina with focal chorioretinitis is irreversibly impaired. If the lesion or recurrence is in the peripheral retina, the impact of the recurrence on the infected individual's vision can be minimal or even asymptomatic, because the impaired vision exists in a small area of the peripheral field. Usually the new lesion is a focal chorioretinitis; however, a more generalized vitritis and anterior uveitis usually develops secondarily as a generalized intraocular inflammatory process. This more generalized intraocular inflammatory process is responsible for the complaint of decreased vision in patients with ocular toxoplasmosis. The secondary inflammatory process can lead to retinal detachment or other ocular morbidity, such as epiretinal membranes.

5.8.3 The clinical features of congenital ocular toxoplasmosis

Congenital infection is more common later in gestation, but disease manifestations are worse if acquired earlier in gestation (Dunn *et al.*, 1999). Classically, congenital disease is associated with bilateral macular scarring, but acquired infection can also result in macular disease and, rarely, bilateral scarring as well (Glasner *et al.*, 1992). Other manifestations include optic neuritis, iritis, neuroretinitis, retinal vasculitis, acute retinal necrosis, recurrent iridocyclitis, and persistent vitritis. Long-term follow-up of congenitally infected children results in identification of further ocular sequelae not present at birth – for example, in one study four of six untreated

congenitally infected children developed scars subsequent to birth during the next 20 years (Koppe et al., 1986). It is estimated that 85 percent of infants untreated and without ocular lesions at birth will subsequently develop ocular toxoplasmosis (Koppe et al., 1974; Wilson et al., 1980). Microphthalmos and microcornea can occur as a consequence of severe congenital eye disease (Suhardjo et al., 2003). Nystagmus and strabismus and amblyopia secondary to congenital toxoplasmosis are more complex than even most expert ophthalmologists realize (O'Neill, 1998). There is a tendency for clinicians not to struggle with the complex care involved in trying to achieve optimal visual outcome in congenital infection. Less initially severe but still disabling disease such as anterior uveitis (Cano-Parra et al., 2000) secondary to T. gondii is likely underdiagnosed because of the limitations of current non-invasive diagnostic tests.

Exposure to Toxoplasma 6 months prior to conception is thought to eliminate the possibility of congenital transmission secondary to lifelong immunity in immunocompetent individuals. Rarely, reactivation of toxoplasmosis in previously infected immunodeficient women can result in congenital transmission of toxoplasmosis (Mitchell et al., 1990). There is one recent case report of treated acquired ocular toxoplasmosis during pregnancy occurring in the mother without any subsequent fetal disease (Ramchandani et al., 2002). The exact mechanism of transmission is not yet understood, but is thought to be secondary to transplacental transmission of the parasite. The severity of ocular manifestations parallels the severity of CNS disease in congenital infection (Roberts et al., 2001).

A recent report highlighted the ophthalmic findings of congenital *Toxoplasma* infection in treated and untreated individuals (Mets *et al.*, 1996): 79 percent of children had retinochoroidal scars, 28 percent of individuals had significant unilateral vision loss, and 29 percent of children had bilateral vision loss. The presence of inactive chorioretinal lesions in congenitally infected newborns indicates that the complete cycle of infection, activation, and resolution of chorioretinal lesions may occur *in utero* (Guerina *et al.*, 1994; Mets *et al.*, 1996). The New England Regional Toxoplasma Working Group detected 100 of 635 000 infants who were seropositive for IgG and IgM against *Toxoplasma*. Of 39 treated children observed for as long as 6 years, 4 had new postnatally developed retinal scars; a separate 9 of 48 patients had retinal lesions at birth (Guerina *et al.*, 1994). In a different study from England, after 20 years of follow-up, 9 of 11 patients with congenital toxoplasmosis had evidence of chorioretinitis and 4 had severe impairment (Koppe *et al.*, 1986).

The largest report of congenital toxoplasmosis in twins highlights that multiple factors beyond time of exposure during gestation influence ocular outcome in congenital infection (Peyron et al., 2003). Although there are possible confounding issues of shared placentas and mortality (as is true of any infectious congenital disease involving twins), if concordance of the disease is more common among monozygotic twin than among dizygotic ones, then genetic susceptibility is likely more important than environmental influence in disease outcome. While there is no rigorous protocol that has been published focusing on a cohort of ocular outcome in twins (Rieger, 1959), it appears there is a lack of identical outcome between eyes and between twins. It is therefore not time or inoculum alone that leads to presence or absence of ocular disease, size of lesions, or location of lesions (Couvreur et al., 1976). There are differences in specific ocular outcomes in both dizygotic and monozygotic twins. The general disease impact with respect to symptomatic involvement and eventual ocular involvement appears more concordant in dizygotic than in monozygotic twins. It is clear, in order definitively to assess patterns of ocular toxoplasmosis in twins, a long rigorous follow-up report remains to be published.

It is unclear why macular lesions commonly occur in congenital infection. Other frequently involved areas in the brain are the periaqueductal, periventricular, and basal ganglia regions. One theory is that, secondary to a high-affinity transport protein for putrescein, *T. gondii* thrives in the putrescein-rich fetal retina (Seabra *et al.*, 2004). A separate theory is that the macula is the first part of the retina that is vascularized, as the vasculogenesis spreads perirpherally from the central posterior retina to the far periphery. The macula is therefore affected because it is the region that has been vascularized for longest and is thus more likely to be infected than the peripheral retina.

5.8.4 The clinical features of ocular presentation in the elderly

Until recently, it was thought that the elderly were an uncommon risk group for severe ocular involvement. In a publication describing an epidemic of toxoplasmosis, ocular involvement in the elderly was identified as being more common than in other age groups (Bowie *et al.*, 1997). There was a statistically significant older mean age of ocular involvement of 56.1 years, with a range of 15–83 years, in the 19 reported ocular cases (Bowie *et al.*, 1997). It is hard to imagine significant confounders to this finding, as young as well as older patients are usually symptomatic with ocular toxoplasmosis.

5.8.5 Atypical presentations common in immunocompromised patients

Typical severe inflammation when extensive areas of retina are involved owing to active ocular toxoplasmosis can be absent in immunocompromised patients. In addition, the disease is clearly associated with replication of active parasites as demonstrated by histopathology (Nicholson and Wolchok, 1976; Yeo *et al.*, 1983). Although severe *Toxoplasma* chorioretinitis does develop in patients with AIDS (Parke and Font, 1986), it is uncommon (Newsome *et al.*, 1984).

5.8.6 Classification systems for uveitis and chorioretinitis

Recently, an attempt has been made to develop consensus regarding uveitis nomenclature within the field of uveitis (Jabs *et al.*, 2005). The three main subdivisions of uveitis are broken down by

anatomic location – anterior, posterior, or intermediate uveitis – and *Toxoplasma* may cause inflammation in any subdivision (either primarily or secondarily). Anterior uveitis refers to inflammation in the front of the eye anterior to the vitreous. Intermediate uveitis refers to inflammation in the vitreous or in the pars plana (tissue located just anterior to the retina). Posterior uveitis refers to inflammation within the retina or choroids.

Freidmann and Knox (1969) described three types of chorioretinal lesions found in a cohort of patients with no other clinical manifestations of T. gondii infection. Of these patients, 56 percent had 'large destructive lesions'. This type of lesion is characterized by its size; a large destructive lesion is usually larger than the optic disk and is likely to be associated with vision loss and/or complications such as retinal detachment, cataracts, cystoid macular edema, glaucoma, and chronic intraocular inflammatory reactions. 'Punctate inner lesions' accounted for 27 percent of the patients; these were described as being smaller and less likely to be associated with vitreous traction. 'Punctate deep lesions' appeared in 17 percent of the patients; this type of lesion is always located in the macula or peripapillary area. Because the infected tissue is separated from the vitreous by uninvolved retinal layers, punctate deep lesions are usually not associated with vitreous inflammation.

5.8.7 Punctate outer retinal toxoplasmosis

Other reports have described a distinctly different category of *T. gondii* lesions known as 'punctate outer retinal toxoplasmosis' (Doft and Gass, 1985; McAuley *et al.*, 1994). This condition consists of multifocal lesions that are gray to white in color and less than 1000 µm in diameter. These lesions appear at the deep level of the retina and retinal pigment epithelium. There is little inflammatory reaction in the vitreous, and involvement is sometimes bilateral. Though these lesions appear as a cluster, there is generally only one focus of active disease at any point in time.

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5.8.8 Atypical *Toxoplasma* chorioretinitis

Unusual manifestations of ocular toxoplasmosis exist. Bilateral, multifocal, and extensive ocular toxoplasmosis can rarely occur, and these cases have usually been in immunocompromised individuals. Toxoplasmosis can mimic acute retinal necrosis (ARN), an explosively blinding chorioretinitis usually caused by herpes viruses. Rarely, toxoplasmosis can mimic viral acute retinal necrosis (Moshfeghi et al., 2004). Since diagnosis is usually made by clinical appearance alone, therapy is usually directed first against the herpes virus, and subsequently a broader differential is entertained if instituted therapy fails to stop progression of disease. An anti-Toxoplasma regimen may be instituted in severe posterior uveitis as empiric therapy.

A dramatic and unusual periarteritis, eponymously named Kyrielieis-type periarteritis, with deposition of focal plaque like deposits, can occur along the major arcades and may persist after resolution of active disease (Schwartz, 1977). Rarely, a very opaque form called frosted branch angiitis (Ysasaga and Davis, 1999) may occur without retinochoroidal lesions (Holland *et al.*, 1999). A neuroretinitis can also occur, with its classic stellate exudative appearance, within the macula as a consequence of *T. gondii* infection (Kucukerdonmez *et al.*, 2002; Perrotta *et al.*, 2003).

5.8.9 Optic nerve involvement in ocular toxoplasmosis

The optic nerve is populated by approximately 1.3 million nerves that originate in the retina ganglion cell layer and connect the eye to the brain. In congenital toxoplasmosis, because eyes available for autopsy are from individuals with severe CNS involvement, it is impossible definitively to determine whether optic nerve changes are secondary to direct infection or secondary to active CNS processes such as *Toxoplasma* encephalitis. Optic nerve atrophy was present in 20 percent of individuals in one congenital toxoplasmosis cohort

(Mets *et al.*, 1996). 'Jensen juxtapapillary retinitis' is an occasionally used eponym-associated label of peripapillary *Toxoplasma* retinitis, and highlights that posterior lesions are more likely to be symptomatic.

5.8.10 Toxoplasma and glaucoma

Ocular toxoplasmosis appears to have a weak but important association with glaucoma. However, there is no prospective study that has examined the relationship between toxoplasmosis and glaucoma. Unlike herpetic uveitis, which is frequently associated with glaucoma, ocular toxoplasmosis in a retrospective study found the highest incidence (38 percent) of elevated intraocular pressure in patients with active ocular toxoplasmosis (Westfall *et al.*, 2005). There was, however, no associated glaucomatous nerve damage. The elevated intraocular pressure appeared to resolve with resolution of the retinochoroiditis. A prospective study will be required to further examine any possible association.

5.9 DIAGNOSTIC TESTS AND PATHOLOGY

5.9.1 Histopathology

Several reports characterizing the destructive retinochoroiditis exist (Hogan, 1951; Wilder, 1952; Zimmerman, 1961; Rao and Font, 1977). Organisms in immunocompetent individuals are identified in the retina and optic nerve, but not in the choroid. Toxoplasma cysts have also been demonstrated in the retinal pigmented epithelium (Nicholson and Wolchok, 1976). Tachyzoites stain well by both Wright and Giemsa stains, and bradyzoites stain well with periodic acid-Schiff stain. Granulomatous choroidal inflammation and scleral thickening can occur adjacent to the retinal lesions. After resolution of active disease, the involved retina shows severe destruction with retinal atrophy and chorioretinal adhesions. In a murine congenital model of ocular toxoplasmosis,

surprisingly the photoreceptors (not the *Toxoplasma* cysts) appeared to be the focal activity of the mononuclear intraocular infiltrate (Dutton *et al.*, 1986b). Wilder's case series highlighted the value of thicker sections in an attempt to identify tachyzoites. She used celloidin, which permits 18-µm sections vs. the 8-µm sections performed with paraffin thin sections (Holland *et al.*, 2002); however, celloidin requires 6 weeks before sectioning and paraffin thin sections have subsequently been shown to be sensitive enough to detect parasites (Holland *et al.*, 1988b). Wilder noted in her report of severe disease that active disease can extend into the sclera.

The original identification of T. gondii as an etiology of ocular disease came through histopathologic examination of ocular tissues in congenital infection, which revealed organisms in the retina and adjacent choroids (Wolf, 1939). Parasites were identified in 10 of 18 eyes of infants and fetuses in a recent report (Roberts et al., 2001). In the report, 8 of 15 eyes had focal retinal lesions, some with retinal necrosis. A lesion 7 mm large was identified in a 22-week-old fetus. Only 1 of 4 cases had bilateral disease. In addition, 10 of 15 eyes had clinical lesions in the peripheral retina only. A vitritis was identified in 7 of the 18 eyes. In 5 of 8 eyes where there was optic nerve present in sections a leptomeningitis was identified, and 3 of the eyes had disruption of the optic nerve architecture. A diffuse choroiditis was identified in all cases. Eyes from a 7-day-old and a 5-day-old infant showed similar findings to those in the fetuses, with more evidence of organization and a retinal detachment. A 2-year-old child demonstrated areas of an end-stage continuum compared to the fetal eyes, with evidence of retinal atrophy, retinal pigmentary epithelial changes, and overlying gliosis. The inflammatory cells present in the eyes consisted of lymphocytes, plasma cells, and macrophages. Immunohistochemical staining showed both T and B cells, with the B cells less in number and confined primarily to the choroid. The T-cell population contained both CD4+ and CD8+ cells. No tissue cysts were identified in the group of eves. However, immunohistochemical staining confirmed the presence of parasites in 10 of 15 eyes. Parasites were not identified in the choroid or substance of the optic nerve of any of the eyes. A key point in understanding the complexity of ocular toxoplasmosis is that normal-appearing retina can harbor *Toxoplasma* (McMenamin *et al.*, 1986).

5.9.2 Ocular biopsies

There is a risk of irreversible blindness with any type of intraocular procedure, owing to possible complications that include bacterial endophthalmitis and retinal detachment. Since most cases of ocular toxoplasmosis can be diagnosed on clinical grounds, the need for an intraocular procedure to make the diagnosis is highly unusual. Although the risks are severe, the actual likelihood of risk of blindness is low (< 1/100). There are two intraocular compartments that can be sampled. The anterior chamber is in the front of the eye, and the aqueous humor is fluid alone without a gel matrix being present. There are several slightly different approaches to performing an anterior chamber paracentesis (van der Lelij and Rothova, 1997). There is still a risk of complications from sampling this fluid; however, the risk is less than that involved in doing a vitreous biopsy. The vitreous biopsy is performed by sampling the posterior chamber or vitreous cavity. Even though the vitreous is 99 percent water by weight, it is a collagen gel matrix, and sampling the vitreous runs the risk of other complications such as retinal detachment. This is in contrast to an anterior chamber paracentesis which would not be likely to cause a retinal detachment. Based on recent widely used medicines (for macular degeneration) that require intraocular injections, consensus on using topical povidone and a lid speculum appears to be an important step in any office-based intraocular procedure to prevent bacterial endophthalmitis (Aiello et al., 2004).

A vitreal biopsy can be performed as an officebased procedure, depending on the local office setting, operating room access, and clinical scenario. The vitreal biopsy involves more risks than an anterior chamber paracentesis because entry into the vitreous, which is a gel-like substance, can cause traction on the retina. There is a wide variety of approaches to performing a vitreal biopsy.

Analysis of T cells recovered after vitrectomy in 10 patients with active suspected *Toxoplasma* chorioretinitis demonstrated the presence of *T. gondii*-associated T-cell clones (Feron *et al.*, 2001). In addition, there was an absence of T-cell clones against retinal antigens. Three of eight patients were positive for *T. gondii* by PCR.

5.9.3 Serology

There is currently clinically a limited role for serologic testing in recurrent ocular toxoplasmosis. The author always obtains a confirmatory IgG ELISA in suspected recurrent disease; however, there is no test available that can confirm that ocular inflammation is in fact due to ocular toxoplasmosis. High-avidity (> 40 percent) antibodies are associated with infections that are over 6 months old (Paul, 1999; Liesenfeld *et al.*, 2001). Documented seroconversion is a scenario where serologic testing is useful in suspected cases of acquired ocular disease. Patients concomitantly infected with HIV and *T. gondii* can have a negative serology (Moshfeghi *et al.*, 2004).

Antibodies against toxoplasmosis may be present in the eye because of damage to the blood-brain barrier leading to passive accumulation of antibody. The Goldmann-Wittner coefficient is an attempt to overcome the false-positive possibility by determining the relative local concentration of antibody compared to systemic levels. While any coefficient greater than 1.0 would indicate increased local production, most authors use coefficients greater than 3.0 as indicative of a more valid and specific threshold value (Baarsma et al., 1991; De Boer et al., 1994, 1996). A Goldmann-Wittner coefficient greater than 3.0 is seen in 50-70 percent of patients with ocular toxoplasmosis. This coefficient provides evidence of local antibody production against T. gondii compared to systemic production, and provides support for a diagnosis of active local ocular disease. The Goldmann-Wittner coefficient is the ratio of (anti-Toxoplasma IgG in the aqueous humor/anti-Toxoplasma IgG in serum) divided by (total IgG in serum/total IgG in aqueous humor).

Ophthalmologists should understand that any infusion solution routinely used to prepare instruments immediately prior to surgery may result in a false-negative result from its resultant dilution. Ophthalmologists therefore need to tell the operating staff not to prime equipment prior to sampling of vitreous.

Surprisingly, most cases with a positive Goldmann–Wittner coefficient have a negative PCR assay for *T. gondii*. Also, most cases with a positive PCR for *T. gondii* have a negative Goldmann–Wittner coefficient. From the above, it can be concluded that the local humoral response appears not to coincide with the local proliferation of parasite. In addition, just as peripheral serology may be negative in immunocompromised patients, local antibody production measurement has been reported to be negative. In one series, seven of nine immunocompromised patients had negative local antibody production but had positive PCR (Fardeau *et al.*, 2002).

5.9.4 Immunoblotting

No universal pattern exists based on immunoblotting to aid the diagnosis of ocular toxoplasmosis. One paper highlighted the antibody binding to molecular sizes below 16 kDa and above 116 kDa (De Marco et al., 2003), a different report highlighted binding of antibodies to a 28-kDa antibody (Klaren et al., 1998), and two further papers did not detail findings (Riss et al., 1995; Villard et al., 2003). There is a broad range of data regarding reported immunoglobulin subtypes in ocular toxoplasmosis. IgA in the aqueous humor has been demonstrated in 26-63 percent of patients, and IgM has been reported in the aqueous humor in from <1 to 11 percent of patients (Ronday et al., 1999; Garweg et al., 2000). IgE has been reported in the vitreous fluid in up to 14 percent of patients (Liotet et al., 1992; Gomez-Marin et al., 2000).

5.9.5 Polymerase chain reaction (PCR)

Since PCR testing requires a small sample volume, it is well suited for the samples available from

most invasive ocular biopsies (Burg *et al.*, 1989; Brezin *et al.*, 1990). Unfortunately the vitreous, being 99 percent water by weight, does not have the dense cellular substrate ideally suited for the presence of *T. gondii* (Brezin *et al.*, 1991). Large, atypical lesions which would be more likely to necessitate biopsy to help substantiate diagnosis also appear to be more likely to have positive vitreous PCR results. In one series, five of seven patients with severe toxoplasmosis had positive vitreous PCR results (Montoya *et al.*, 1999).

Should a sensitive, specific test requiring intraocular sampling be developed for the eye, sampling aqueous humor would be safest. However, currently PCR testing of the aqueous has a low yield in ocular toxoplasmosis (Aouizerate *et al.*, 1993; Bou *et al.*, 1999; Montoya *et al.*, 1999; Figueroa *et al.*, 2000). The yield of positive PCR testing has been reported to range from 17 percent in patients with chorioretinitis (Brezin *et al.*, 1991) to 100 percent of patients with large lesions (Fardeau *et al.*, 2002). In elderly patients, larger lesions had a higher yield of aqueous PCR sampling compared to smaller lesions (60 percent vs. 25 percent; Labalette *et al.*, 2002).

When a fulminant vitritis transforms the normally transparent vitreous to an opaque structure, it can be impossible to examine the retina. Without the possibility of detecting clinical features on fundus examination, more dependence on ancillary tests is necessary. A multiplex PCR has been established to help to differentiate between the more common causes of retinitis (Dabil et al., 2001). The T. gondii-based primer is based on the repetitive B1 gene, which has been found to have sensitivity of 60-70 percent (Montoya et al., 1999). The vitreous must be boiled for 15 minutes to remove PCR reaction inhibitors. When compared to the lower sensitivity initially reported, modifications to this PCR, including more recent primers, have been reported with a sensitivity approaching one tachyzoite (Jones et al., 2000).

The most common use of PCR in ocular disease is to differentiate *T. gondii* from herpes family viruses (van Gelder, 2003). Short tandem multiple pathogen PCR analysis has been validated as a possible approach, given the inherent limited sample from ocular tissues (Dabil *et al.*, 2001). While PCR can be less sensitive for ocular toxoplasmosis, it appears to be very sensitive for herpetic ocular disease, with reported sensitivities as high as 97 percent (Abe *et al.*, 1998; Ganatra *et al.*, 2000).

5.9.6 Clinical tissue culture systems

Tissue culture systems developed for viral culture are widely available in most eye centers, and have been used to culture *T. gondii* (Miller *et al.*, 2000). Because it can take several weeks before a result becomes positive, therapy should likely be initiated empirically while awaiting results.

5.9.7 Differential diagnosis

Congenital toxoplasmosis must be differentiated from other possible causes of the classic clinical acronym 'TORCH' for a series of etiologies that share similar signs and symptoms. The acronym includes Toxoplasma, rubella, cytomegalovirus, syphilis, and herpes simplex virus. However, emerging pathogens such as West Nile Virus must be considered as part of any differential in known congenital infection (Alpert et al., 2003). Recurrent toxoplasmosis with its unilateral active lesion associated with multiple adjacent chorioretinal scars with the appropriate clinical history is virtually pathognomonic. However, clinical syndromes such as serpiginous chorioretinitis, and other infectious etiologies such as cytomegalovirus, may occasionally be considered. For the many other possible and unusual manifestations of ocular toxoplasmosis, such as pars planitis, the differential diagnosis is even broader, and includes autoimmune disorders such as multiple sclerosis and infections such as Lyme disease. Importantly, there are likely many cases of unusual manifestations of ocular toxoplasmosis that remain undiagnosed because of the limits of non-invasive assays.

5.10 THE TREATMENT AND MANAGEMENT OF OCULAR TOXOPLASMOSIS

Despite Toxoplasma being the most common cause of posterior uveitis in the United States and the world, there is no consensus on best treatment, and controversy exists as to whether treatment should be initiated. In addition, there is no treatment that prevents recurrences. Part of the lack of evidence for a best treatment is that the inflamed active component of ocular toxoplasmosis resolves in immunocompetent individuals without treatment. In addition, there is a variable course depending on host factors, environment, and parasite. A very controversial review of the literature highlighted that only three designed prospective randomized placebo-controlled studies exist (Stanford et al., 2003), and much of the literature was deemed inappropriate for analysis because of a lack of placebo. The conclusion of this meta-analysis went against what most would consider to be the standard of care. Given the known clinical experience with this disease, most clinical scientists would not have sufficient ethical equipoise to design a placebo trial for the management of ocular toxoplasmosis.

A recent survey highlights the uncertainty regarding the treatment and understanding of toxoplasmosis (Lum et al., 2005). The formal survey was completed in the year 2000; 1000 ophthalmologists in the United States were contacted, and 48 percent of them responded. During the 2 years of 1999 and 2000, there were an estimated 253 000 visits to ophthalmologists in the United States for ocular toxoplasmosis, 24000 of which were for active disease. There was a surprising lack of understanding among surveyed respondents regarding the importance of acquired disease (50 percent), the elderly as a high-risk group (16 percent), and the lack of likelihood of transmission to the fetus from a recurrence of ocular toxoplasmosis during pregnancy (30 percent). Only 19 percent of respondents, compared to 15 percent of uveitis subspecialists, treated all patients with ocular toxoplasmosis

(Holland and Lewis, 2002). Surprisingly, a zone of recurrence called the papillomacular bundle, which is a vital area for vision, would only merit treatment by 51 percent of the respondents. In the author's opinion, this area of recurrence should always warrant treatment.

There are many different regimens that are used in the treatment of ocular toxoplasmosis. A recent survey of uveitis subspecialists reported that 9 different commercially available drugs were used in 24 different possible combinations as the treatment of choice in the treatment of typical ocular toxoplasmosis (Holland and Lewis, 2002). The following 17 different oral drugs had been used by the 80 respondents as treatment in all types of ocular toxoplasmosis, in descending order of frequency:

	No. of	Percentage of
Drug	respondents	respondents
Clindamycin	74	94
Pyrimethamine	71	90
Sulfadiazine	64	81
Trimethoprim/ sulfamethoxazole	64	81
Sulfadiazine/ sulfamerazine/ sulfamethazine ('triple sulfa')	37	47
Doxycycline	27	34
Atovaquone	26	33
Tetracycline	25	32
Minocycline	20	25
Azithromycin	15	19
Sulfasoxazole	14	18
Pyrimethamine/ sulfadoxine	29	36
Clarithromycin	6	8
Spiramycin	6	8
Trimethoprim	6	8
Dapsone	5	6
Trimetrexate	1	1

Comparison of results between a 1991 survey on uveitis specialists and one undertaken in 2001
indicates a trend toward more aggressive treatment of uveitis among respondents. There was decreased use of clindamycin between the two surveys. The initial enthusiasm for clindamycin was because of the finding that clindamycin appeared both to achieve good intraocular concentrations and to enter into cysts well (Tabbara and O'Connor, 1975). However, the decrease in use of clindamycin is presumed to be due to the lack of evidence of improved outcome and the fear of side effects such as pseudomembranous enterocolitis.

The most commonly used treatment regimen is a combination of sulfadiazine, pyrimethamine, corticosteroids, and folinic acid (Holland and Lewis, 2002). This regimen has demonstrated *in vitro* synergy for its activity against *T. gondii*. The plasma half-life of pyrimethamine in adults is 100 hours, and in children is about 60 hours (McLeod *et al.*, 1992). The current author and most experts consider the combination of pyrimethamine and sulfadiazine to be the gold standard for the treatment of ocular toxoplasmosis.

In a recent study from France, where serologic testing for T. gondii is a routine part of pre-natal care, 18 of 24 consecutive congenitally infected patients were examined for ocular outcome with treatment (Brezin et al., 2003). An oral regimen was used to treat mothers prenatally. Pyrimethamine (50 mg per day) was alternated throughout gestation with 4 weeks of concomitant sulfadiazine (3 g per day) and folinic acid, followed by a 2-week cycle of spiramycin (9 million IU per day). Postnatal treatment was continued for 1 year with a regimen of 1 mg/kg per day of pyrimethamine, 50 mg/kg per day of sulfadiazine, and 50 mg/week of folinic acid. The ocular outcome was that 61 percent had no lesions, peripheral lesions were seen in nine eyes of five children (four eyes also had posterior pole lesions), and posterior pole lesions were detected in six eyes of five children (all of which had good visual acuity). Only one patient had severe visual impairment, which was associated with sensory deprivation nystagmus.

In a different study where 15 of 39 cases of congenital *Toxoplasma* infection did not result in termination of gestation, the treatment regimen

was less aggressive: 3 g of spiramycin per day was administered when infection was suspected and pyrimethamine plus sulfonamides was added when diagnosis in the fetus was confirmed. With a shorter median follow-up of 12 months, only 2 patients had eye lesions (Daffos *et al.*, 1988).

For infants, the pyrimethamine dose is usually 1 mg/kg per day, and the sulfadiazine dose is 100 mg/kg per day in two equal doses. Folinic acid is given 10 mg TIW with apple juice. This infant regimen is derived from the Chicago Collaborative Treatment Trial, which produced a helpful dispensing aide based on weekly weight assessment (McAuley et al., 1994). In addition, treatment is not dictated by presence or absence of eye involvement alone in congenital toxoplasmosis, as extended treatment appears to be indicated to provide optimal outcome for the multiple systemic complications (Remington et al., 2001). The regimen can result in prompt resolution of active ocular toxoplasmosis in newborns (Mets et al., 1996).

The most common side effect from the use of pyrimethamine is bone-marrow toxicity. A peripheral complete blood count should be performed as frequently as twice a week. Folinic acid is commonly used to help ward off the toxicity associated with pyrimethamine therapy (80 percent of respondents) (Holland and Lewis, 2002). Folinic acid can be used at doses of 5 mg (two to seven times daily per week). Folinic acid does not cross the cellular membrane of T. gondii, and therefore has no impact on pyrimethamine efficacy (Allegra et al., 1987). Sulfadiazine can cause a crystalluria, which usually promptly responds to alkalinazation of the urine, and there is one report in the ophthalmic literature of acute ureteric obstruction soon after initiation of therapy for ocular toxoplasmosis (Smith et al., 2001).

5.10.1 Chronic suppressive therapy

There is one paper that details the success of chronic long-term therapy in the treatment of ocular toxoplasmosis(Silveira *et al.*, 2002); however, there are no large-scale multicenter studies that validate the use of chronic suppressive therapy in

ocular toxoplasmosis – although many data exist regarding improved outcome with chronic suppressive therapy in central nervous system toxoplasmosis in patients with AIDS. Extrapolation based on the anatomic similarities between the eye and the brain suggests that suppressive therapy could be useful; however, further study will be required to validate chronic suppressive therapy broadly. If an individual has serious visual morbidity from frequent recurrences or very visually threatening lesions, the current author believes that chronic suppressive therapy should likely be instituted. The duration and optimal regimen of chronic suppressive therapy remains unknown.

5.10.2 Corticosteroids

Intravitreal, topical, oral, and periocular corticosteroids have been used as part of the regimen to treat ocular toxoplasmosis. Topical corticosteroids were used by 80 percent of respondents presumably to prevent presumed complications of anterior segment inflammation such as posterior synechiae (scarring of the iris to the underlying lens). Only 17 percent of respondents used corticosteroids in all patients regardless of the severity of the inflammation; 71 percent of uveitis specialist respondents would use therapy in severe vitreous inflammation. Highlighting how ocular toxoplasmosis is secondary to actively replicating parasites, there are several reports regarding poor outcome with patients treated with corticosteroids alone and without a concomitant antiparasitic regimen (O'Connor and Frenkel, 1976; Nozik, 1977; Sabates et al., 1981).

5.10.3 Laser treatment

In the past, laser treatment was used on the retina surrounding *Toxoplasma* scars (Spalter *et al.*, 1966). The theory was that the destruction of retina by local laser treatment would dramatically decrease (if not eliminate) the risk of recurrence. Since *T. gondii* bradyzoites have been demonstrated in normal-appearing retina, the practice of laser treatment of the retina as a means of prophylaxis against recurrence is rarely if ever employed today.

5.10.4 Subconjunctival therapy

Clindamycin has been administered subconjunctivally with an injection of 50 mg every other day for 30 days (Ferguson, 1981). The benefit of subconjunctival injection as a route of local medicine administration is that if the medicine were to penetrate the sclera and achieve sufficient intraocular concentrations, it would be very unlikely to develop serious route-of-administration complications with a subconjunctival injection as compared to an intravitreal injection. Of note, a larger amount of subconjunctival clindamycin (150 mg) has been shown to result in corneal edema (Tabbara and O'Connor, 1975).

5.10.5 Intravitreal therapy

Intravitreal therapy has the risk of irreversible blindness with each administration of any medicine, as well as other potential complications. However, recently, intravitreal therapy has been the mode of administration of medicines for a variety of eye diseases. The benefit of intravitreal therapy is that it has excellent bioavailability and almost no risk of systemic side effects. Intravitreal clindamycin/ dexamethasone, clindamycin/triamcinolone acetonide with systemic anti-*Toxoplasma* therapy (Aggio *et al.*, 2005), and liposomal encapsulated clindamycin (Peyman *et al.*, 1988) have all been used in ocular toxoplasmosis.

5.11 CONCLUSIONS

The devastation caused by ocular toxoplasmosis, despite being widespread, remains without appropriate attention. Perhaps this is because ocular toxoplasmosis is a disease that crosses several disciplines: epidemiology, infectious disease, ophthalmology, pediatrics, internal medicine, pathology, and parasitology. Also, ocular toxoplasmosis is a disease where the active component will resolve; however, visual morbidity remains and is often permanent after inflammation has resolved. With the recent identification of unique intracellular targets (Roberts *et al.*, 1998) to kill

T. gondii effectively, with little likelihood of any impact on our own cellular machinery, the future of care of patients with ocular toxoplasmosis will likely change dramatically. Indeed, it is possible in the future that regimens may not only treat active disease but also effectively kill all stages of the parasite, eliminating the frustrating possibility of recurrence.

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Toxoplasmosis in Wild and Domestic Animals

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6.1 Introduction

6.2 Toxoplasmosis in wildlife

6.3 Toxoplasmosis in zoos

6.4 Toxoplasma gondii and endangered species

6.1 INTRODUCTION

Toxoplasma gondii is widely distributed in wild and domestic animals. This chapter reviews toxoplasmosis in wild and domestic animals. Coverage in wild animal species is limited to confirmed cases of toxoplasmosis, cases with parasite isolation, cases with parasite detection by PCR, and experimental infection studies (Figures 6.1–6.3). Studies concerning serological prevalence have not been included for the majority of host species. This is because many serological tests (latex agglutination, indirect hemagglutination) have been demonstrated to underestimate the prevalence of *T. gondii*.

6.2 TOXOPLASMOSIS IN WILDLIFE

6.2.1 Felids

Congenital toxoplasmosis has been reported in bobcat (*Felis rufus*) kits (Dubey *et al.*, 1987).

6.5 Toxoplasmosis in pets6.6 Domestic farm animals6.7 Fish, reptiles, and amphibians *References*

Toxoplasmic meningoencephalitis has been observed in a 6-month-old bobcat (Smith *et al.*, 1995), and *Toxoplasma gondii* has been isolated from the tissues of adult bobcats (Lindsay *et al.*, 1997a; Dubey *et al.*, 2004a). Bobcats are important in maintaining *T. gondii* in wild herbivores in many areas of the United States (Figure 6.1).

Oocysts excreted by cougars (*Felis concolor*) were thought to be the source of a large waterborne outbreak of human toxoplasmosis in Victoria, British Columbia, Canada, and oocysts were isolated from the feces of cougars collected around the watershed (Aramini *et al.*, 1998). Experimental infections resulting in oocyst excretion have been demonstrated in jaguarundi (*F. yagouaroundi*), ocelot (*F. pardalis*), bobcats (*Lynx rufus*), and cheetah (*Acinonyx jubatus*) (Jewell *et al.*, 1972; Miller *et al.*, 1972). In general, these felids are not as efficient at producing oocysts as are domestic cats.

6.2.2 Canids

Acute toxoplasmosis has been reported in arctic foxes (*Alopex lagopus*) (Sørensen *et al.*, 2005),



FIGURE 6.1 Fatal toxoplasmic encephalitis in a naturally infected bobcat. H & E stain. (A) Necrosis and inflammation of a blood vessel (arrow). Bar = $50 \mu m$.

- (B) Tachyzoites (arrows) in a capillary. Bar = $10 \mu m$.
- (C) Vasculitis and suppurative encephalitis. Bar = $100 \,\mu m$.
- (D) An abscess with degenerating neutrophils and tachyzoites (arrows). Bar = $10 \,\mu m$.

Fennec foxes (*Fennecus zerda*) (Kottwitz *et al.*, 2004), gray foxes (*Urocyon cinereoargenteus*) (Davidson *et al.*, 1992; Dubey and Lin, 1994; Kelly and Sleeman, 2003), and red foxes (*Vulpes vulpes*) (Reed and Turek, 1985; Dubey *et al.*, 1990; Kelly and Sleeman, 2003). Co-infection with canine distemper virus is often associated with clinical toxoplasmosis in gray foxes (Davidson *et al.*, 1992; Kelly and Sleeman, 2003) and red foxes (Reed and Turek, 1985). Clinical toxoplasmosis has not been documented in wolves, coyotes, hyenas, or dingos. *Toxoplasma gondii* has been isolated from red

foxes (Smith and Frenkel, 1995; Dubey *et al.*, 2004a), gray foxes (Dubey *et al.*, 2004a), and coyotes (Lindsay *et al.*, 1997a; Dubey *et al.*, 2004a).

6.2.3 Bears

Clinical toxoplasmosis has not been reported from bears. Viable *T. gondii* has been isolated from black bears (*Ursus americanus*) (Dubey *et al.*, 1995a). Serological surveys indicate that *T. gondii* infections occur in polar bears (*Ursus maritimus*) (Rah *et al.*, 2005) and in grizzly bears (*Ursus arctos*) (Chomel *et al.*, 1995). Bear meat should be considered a potential source of *T. gondii*.

6.2.4 Raccoons

Many serosurveys indicate that *T. gondii* is highly prevalent in raccoons (*Procyon lotor*) (reviewed by Hancock *et al.*, 2005). Encysted *T. gondii* has been isolated from the tissues of naturally infected raccoons (Lindsay *et al.*, 1997a; Dubey *et al.*, 2004a). Clinical toxoplasmosis has not been immunohistochemically confirmed from raccoons, and they are resistant to experimental infection (Dubey *et al.*, 1993a).

6.2.5 Squirrels

Acute toxoplasmosis has been reported in gray squirrels (*Sciurus carolinensis*) (Soave and Lennette, 1959; Roher *et al.*, 1981; Dubey *et al.*, 2006a), thirteenlined ground squirrels (*Citellus tridecemlineatus*) (van Pelt and Dieterich, 1972), and Korean squirrels (*Tanias sibericus*) (Carrasco *et al.*, 2006). *Toxoplasma gondii* has been isolated from gray squirrels (Walton and Walls, 1964; Smith and Frenkel, 1995), and Formosan giant flying squirrels (*Petaurista petaurista grandis*) (Cross and Santana, 1969).

6.2.6 Rabbits and hares

Fatal toxoplasmosis has been reported in three domestic (Oryctolagus cuniculus) rabbits from two different sources in the United States (Dubey et al., 1992a). The rabbits died after an acute illness characterized by fever, lethargy, and diarrhea in one rabbit, and no clinical signs in the other two rabbits. The most striking lesion in all three rabbits was the foci of necrosis of the spleen and liver associated with massive presence of multiplying tachyzoites (Dubey et al., 1992a). Similar findings were present in 2-18-month-old domestic rabbits from 15 flocks in Germany. Necropsy examinations of 49 rabbits revealed lesions of a generalized granulomatousnecrotizing toxoplasmosis within the spleen, liver, lungs, and lymph nodes (Bergmann et al., 1980). Both authors of the current chapter have inoculated domestic rabbits orally and subcutaneously with

T. gondii oocysts (usually 10 000/rabbit) to generate immune serum for immunohistochemistry. All inoculated rabbits had fatal toxoplasmosis, or would have developed it had they not been put down for humane reasons.

Brown hares (Lepus europaeus) develop fatal toxoplasmosis after experimental infection with as few as 10 oocysts, and in a study all inoculated hares died within 8 to 19 days after ingesting oocysts (Sedlák et al., 2000). The typical pathological findings in hares are hemorrhagic enteritis, enlargement and hyperemia of mesenteric lymph nodes, splenomegaly, and multiple necrotic lesions in the parenchyma of the liver and other organs (Sedlák et al., 2000). Mountain hare (Lepus timidus) experimentally inoculated with 50 T. gondii oocysts and examined 7 days later had gross lesions in the mesenteric lymph nodes and liver (Gustafsson et al., 1997). Histologically, the hares had extensive necrotic areas in the small intestine, mesenteric lymph nodes, and liver, and less prominent foci of necrosis in various other organs (Gustafsson et al., 1997).

6.2.7 Skunks

Toxoplasma gondii genotype III was isolated from three of six asymptomatic striped skunks (*Mephitis mephitis*) from Mississippi (Dubey *et al.*, 2004b). Two of the three isolated were mouse pathogenic, even thought they were molecularly consistent with the mouse avirulent genotype III.

6.2.8 Fisher

Toxoplasma gondii was detected by PCR from brain and skeletal muscle of a free-ranging juvenile fisher (*Martes pennanti*) from Maryland (Gerhold *et al.*, 2005). Clinically this animal had encephalitis, but it was not associated with the *T. gondii* infection.

6.2.9 Beavers

Toxoplasma gondii has been isolated from beaver (*Castor canadensis*) tissue (Dubey, 1983; Smith and Frenkel, 1995), but clinical toxoplasmosis has not been observed in these animals. Of 62 samples from beavers in Massachusetts, 6 (10 per cent)

were positive in the direct agglutination test (Jordan *et al.*, 2005).

6.2.10 Insectivores

Little is known about toxoplasmosis in insectivores. The prevalence of *T. gondii* using the Sabin– Feldman dye test was < 1 percent in 578 insectivores from the Czech Republic (Hejliček *et al.*, 1997). Fatal toxoplasmosis was diagnosed in a juvenile male common mole (*Talpa europaea*) from Germany (Geisel *et al.*, 1995).

6.2.11 Bats

There are no reports of clinical toxoplasmosis in bats. Isolation of *T. gondii* was reported from *Vespertilio pipistrellus* and the red night bat *Nyctalus noctula* from Alma-Ata, Kazakhstan, USSR (Galuzo *et al.*, 1970). Inoculation of RH *T. gondii* did not induce clinical disease in red night bats in these studies.

6.2.12 White-tailed and mule deer

Toxoplasma gondii is prevalent in deer from North America. Consumption of venison has been linked with clinical toxoplasmosis in humans (Sacks et al., 1982; Ross et al., 2001). Clinical toxoplasmosis has not been described from naturally infected deer in North America. Toxoplasma gondii has been isolated from the tissues of white-tailed deer (Odocoileus virginianus) (Lindsay et al., 1991b, 1997a; Dubey et al., 2004a) and mule deer (Odocoileus hemionus) (Dubey, 1982). The fetuses from T. gondii-positive white-tailed and mule deer were negative for T. gondii antibodies in one study, suggesting that seropositive dams do not transmit the infection to their fetuses (Lindsay et al., 2006). Acute toxoplasmosis and death can occur in mule deer experimentally inoculated with T. gondii oocysts (Dubey et al., 1982).

6.2.13 Other deer

Congenital toxoplasmosis has been observed in reindeer (*Rangifer tarandus*) from a private collection in the United States (Dubey *et al.*, 2002a).

Yearling reindeer may develop enteritis and die after experimental oral infection with *T. gondii* oocysts (Oksanen *et al.*, 1996). *Toxoplasma gondii* has been isolated from Roe deer (*Capreolus capreolus*) (Entzeroth *et al.*, 1981).

6.2.14 Other wild ruminants

Elk (*Cervus canadensis*) are resistant to clinical disease following oral infection with oocysts but *T. gondii* can be isolated from many of their tissues, indicating that elk are a potential source of infection for humans (Dubey *et al.*, 1980).

Toxoplasma gondii has been isolated from naturally infected pronghorn antelope (Dubey, 1981). Acute toxoplasmosis and death can occur in pronghorn antelopes experimentally inoculated with *T. gondii* oocysts (Dubey *et al.*, 1982).

Toxoplasmic encephalitis has been observed in a 4-month-old Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) (Baszler *et al.*, 2000).

Toxoplasma gondii has been isolated from naturally infected moose (*Alces alces*) (Dubey, 1981).

6.2.15 Sea otters and other marine mammals

Toxoplasmosis was recognized as a significant cause of mortality in southern sea otters (Enhydra lutris nereis) in the early 1990s (Cole et al., 2000). Encephalitis is the primary cause of T. gondiiassociated death in these sea otters (Kreuder et al., 2003). This was unexpected, as sea otters do not ingest the usual intermediate hosts of T. gondii, and their location in seawater keeps them segregated from cats. Definitive proof that T. gondii was killing the sea otters came when viable T. gondii was isolated from the tissues of sea otters (Cole et al., 2000; Lindsay et al., 2001a) and isolated parasites from sea otters were shown to retain the ability to make oocysts when fed to cats (Cole et al., 2000). Initial isolates were all type II genotypes of T. gondii (Cole et al., 2000), but a novel new genotype X was identified in sea otter a few years later (Miller et al., 2004). It has been postulated that T. gondii oocysts excreted in the feces of feral cats living along the Pacific coast enter the marine

environment and are ingested by sea otters when they feed on paratenic hosts (Cole et al., 2000; Conrad et al., 2005), and this is supported by the fact that coastal freshwater runoff is a risk factor for T. gondii infection in southern sea otters (Miller et al., 2002). Toxoplasma gondii oocysts will sporulate in seawater and remain infectious for at least 6 months (Lindsay et al., 2003), and viable T. gondii and T. gondii DNA can be recovered from experimentally inoculated bivalves (Lindsay et al., 2001b, 2004; Arkush et al., 2003), further supporting these assumptions. Toxoplasma gondii also cause deaths in other marine mammals off the Pacific coast of the United States, often in the same areas as the sea otters (Miller et al., 2001). Toxoplasma gondii genotype X has also bee isolated from Pacific harbor seals (Phoca vitulina) and California sea lions (Zalophus californianus) (Conrad et al., 2005).

Toxoplasmosis is frequently reported from dolphins worldwide (Dubey *et al.*, 2003a). Congenital toxoplasmosis has been reported in Indo-Pacific bottle-nosed dolphins (*Tursiops aduncus*) (Jardine and Dubey, 2002). Disseminated toxoplasmosis with transplacental fetal infection has been seen in a pregnant Risso's dolphin (*Grampus griseus*) (Resendes *et al.*, 2002). Acute cases of toxoplasmosis have been seen in Indo-Pacific humpbacked dolphins (*Sousa chinensis*) (Bowater *et al.*, 2003), spinner dolphins (*Stenella longirostris*) (Migaki *et al.*, 1990), and Atlantic bottle-nosed dolphins (*Tursiops truncatus*) (Inskeep *et al.*, 1990).

Toxoplasmosis has been reported from several additional species of marine mammals, such as the beluga whale (Delphinapterus leucas) (Mikaelian et al., 2000), California sea lion (Zalophus californianus) (Migaki et al., 1977), northern fur seal (Callorhinus ursinus) (Holshuh et al., 1985), elephant seal (Mirounga angustirostris) (Dubey et al., 2004c), Hawaiian monk seal (Monachus schauinslandi) (Honnold et al., 2005), Antillean manatee (Trichechus manatus manatus) (Dubey et al., 2003a), and West Indian manatee (Trichechus manatus) (Buergelt and Bonde, 1983). Experimental infection of grey seals (Halichoerus grypus) with up to 10000 T. gondii oocysts did not induce overt clinical disease (Gajadhar et al., 2004). Mild behavioral changes were the only adverse effects, and T. gondii was isolated from brain and muscles of the experimentally infected seals.

6.2.16 New World monkeys

Toxoplasmosis can be a problem in exhibited New World monkeys (Table 6.1). Many reports of acute disease have come from squirrel monkeys (*Saimiri sciureus*) (Anderson and McClure, 1982; Dickson *et al.*, 1983; Cunningham *et al.*, 1992; Dietz *et al.*, 1997; Inoue, 1997; Epiphanio *et al.*, 2003) and golden lion tamarins (*Leontopithecus rosalia*) (Dietz *et al.*, 1997; Pertz *et al.*, 1997; Juan-Salles *et al.*, 1998; Epiphanio *et al.*, 2003). Squirrel monkeys and Panamanian night monkeys (*Aotus lemurinus*) are highly susceptible to oral tissue cyst inoculation, and develop acute fatal disease (Harper *et al.*, 1985; Escajadillo and Frenkel, 1991; Furuta *et al.*, 2001).

TABLE 6.1 Summary of host species reports of clinical toxoplasmosis in New World primates

Cotton-top tamarin (Saguinus oedipus) Yellow-handed marmoset (Saguinus midas midas) Black marmoset (Saguinus midas niger) Emperor marmoset (Saguinus imperator) Red-bellied white-lipped tamarin (Saguinus labiatus) Black lion tamarin (*Leontopithecus chrysopygus*) Golden-headed lion tamarins (Leontopithecus chrysomelas) Golden lion tamarins (Leontopithecus rosalia) Squirrel monkeys (Saimiri sciureus) Pygmy marmoset (Callithrix pygmaea) Common marmoset (Callithrix jacchus) Black ear-tufted marmoset (Callithrix pencillata) Pale-headed saki (Pithecia pithecia) Night monkey (Aotus trivirgatus) Howler monkey (Alouatta fusca) Woolly monkey (Lagothrix lagotricha)

Sources: Anderson and McClure, 1982; Dickson *et al.*, 1983; Cunningham *et al.*, 1992; Dietz *et al.*, 1997; Inoue, 1997; Bouer *et al.*, 1999; Epiphanio *et al.*, 2001, 2003.

6.2.17 Old World monkeys

Toxoplasmosis is reported infrequently in Old World monkeys. A case of concurrent central nervous system toxoplasmosis and simian immunodeficiency virus-induced AIDS encephalomyelitis has been seen in a Barbary macaque (*Macaca sylvana*) (Sasseville *et al.*, 1995). Rhesus monkeys (*Macaca mulatta*) and stump-tailed macaques (*Macaca arctoides*) have been are used as experimental models for human congenital toxoplasmosis (Wong *et al.*, 1979; Schoondermark-van de Ven *et al.*, 1993), and cynomolgus monkeys (*Macaca fascicularis*) have been used as a model for recurrent toxoplasmic retinochoroiditis (Holland *et al.*, 1988).

6.2.18 North American marsupials

Toxoplasma gondii has been isolated from North American opossums (*Didelphis virginiana*) (Smith and Frenkel, 1995). North American opossums are more resistant to experimental toxoplasmosis than are Australian marsupials (Patton and Funk, 1992).

6.2.19 Australian marsupials

Toxoplasma gondii infection is usually life-ending in marsupials from Australia or New Zealand. Outbreaks of toxoplasmosis often occur in these animals when housed in zoos (see below). These animals evolved in the absence of cats and *T. gondii*, and this may be why they are so highly susceptible.

Canfield *et al.* (1990) summarized clinical signs, necropsy findings, and histopathological changes for 43 macropods (species not given), 2 common wombats (*Vombatus ursinus*), 2 koalas (*Phascolarctos cinereus*), 6 possums (species not given), 15 dasyurids (species not given), 2 numbats (*Myrmecobius fasciatus*), 8 bandicoots (species not given), and 1 bilby (*Macrotis lagotis*). The animals either died suddenly without clinical signs, or exhibited signs associated with respiratory, neurological, or enteric disease. At necropsy, many had no visible lesions. Common necropsy findings included pulmonary congestion, edema and consolidation, adrenal enlargement and reddening, hemorrhage and ulceration of stomach and small intestine, and lymphadenomegaly and splenomegaly (Canfield *et al.*, 1990). Congenital toxoplasmosis apparently occurs in black-faced kangaroos (*Macropus fuliginosus melanops*), based on the finding of *T. gondii* in the tissues of an 82-day-old joey that died from toxoplasmosis (Dubey *et al.*, 1988a). *Toxoplasma gondii* was seen in the heart, kidney, liver, lung, lymph node, spleen, small intestine, and stomach from two koalas (*Phascolarctos cinereus*) that died suddenly in a fauna park in Sydney, Australia (Hartley *et al.*, 1990).

Experimental studies support the assumption that Australian marsupials are highly susceptible to toxoplasmosis. Eastern barred bandicoots (Perameles gunnii) developed acute toxoplasmosis when fed 100 T. gondii oocysts and died 15 and 17 days post-infection (Bettiol et al., 2000). Lesions consistent with acute toxoplasmosis were present in their tissues. The authors indicated that T. gondii may be a cause for a reduction in wild populations of eastern barred bandicoots. Tammar wallabies (Macropus eugenii) fed 500, 1000, or 10 000 T. gondii oocysts died of acute toxoplasmosis 9 to 15 days after challenge (Reddacliff et al., 1993). The lesions consisted of foci of necrosis and inflammation in the intestines, lymphoid tissue, adrenal cortex, heart, skeletal muscle, and brain, and severe generalized pulmonary congestion and edema.

6.2.20 African wildlife

Surprisingly little is known about *T. gondii* and toxoplasmosis from African mammals. Clinical disease has not been reported from free-ranging elephants, hippopotamus, giraffes, gazelle, wildebeests, impala, chimpanzees, baboons, orangutans, and gorillas, and nor has the parasite been isolated from the tissues of these mammals. *Toxoplasma gondii* has been isolated from domestic chickens and ducks from Africa, and this is discussed below.

6.2.21 Wild birds

Dubey (2002) reviewed the literature on *T. gondii* in wild birds. Table 6.2 lists the wild avian hosts

Pigeon (Columba livia)

TABLE 6.2 Host records for Toxoplasma gondii isolation from wild birds

Anseriformes Mallards (Anas platyrhynchos) Pochard (Aythya ferrina) Tufted ducks (Aythya fuligula) Pintail (Anas acuta) Gadwall (Anas strepera) Canada goose (Branta canadensis) Accipitriformes Goshawk (Accipiter gentilis) Cooper's hawk (Accipiter cooperi) Common buzzard (Buteo buteo) Kestrel (Falco tinnunculus) American kestrel (Falco sparverius) Pallid harrier (Circus macrourus) Black vulture (Aegypius monachus) Red-tailed hawk (Buteo jamaicensis) Red-shouldered hawk (Buteo lineatus) Galliformes Partridge (Perdix perdix) Pheasant (Phasianus colchicus) Wild turkey (Meleagris gallopavo) Gruiformes Coot (Fulica atra) Charadriformes Blackheaded gull (Larus ridibundus) Common tern (Sterna hirundo) Columbiformes Collared dove (Streptopelia decaocto) Laughing dove (Streptopelia senegalensis) Woodpigeon (Columba palumbus)

Ruddy ground dove (Columbina talpacoti) Strigiformes Ferruginious pygmy owl (Glaucidium brasilianum) Little owl (*Athene noctua*) Great horned owl (Bubo virginianus) Barred owl (Strix varia) **Passeriformes** Great grey shrike (Lanius excubitor) Yellowhammer (Emberiza citrinella) Chaffinch (Fringilla coelebs) House sparrow (Passer domesticus) Tree sparrow (Passer montanus) Jay (Garrulus glandarius) Starling (Sturnus vulgaris) Palm tanager (Thraupis palmarum) Blackbird (Turdus merula) Mistle thrush (Turdus viscivorus) Song thrush (Turdus philomelos) Robin (Erithacus rubecula) Great tit (Parus major) Nuthatch (Sitta europea) Treecreeper (Certhia familiaris) Greenfinch (Chloris chloris) American crow (Corvus brachyrhynchos) Carrion crow (Corvus corone) Jackdaw (Corvus monedula) Rook (Corvus frugilegus)

Source: Dubey, 2002.

from which viable *T. gondii* has been isolated, and Table 6.3 lists the avian species which have been reported to suffer from clinical toxoplasmosis.

Toxoplasma gondii is readily isolated from the hearts and breast muscles of raptors (Lindsay *et al.*, 1993). Necrotizing myocarditis caused by *T. gondii*

has been observed in a bald eagle (*Haliaeetus leucocephalus*) from New Hampshire (Szabo *et al.*, 2004). Severe toxoplasmic hepatitis was seen in an adult barred owl (*Strix varia*) from Quebec, Canada (Mikaelian *et al.*, 1997). No clinical signs were seen in three red-tailed hawks (*Buteo jamaicensis*) fed *T. gondii* tissue cysts (Lindsay *et al.*, 1991a), although

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Columbiformes	Psittaciformes		
Common pigeon (Columba livia)	Budgerigars (Melopsittacus undulatus)		
Crown pigeons (Goura sp.)	Regent parrot (Polytelis anthopeplus)		
Torres Strait pigeon (Ducula spilorrhoa)	Superb parrot (Polytelis swansonii)		
Wonga pigeon (Leucosarcia melanoleuca)	Red lory (Eos bornea)		
Bleeding-heart dove (Gallicolumba	Swainson's lorikeet (Trichologlossus moluccanus)		
luzonica)	Crimson rosella (Platycercus elegans)		
Nicobar pigeon (Caloenas nicobarica)	Strigiformes		
Luzon bleeding-heart pigeon (<i>Gallicoluba</i>	Barred owl (Strix varia)		
Orange breasted green pigeon (Tranch	Galliformes		
bicinta)	Wild turkeys (Meleagris gallapavo)		
Crested wood partridge (Rolulus roul roul)	Partridges (Perdix perdix)		
Yellow-headed rockfowl (Picathartes	Capercaillie (<i>Tetrao urogallus</i>)		
gymnocephaus)	Erckel's francolin (Francolinus erckelii)		
Passeriformes	Anseriformes		
Canaries (Serinus canarius)	Magpie geese (Anseranas semipalmata)		
Greenfinches (Carduelis chloris)	Hawaiian nene goose (Nesochen sandicensis)		
Goldfinches (Carduelis carduelis)	Sphenisciformes		
Sirkins (Carduelis spinus)	Humboldt penguin (Spheniscus humboldti)		
Linnets (Carduelis cannabina)	Megellanic penguin (Spheniscus magellanicus)		
Bullfinches (Pyrrhula pyrrhula)	Black-footed penguin (Spheniscus demersus)		
Hawaiian crow (Corvus hawaiiensis)	Little penguin (<i>Eudyptula minor</i>)		
Satin bowerbird (Ptilornorhyncus violaceus)	Indian Pangolin (Manis crassi caudato)		
Regent bowerbird (Sericulus chrysocephalus)	Pelecaniformes		
Red-whiskered bulbul (Pycnonotus jocosus)	Red-footed booby (Sula sula)		

Source: Dubey, 2002.

Toxoplasma gondii was isolated from all three red-tailed hawks. No clinical signs were seen in great horned owls (*Bubo virginianus*), barred owls (*Strix varia*) or screech owls (*Asio otus*), fed *T. gondii* tissue cysts (Dubey *et al.*, 1992b), but parasites were isolated from the tissues of the owls at necropsy. *Toxoplasma gondii* was not re-isolated from a sparrow hawk (*Falco sparverius*) that had been experimentally infected (Miller *et al.*, 1972).

Viable *T. gondii* was isolated from the hearts of 8 of 16 wild turkeys (*Meleagris gallopavo*) from Alabama (Lindsay *et al.*, 1994). Fatal systemic toxoplasmosis has been seen in wild turkeys from Georgia (Howerth and Rodenroth, 1985) and West Virginia (Quist *et al.*, 1995).

Toxoplasma gondii genotype III was isolated from one of four Canada geese (*Branta canadensis*) from Mississippi (Dubey *et al.*, 2004b).

6.3 TOXOPLASMOSIS IN ZOOS

Toxoplasmosis is a zoo management problem because wild felids can excrete *T. gondii* oocysts in their feces (Jewell *et al.*, 1972; Miller *et al.*, 1972; Lukesová and Literák, 1998) and because of the occurrence of feral cats in zoos (Gorman *et al.*, 1986). Oocysts excreted by these felids can make their way into highly susceptible species.

Mammalian species that frequently develop toxoplasmosis in zoos include Australian marsupials (Dobos-Kovács, 1974; Boorman *et al.*, 1977; Dubey *et al.*, 1988a; Hartley *et al.*, 1990), New World and arborial monkeys (Cunningham *et al.*, 1992; Dietz *et al.*, 1997; Pertz *et al.*, 1997; Juan-Salles *et al.*, 1998; Epiphanio *et al.*, 2000), lemurs (Dubey *et al.*, 1985; Spencer *et al.*, 2004), and Pallas cats (Riemann *et al.*, 1974; Dubey *et al.*, 1988b; Kenny *et al.*, 2002; Basso *et al.*, 2005) (Figure 6.2). Lesions in these animals are consistent with acute toxoplasmosis, and are usually most severe in visceral tissues such as the lungs, liver, and spleen.

Sporadic cases of acute toxoplasmosis have been reported in exhibited dik-dik (*Madoqua* guentheri smithi) (Dubey et al., 2002b), slendertailed meerkats (*Suricata suricatta*) (Juan-Salles et al., 1997), and porcupine (*Coendou mexicanus*) (Morales et al., 1996). A case of abortion due to *T. gondii* has been reported in a Greenland muskox (*Ovibos moshatus wardi*) (Crawford et al., 2000).

Abortion and neonatal death have been observed in captive nilgais (*Boselaphus tragocamelus*). *Toxoplasma gondii* DNA was demonstrated in the tissues of the nilgais using PCR (Sedlák *et al.*, 2004). Fatal toxoplasmosis was diagnosed in a captive, adult female saiga antelope (*Saiga tatarica*). *Toxoplasma gondii* was detected in the liver, lung, spleen, kidney, and intestine, and confirmed by PCR (Sedlák *et al.*, 2004).

Acute toxoplasmois has been seen in captive Cuvier's gazelle (*Gazella cuvieri*), slender-horned gazelle (*G. leptoceros*), dama gazelle (*G. dama*), and gerenuk (*Litocranius walleri*) housed in North American zoos (Stover *et al.*, 1990; Junge *et al.*, 1992). These infections are disseminated, and most lesions are in the liver (Figure 6.3), lungs, lymph nodes, adrenal glands, spleen, intestines, and brain.

Outbreaks of toxoplasmosis also occur in avian species exhibited in zoos (Poelma *et al.*, 1972; Hubbard *et al.*, 1986). Toxoplasmosis in canaries has been reported from aviaries worldwide (reviewed by Dubey, 2002). *Toxoplasma gondii* genotype III was isolated from five of five blackwinged lorys (*Eos cyanogenia*) from an acute toxoplasmosis outbreak in an aviary in South Carolina (Dubey *et al.*, 2004b).

Management and husbandry programs can be designed to help achieve prevention of toxoplasmosis in highly susceptible species in zoos and aviaries. Felids should never be fed fresh, unfrozen meats because of the possibility of contamination with *T. gondii* tissue cysts. Meat that has been frozen



FIGURE 6.2 Necrosis associated with T. gondii in small intestine. H & E stain.

- (A) Necrosis of lamina propria (arrows) of villi 7 days after feeding oocysts to a mouse. The surface epithelium is not affected. Numerous tachyzoites are present in lesions, but are not visible at this magnification. Bar = $100 \,\mu\text{m}$.
- (B) Necrosis of the lamina propria cells including blood vessels in a naturally infected Pallas cat. Numerous tachyzoites (small arrows) are present. The surface epithelium (large arrow) was not affected. Bar = 10 μm.



FIGURE 6.3 Section of liver from a gazelle with toxoplasmosis showing a central area of hepatitis. Note *T. gondii* (arrows) in hepatocytes at the periphery of the lesion. H & E stain. Bar = $25 \mu m$.

solid and then thawed can be safely fed, because freezing kills *T. gondii* tissue cysts (Kotula *et al.*, 1991). Feral cats should be actively controlled in zoos to prevent them from shedding oocysts. Highly susceptible species should not be housed near felids.

Outdoor aviaries are at risk because of oocysts excreted by domestic cats. Aviaries should be designed to exclude cat feces and transport hosts (flies, roaches, etc.) which may bring in *T. gondii* on or in their bodies.

6.4 TOXOPLASMA GONDII AND ENDANGERED SPECIES

Toxoplasmosis can adversely affect endangered avian and mammalian species. The 'Alala (Hawaiian

crow, Corvus hawaiiensis) is an endangered species, and only about 25 were left in captivity and the wild in 2000 (Work et al., 2000). Tragically, these birds are highly susceptible to fatal toxoplasmosis, and develop disease after being introduced back in to the wild. Toxoplasmosis appears to pose a significant threat and management challenge to reintroduction programs for 'Alala in Hawaii (Work et al., 2000). Captive breeding groups of golden lion tamarins (Leontopithecus rosalia) have developed acute toxoplasmosis and suffered many fatalities both in North American and in European zoos (Pertz et al., 1997; Juan-Salles et al., 1998). These arboreal monkeys are endangered, and attempts to breed them in captivity for eventual release in the wild are hampered because it is difficult to keep them from being exposed to T. gondii.

6.5 TOXOPLASMOSIS IN PETS

6.5.1 Cats

Most cats are asymptomatic during a primary T. gondii infection. Fever (40.0-41.7°C) is present in many cats with clinical toxoplasmosis. Clinical signs of dyspnea, polypnea, and icterus, and signs of abdominal discomfort were the most frequent findings in 100 cats with histologically confirmed toxoplasmosis (Dubey and Carpenter, 1993). Uveitis and retinochoroiditis are also common clinical signs in cats with toxoplasmosis. Gross and microscopic lesions are found in many organs, but are most common in the lungs. Gross lesions in the lungs consist of edema and congestion, failure to collapse, and multifocal areas of firm, white to vellow, discoloration. Pericardial and abdominal effusions may be present. The liver is the most frequently affected abdominal organ, and diffuse necrotizing hepatitis may be visible grossly. Gross lesions associated with necrosis can also be observed in the mesenteric lymph nodes and pancreas.

All ages, sexes, and breeds of domestic cats are susceptible to *T. gondii* infection (Dubey *et al.*, 1977). Transplacentally or lactogenically infected kittens will excrete oocysts but the prepatent period is usually 3 weeks or more because the kittens are infected with tachyzoites (Dubey *et al.*, 1995b). Domestic cats under 1 year of age produce the greatest numbers of *T. gondii* oocysts. Cats that are born and raised outdoors usually become infected with *T. gondii* shortly after they are weaned and begin to hunt. *Toxoplasma gondii*naïve adult domestic cats will excrete oocysts if fed tissue cysts, but they will usually excrete fewer oocysts and for a shorter period of time than will recently weaned kittens.

Intestinal immunity to T. gondii is strong in cats that have excreted oocysts (Dubey, 1995). Primary T. gondii infection in cats does not cause immunosuppression (Lappin et al., 1992; Davis and Dubey, 1995). Serum antibody does not play a significant role in resistance to intestinal infection, and intestinal immunity is most likely cell mediated. Oocysts begin to be excreted in the feces before IgM, IgG, or IgA antibodies are present in the serum (Lappin et al., 1989). Partial development of the enteroepithelial stages occurs in the intestines of immune cats, but oocyst production is prevented (Davis and Dubey, 1995). Most cats that have excreted oocysts once do not re-excrete oocysts if challenged within 6 months to 1 year. Intestinal immunity will last up to 6 years in about 55 percent of cats (Dubey, 1995).

Vaccination of cats against intestinal T. gondii infection has been successfully achieved using a mutant strain (T-263) of the parasite (Frenkel et al., 1991; Freyre et al., 1993). Oral administration of strain T-263 bradyzoites results in intestinal infection but does not result in oocyst production in cats. These vaccinated cats do not excrete oocysts when challenged with oocyst-producing strains of T. gondii. The T-263 strain is safe to use in healthy cats, but is not recommended for use in pregnant cats, FeLV positive cats or immunocompromised cats (Choromanski et al., 1994, 1995). It has only limited ability to persist in the tissues of cats, and cannot survive more than three back-passages in cats. No reversion to oocyst excretion or increase in virulence has been observed in over 200 inoculated cats. The T-263 strain is rapidly cleared from the mouth of inoculated cats.

Few studies have examined isolation of *T. gondii* from naturally infected cats (Dubey *et al.*, 2004b, 2004d; Pena *et al.*, 2006). A recent study produced 37 isolates from 54 cats from Parana, Brazil (Dubey *et al.*, 2004d). Of the 37 isolates, 15 were genotype I and 22 were genotype III. *Toxoplasma gondii* was more frequently isolated from the hearts than from the brains of these cats (Dubey *et al.*, 2004d). *Toxoplasma gondii* genotype II was isolated in two of two cats from Mississippi (Dubey *et al.*, 2004b). Pena *et al.* (2006) isolated *T. gondii* from tissues of 47 of 71 seropositive cats from São Paolo, Brazil; 34 isolates were type I, 12 were type III, and 1 was mixed type I and III.

It is logical to assume that cat owners and veterinarians would be at a greater risk for developing toxoplasmosis; however, serological studies do not confirm this assumption. In one study in AIDS patients, it was conclusively shown that owning cats did not increase the risk of developing toxoplasmosis (Wallace et al., 1993). The role of cat ownership and exposure to T. gondii is, however, not completely clear at present. Many studies have been conducted to determine the association between cat ownership or cat exposure and the prevalence of T. gondii infection in humans. Many studies do not find a positive relationship, while many do. It must be stressed that preventing exposure to cats is not the same as preventing exposure to T. gondii oocysts. Pregnant women or immunocompromised individuals should not change the cat's litter box. If feces are removed daily this will also help to prevent exposure by removing oocysts before they can sporulate. Toxoplasma gondii oocysts can survive in the soil for years, and can be disseminated from the original site of deposition by erosion, by other mechanical means, and by phoretic vectors. Inhalation of oocysts stirred up in the dust by horses has been associated with an outbreak of human toxoplasmosis at a riding stable (Teutsch et al., 1979). Oocysts are not likely to remain in the air for extended periods of time. Washing fruits and vegetables and wearing gloves while gardening are means of preventing exposure to oocysts.

Toxoplasma gondii oocysts were not isolated from the fur of oocyst-excreting cats (Dubey, 1995),

and therefore it is unlikely that infection can be obtained by petting a cat. Tachyzoites are not likely to be present in the oral cavity of cats with active *T. gondii* infection, and none would be present in a chronic infection; therefore, it is unlikely that a cat bite would transmit *T. gondii* infection. Cat scratches are also unlikely to transmit *T. gondii* infection.

6.5.2 Dogs

Toxoplasma gondii was once confused with *Neospora caninum* as a cause of disease in dogs, and many reports of toxoplasmosis in dogs are actually neosporosis (Dubey and Lindsay, 1996; Lindsay and Dubey, 2000). True toxoplasmosis does occur in dogs (Dubey *et al.*, 1989). Clinical toxoplasmosis in dogs is often associated with immunosuppression induced by canine distemper virus infection. Clinical signs are usually most apparent in the respiratory and hepatic systems, and probably result from reactivation of latent infections (Dubey *et al.*, 1989). Transplacental infection has not yet been confirmed in naturally infected dogs. Dogs are resistant to experimental infection with tissue cysts and oocysts (Lindsay *et al.*, 1996, 1997b).

A role for dogs in the transmission of T. gondii to humans has been postulated, based on serological surveys and observations that dogs ingest cat feces and often roll in cat feces and other foul-smelling substances (Frenkel et al., 2003). It is believed that dogs can bring oocysts to a home after ingesting them, and deposit them in or around the home when they defecate. Experimentally infective T. gondii oocysts can be found in dog feces for up to 2 days after the dog ingests oocysts (Lindsay et al., 1997b). Toxoplasma gondii oocysts will not sporulate when placed on dog fur (Lindsay et al., 1997b). Recently, Schares et al. (2005) found viable T. gondii oocysts in 2 of 24089 dogs in Germany. The role of dogs as potential transport hosts for T. gondii needs further examination.

6.5.3 Ferrets

Congenital toxoplasmosis has been observed in farmed-raised ferrets (*Mustela putorius furo*) from

New Zealand (Thornton and Cook, 1986). Thirty percent of the kits on the farm died acutely and had lesions of disseminated toxoplasmosis. An epizootic of toxoplasmosis occurred among a population of endangered black-footed ferrets (*Mustela nigripes*) at a zoo in the United States (Burns *et al.*, 2003); 22 adults and 30 kits died from acute toxoplasmosis, and an additional 13 adults died from chronic toxoplasmosis after the initial outbreak.

6.6 DOMESTIC FARM ANIMALS

6.6.1 Mink

Acute toxoplasmosis with abortions has been reported in farmed mink (*Mustela vison*) from Europe and the United States (Frank, 2001; Smielewska-Los and Turniak, 2004). The practice of feeding non-frozen slaughter offal was blamed for acute toxoplasmosis in one report (Smielewska-Los and Turniak, 2004). *Toxoplasma gondii* has been isolated from wild mink from the United States (Smith and Frenkel, 1995).

6.6.2 Horses

Horses are resistant to experimental infection with 1×10^4 (Dubey, 1985) or 1×10^5 oocysts (Al-Khalidi *et al.*, 1980). *Toxoplasma gondii* can persist in edible tissues of horses for up to 476 days (Dubey, 1985). Although *T. gondii* has been isolated from tissues of horses, there is no confirmed report of clinical toxoplasmosis in horses (Al-Khalidi and Dubey, 1979; Dubey, unpublished).

6.6.3 Swine

Toxoplasma gondii can cause mortality in neonatal pigs, but abortion is rare. Pigs raised on dirt are more likely to have *T. gondii* in their tissues. Diagnosis of *T. gondii* abortion in sows is best done by examining fetal fluids for antibodies using the modified agglutination test. Undercooked pork is a source of human infection, and viable tissue cysts can remain in pork for up to 865 days (Dubey, 1988).

6.6.4 Cattle

Clinical toxoplasmosis in cattle is rare, and abortions are uncommon. Many reports of bovine abortion due to *T. gondii* are actually due to *N. caninum* (Dubey and Lindsay, 1996). Attempts to isolate *T. gondii* from seropositive cattle are often unsuccessful, indicating that beef may not be a significant source of human infection (Dubey *et al.*, 2005a). For example, no *T. gondii* was isolated from 2094 samples of beef obtained from retail markets in the United States (Dubey *et al.*, 2005a). However, viable tissue cysts can remain in cattle for up to 1191 days (Dubey and Thulliez, 1993). Additional studies are needed to document these experimental findings fully.

6.6.5 Sheep

Toxoplasma gondii is a common cause of abortion in ewes, and an important production problem. Multiple abortions can occur in a flock, indicating a common oocyst source for ewes. Ewes develop solid immunity after aborting *T. gondii*-infected fetuses. A vaccine to prevent abortion in ewes is available in several countries (Buxton and Innes, 1995). Diagnosis of *T. gondii* abortion in ewes is best done by examining fetal fluids for antibodies using the modified agglutination test. Undercooked lamb and mutton are sources of human infection.

6.6.6 Goats

Toxoplasma gondii is a common cause of abortion in does. Multiple abortions can occur in a flock, indicating a common oocyst source for does. Does develop immunity after aborting *T. gondii*-infected fetuses, but repeat abortions can occur. Diagnosis of *T. gondii* abortion in goats is best done by examining fetal fluids for antibodies using the modified agglutination test. Undercooked goat meat is a source of human infection.

6.6.7 Buffaloes

Naturally occurring clinical toxoplasmosis has not been observed in buffaloes (*Bison bison*, *Bubalus* *bubalis, Syncerus caffer*), and viable *T. gondii* has not been isolated from buffaloes.

6.6.8 Camels

Acute toxoplasmosis was observed in a 6-year-old camel (*Camelus dromedarius*) (Hagemoser *et al.*, 1990). Dyspnea was the main clinical sign, and many tachyzoites were found in its lungs and plural exudates. *Toxoplasma gondii* has been isolated from camel meat, using cat bioassays (Hilali *et al.*, 1995).

6.6.9 Llamas, alpacas, and vicunas

Experimental studies indicate that llamas (*Lama glama*) are resistant to clinical toxoplasmosis even if challenged during pregnancy (Jarvinen *et al.*, 1999). Naturally occurring toxoplasmosis has not been reported in llamas, alpacas (*Lama pacos*), or vicunas (*Lama vicugna*).

6.6.10 Chickens

Chickens (Gallus domesticus) that are raised on the ground are a potential source of T. gondii due to high level of exposure to oocysts. All three T. gondii genotypes have been isolated from the tissues of naturally infected chickens (Table 6.4). Chickens usually do not develop clinical signs even after oral inoculation of large numbers of oocysts (Dubey et al., 1993b; Kaneto et al., 1997). Egg production may be adversely affected in laying hens fed large numbers of oocysts, but T. gondii is not readily transmitted to the eggs of these hens (Biancifiori et al., 1986). Clinical toxoplasmosis does not occur on modern chicken farms where birds are raised indoors, and chickens raised in modern production facilities in confinement indoors are not likely to have viable T. gondii in their tissues. None of 2094 samples from commercial chickens in retail markets from the United States contained viable T. gondii in a recent survey (Dubey et al., 2005a).

6.6.11 Turkeys

Domestic turkeys (*Meleagris gallopavo*) fed *T. gondii* oocysts remained clinically normal except TOXOPLASMOSIS IN WILD AND DOMESTIC ANIMALS

Location	Number examined	Genotype*	Reference
Argentina (La Plata)	9	1 T1; 1 T2; 7 T3	Dubey <i>et al.</i> , 2003b
Argentina (Santiago del Estero, Entro Rios)	17	4 T1; 3 T2; 10 T3	Dubey <i>et al.</i> , 2005b
Austria	67	67 T2	Dubey <i>et al</i> ., 2005c
Brazil (Amazon)	24	4 T1; 10 T3	Dubey <i>et al</i> ., 2006b
Brazil (Parana)	13	7 T1; 6 T3	Dubey <i>et al.</i> , 2003c
Brazil (Rio de Janeiro)	48	34 T1; 13 T3; 1 mixed	Dubey <i>et al</i> ., 2003d
Brazil (São Paulo)	25	17 T1; 8 T3	Dubey <i>et al</i> ., 2002c
Burkina Faso and Mali	6	2 T2; 4 T3	Dubey <i>et al</i> ., 2005d
Chile	22	17 T2; 4 T3; 1 mixed	Dubey <i>et al.</i> , 2006c
Colombia	24	7 T1; 17 T3	Dubey <i>et al</i> ., 2005e
Costa Rica	32	5 T1; 1T3; 26 mixed	Dubey <i>et al</i> ., 2006d
Congo	10	1 T1; 1 T2; 8 T3	Dubey <i>et al</i> ., 2005d
Egypt	20	3 T2; 17 T3	Dubey <i>et al</i> ., 2003e
Grenada	36	5 T1; 1 T2; 29 T3; 1 mixed	Dubey <i>et al.</i> , 2005f
India	7	2 T2; 5T3	Sreekumar et al., 2003
Kenya	1	1 T2	Dubey <i>et al</i> ., 2005d
Mexico	6	1 T1; 5 T3	Dubey <i>et al.</i> , 2004e
Nicaragua	48	6 T1; 3 T2, 6 T3; 33 mixed	Dubey <i>et al.</i> , 2006e
Peru	10	7 T1; 3 T3	Dubey <i>et al</i> ., 2004f
Portugal	12	8 T2; 4 T3	Dubey <i>et al.</i> , 2006f
Sri Lanka	12	6 T2; 6 T3	Dubey <i>et al.</i> , 2005g
Venezuela	13	10 T2; 3 T3	Dubey <i>et al.</i> , 2005h

*Genotype I = T1; genotype II = T2; genotype III = T3.

for a few that developed pneumonia associated with *Aspergillus*-like fungi (Dubey *et al.*, 1993c). Tissue cysts are present in the breast and leg muscles of some inoculated turkeys. Clinical toxoplasmosis does not occur on modern turkey farms.

6.6.12 Ducks and geese

Domestic ducks (*Anas platyrhynchos*) fed *T. gondii* oocysts do not develop clinical toxoplasmosis (Sedlák *et al.*, 2004). Viable *T. gondii* genotype III has been isolated from the tissues of a naturally infected domestic duck (Dubey *et al.*, 2003e).

Toxoplasma gondii has not been isolated from domestic geese (*Asner asner*), and no reports of experimental infections have been reported.

6.7 FISH, REPTILES, AND AMPHIBIANS

Toxoplasmosis does not occur in fish, reptiles, or amphibians. Reports of infections in these animals in nature are erroneous. Reptiles can be manipulated to make them susceptible to *T. gondii*, but they have to be experimentally infected and kept at temperatures of around $37-40^{\circ}$ C.

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Toxoplasma Animal Models and Therapeutics

U. Reichard and U. Gross

7.1 Introduction7.2 Congenital toxoplasmosis

7.3 Ocular toxoplasmosis

7.4 Cerebral toxoplasmosis *References*

7.1 INTRODUCTION

This chapter discusses animal models of toxoplasmosis with special regard to pharmacological applications, and thereby tries to update existing reviews (Darcy and Zenner, 1993; Derouin *et al.*, 1995).

Virtually all mammals can be infected with toxoplasmosis. However, different animal species differ markedly in their resistance to *Toxoplasma* infection – for example, rats are highly resistant but mice in general are very susceptible (Fujii *et al.*, 1983). In addition, the outcome of infection is dependent not only on the animal species but also on the animal strain. The genetic background seems to be of importance since, after infection with *Toxoplasma*, striking differences in susceptibility of various strains of inbred and outbred mice have been observed (Araujo *et al.*, 1976; Williams *et al.*, 1978; McLeod *et al.*, 1984, 1989; Suzuki *et al.*, 1991, 1994; Brown *et al.*, 1995). We know today that, at least in part, such animal strain-dependent variation in susceptibility may be attributed to certain chromosomal regions and even to the presence of certain single genes that may influence the parasite burden dramatically (Brown and McLeod, 1990; Deckert-Schluter *et al.*, 1994; Brown *et al.*, 1995; Johnson *et al.*, 2002). Nevertheless, many questions in this respect still remain to be solved, which may be exemplarily underlined by the intriguing finding that the control of host genetic resistance against acute infection itself differs according to the virulence and genotype of the *T. gondii* strain used (Suzuki *et al.*, 1995).

The situation becomes even more complex because these differences are not uniform with respect to the strains but are also a function of the mode of inoculation. This means that, for instance, a mouse strain that is highly susceptible to intraperitoneal (i.p.) infection compared to another mouse strain may not necessarily be highly susceptible to oral infection, and vice versa. Indeed, almost mirror-image susceptibility between oral and i.p. challenge has been found with inbred mouse strains (Johnson, 1984), suggesting at least partly independent modes of defense involved in each infection route. In this context it is important to be aware that, in general, two infection modes are used: (1) injection of tachyzoites grown in culture or in mice i.p., s.c., or i.v., and (2) oral administration of tissue cysts of T. gondii obtained mostly from brains of chronically infected mice (McLeod et al., 1984) (oocysts are rarely used for infection). Whereas the latter model is inherently less reproducible due to the variable size and content of the cysts, it has the advantage that it follows the natural route of infection. As tachyzoites are not resistant to the acid pH in the stomach, they are only poorly infective when given orally (Dubey, 1998).

In addition to host factors, the outcome of a challenge with Toxoplasma is largely influenced by the nature of the infectious agent itself, and one of the most common characteristics of many Toxoplasma strains is the variation in virulence. Depending on the time before animals succumb to infection or the percentage of animals that do succumb, highly virulent, moderately virulent, and less virulent strains have been characterized (Kaufman et al., 1958). However, strain-dependent virulence does not appear as a static feature, as it can be enhanced by the continuous passage of the parasite in laboratory animals. For example, when the RH strain was initially isolated, mice succumbed to infection 17-21 days in the first passage, 7-8 days in the second, and 3-5 days in the third, and thereafter (Sabin, 1941). In any case, the virulence of T. gondii strains is commonly assessed according to the outcome of a systemic i.p. infection in mice, and studies of the population genetic structure have shown that most T. gondii strains belong to three clonal lineages, of which type I includes mouse-virulent strains, and types II and III include mouse avirulent strains (Sibley and Boothroyd, 1992; Howe and Sibley, 1995). In fact, type I strains may be lethal in mice when a single infectious organism is injected (Howe et al., 1996).

Figure 7.1 summarizes the factors that influence the outcome of *Toxoplasma* in animals.

7.2 CONGENITAL TOXOPLASMOSIS

An ideal animal model for congenital toxoplasmosis, useful for drug studies as well as for immunological studies and vaccine design, would be one that mimics



Laboratory animal: species; genetic background of the strain (inbred, outbred, genetically altered strains, etc.) FIGURE 7.1 Factors influencing the outcome of Toxoplasma infection in animals.

- the nature of the animal placenta, as the organ where transmission takes place
- the duration of pregnancy

several conditions:

- the duration of parasitemia
- the size of the animal (for review, see Derouin *et al.*, 1995).

However, the latter point is not as important as it used to be, since technical developments during the last few decades (such as, for example, the quantitative real-time PCR) have greatly enhanced the assessment of infection in small animals (for examples, see Flori *et al.*, 2002, 2003).

Unlike most other organs, the placenta shows a wide variation in structure among different mammalian species, and may be classified according to the number of maternal and fetal cell layers (Loke, 1982). While the placentas of carnivores and ruminants in general show four to six layers, the placenta of humans consists of only three - a fetal trophoblastic, a mesenchymal, and an endothelial cell layer. Such a relatively thin interface suggesting significance for parasite transmission is called a hemochorial placenta type, and this type is also present in primates and rodents (Loke, 1982; Darcy and Zenner, 1993). Indeed, most studies on congenital toxoplasmosis were conducted using rodents - although, of course, these animals were generally not primarily chosen because of the nature of their placenta but rather for practical reasons connected with the easier keeping and handling of small laboratory animals. However, major limitations when working with small animals lie in the assessment of the fetal infection itself, and in most cases assessment is confined to the evaluation of the fetal transmission rate by direct observation or mouse subinoculation. Except for simple models for congenital chorioretinitis, studies involving the anatomical assessment of the fetal infection require larger animals, such as pigs, sheep, or even primates. In the following sections, different animal species and their use in models of congenital toxoplasmosis are discussed. A summary is provided in Tables 7.1 and 7.2.

7.2.1 Mouse

As indicated by numerous articles that have appeared during the recent decades, mice are well studied animals in congenital toxoplasmosis (cf. Beverley, 1959; Remington et al., 1961; Beverley and Henry, 1970; Hay et al., 1981, 1984; Roberts and Alexander, 1992; Roberts et al., 1994). This may be somewhat astonishing because, in general, under natural conditions, vertical transmission seems to occur during chronic infection and through successive generations of mice (Beverley, 1959; Remington et al., 1961). Thus, at first glance the mouse model may not be best suited to mimic the situation in humans. However, other findings prove that whether transmission to the fetus during a chronic infection of the mother occurs is largely dependent on the parasite strain as well as on the mouse strain used - for example, in mice that were latently infected with 11 different Toxoplasma strains, placental transmission succeeded with only 6 strains (Werner et al., 1977), and, indeed, chronically infected BALB/c mice do not allow vertical disease transmission at all (Roberts and Alexander, 1992). In addition, no congenital transmission was detected in the litters of chronically infected BALB/K mice, although the mothers themselves were found to have extremely high cyst counts (Roberts and Alexander, 1992). The explanation for this contradictive outcome of vertical transmission of chronically infected mice most likely lies in their genetic background (as known for the low susceptibility of BALB mice in general), and this view may be supported by the fact that, in contrast to the inbred BALB mice, most earlier studies used outbred animals or NMRI mice. As a consequence, more recent investigations on congenital toxoplasmosis tend to use a BALB mouse model rather than models using other inbred or outbred mouse strains (Thouvenin et al., 1997; Fux et al., 2000; Elsaid et al., 2001; Abou-Bacar et al., 2004a, 2004b; Beghetto et al., 2005). On the other hand, as a model for evaluating

therapeutics the mouse model has been rarely used (Nguyen and Stadtsbaeder, 1985; Fux et al., 2000); it has been used rather as a model for studying pathogenetic mechanisms and, more recently, for evaluation of vaccine approaches (McLeod et al., 1988; Roberts et al., 1994; Elsaid et al., 2001; Ali et al., 2003; Couper et al., 2003; Letscher-Bru et al., 2003; Mevelec et al., 2005). However, Fux et al. were able to show the positive effects of minoxycycline treatment on congenital Toxoplasma transmission (Fux et al., 2000). Considering the advantages of mice as laboratory animals, with their easy handling, and the availability of new, sensitive PCR-based detection methods that allow better diagnostics in small animals, a BALB/c mouse model, if further developed, may have its place as a first-line screening model for testing new chemotherapeutic agents against congenital toxoplasmosis.

7.2.2 Rat

Like man and primates, rats are relatively resistant to T. gondii with respect to a clinical manifestation of the infection. While transmission during acute infection of maternal rats induced by intracerebral or i.v. infection was first reported during the early 1950s (Schultz and Bauer, 1952; Hellbrügge and Dahme, 1953; Hellbrügge et al., 1953, 1955), it has been shown more recently that oral ingestion of oocysts or tissue cysts also leads to fetal transmission (Dubey and Shen, 1991; Zenner et al., 1993; Freyre et al., 2001a, 2003a). In general, transmission rates seem to be high, and were reported to lie mostly between 30 and 90 percent. However, there was great variation, which was attributed to the Toxoplasma strains and to the rat strains used (Freyre et al., 2003a). Indeed, a wide variability in the formation of Toxoplasma cysts in rats of the same outbred strain and age, inoculated with the same strain, stage and dose of Toxoplasma, using the same route, was observed (Frevre et al., 2001a). Such an individual resistance of rats belonging to the same outbred strain may be attributed to the individual genetic background of the rat (Freyre et al., 2001a). In an experimental study design, for example, for drug efficacy testings, such a lack of individual reproducibility may be overcome either by a comparatively high number of animals per group or by the use of inbred animals.

Except in rare instances, when unnaturally high doses of several million organisms were used for infection (Hellbrügge, 1955), T. gondii was either not transmitted at all or was transmitted only extremely rarely from chronically infected rats to fetus, irrespective of the route of inoculation, stage, strain or size of inoculum (Remington et al., 1958, 1961; Dubey and Shen, 1991; Zenner et al., 1993; Dubey et al., 1997). In contrast to the situation in mice in which the organism is transmitted repeatedly during chronic infection, as in humans, vertical transmission in rats virtually does not occur. Thus, with respect to the clinical course and in utero transmission, toxoplasmosis in rats and humans is similar, and the infection in rats may serve as a proper model - especially for human congenital toxoplasmosis (for review, see also Dubey and Frenkel, 1998).

In spite of the obvious analogies concerning transmission, transmission rates, and rates of clinical manifestation, rat models – with rare exceptions (Usmanova, 1965) – have so far not been used for drug testing in congenital toxoplasmosis. This may be due to the fact that infected litters usually appear healthy. However, regarding the *T. gondii* strains, stage, and routes of inoculation, and probably also the rat strains, rats may serve as excellent models – especially when emphasis lies on placental transmission. In any case, as total protection against congenital toxoplasmosis can be achieved regardless of the *Toxoplasma* strain, rats may be attractive models for future vaccine candidates against the disease (Zenner *et al.*, 1993, 1999a).

7.2.3 Guinea pig

A guinea pig model for congenital toxoplasmosis has been described in various studies (Adams *et al.*, 1949; Huldt, 1960; Berard-Badier *et al.*, 1968; Wright, 1972; Haumont *et al.*, 2000; Flori *et al.*, 2002, 2003). As in humans, the guinea pig placenta is of the hemomonochorial type (Darcy and Zenner, 1993), suggestive of similar modes of transmission. However, the transmission rate and sensitivity of guinea pigs to T. gondii after i.p. or oral infection is much higher, being about halfway between those of the rat and the mouse. In addition, congenital transmission during chronic maternal pregnancy has been observed (Wright, 1972; Flori et al., 2002). As a possible advantage in comparison to mice and rats, guinea pigs have an approximately three-fold longer gestation duration of 65 days in total - long enough to enable comparative studies with different inoculation times and comparative chemotherapy studies (Flori et al., 2002). For this application, the guinea pig model may be best suited. However, except for rare instances (Youssef et al., 1985), in spite of its potential advantages, a guinea pig model for congenital toxoplasmosis has not yet been employed for drug testing.

7.2.4 Primate

With respect to hemochorial placentation (Ramsey and Harris, 1966; Darcy and Zenner, 1993), fetal blood sampling, and assessment of fetal infection, in general a primate model should actually best meet the requirements for the study of the effect of medication in the infected fetus. Therefore, a first study was conducted with *Macaca arctoides* as a model for primates (Wong

et al., 1979). Data obtained with this model suggested that although certain developmental stages of the Toxoplasma organism and of the fetus may favor the occurrence of congenital infection, the transmission rate in general seems to be low and very little neonatal disease results (Wong et al., 1979). In contrast, a more applicable model was established by Schoondermark-van de Ven et al. with rhesus monkeys (Macaca mulatto) (Schoondermarkvan de Ven et al., 1993). Herein, the frequencies of transmission which were found in the rhesus monkey after maternal infection in the second and third trimesters of gestation equal those observed in humans (Schoondermark-van de Ven et al., 1993). The rhesus model was then used to prove the efficacy of spiramycin or the combination of pyrimethamine and sulfadiazine for the treatment of congenital T. gondii infection (Schoondermarkvan de Ven et al., 1994a; Schoondermark-van de Ven et al., 1995). The results showed that both regimens were clearly effective in reducing the number of parasites in the infected fetus, proven by PCR and mouse inoculation with amniotic fluid. However, spiramycin was less active (Schoondermark-van de Ven et al., 1994a) and was not found anywhere in the fetal brain (Schoondermark-van de Ven et al., 1994b).

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Animal species	Specific comments	Pharmacological studies (literature examples)		
Mouse	Hemotrichorial placenta; vertical transmission during chronic infection possible; rare vertical transmission in inbred BALB mice	Nguyen and Stadtsbaeder, 1985; Fux <i>et al.</i> , 2000		
Rat	Hemotrichorial placenta; rare vertical transmission during chronic infection; strain-dependent variation in transmission rates	Usmanova, 1965		
Guinea pig	Hemomonochorial placenta; vertical transmission during chronic infection observed; 3-fold longer gestation time than in mice and rats	Youssef <i>et al.</i> , 1985		
Primate	Hemomonochorial placenta; transmission rates resemble those in humans	Schoondermark-van de Ven <i>et al.</i> , 1994a, 1994b, 1995		

 TABLE 7.1 Pharmacological studies on congenital toxoplasmosis
TABLE 7.2 Congenital models of toxoplasmosi.

Species and strain of animal used*	<i>Toxoplasma</i> strain administered*	<i>Toxoplasma</i> stage and route of infection	Administration time point and <i>Toxoplasma</i> dose in relation to pregnancy	Outcome of animal infection I (with regard to fetal transmission in acutely infected animals)	Outcome of animal infection II (with regard to transmission in chronically infected animals)	Publication**
Mouse: NIH strains	RH, 113-CE, Beverley, M7727	Tachyzoites (RH) and cysts (113-CE, Beverley, M7727) i.p. or s.c.	Undefined numbers for acute and chronic infections: acute (RH) on day 16 of pregnancy; chronic (113-CE, Beverley, M7727) 2–15 months prior to delivery	RH: no infection of young if delivery before the 5th day after infection; 56% infection on day 7 after i.p. inoculation	Transmission of chronically infected mice occurred, most frequently with the Beverley strain	Remington <i>et al.</i> , 1961
Mouse: NMRI	S93, K8, 558/72, Witting, Gail, KSU, 1070, 162/74, 248/70, MO, ALT	Cysts i.p. prior to pregnancy; cysts orally during preg- nancy only in a primary infection trial with <i>Toxoplasma</i> ALT-strain	20 cysts 4–8 weeks before mating; 20 cysts on day 10 of pregnancy	Primary infection during pregnancy (ALT-strain only): transmission rate 28%	Infection prior to pregnancy: S93, K8, 558/72, Witting, Gail: no transmission; KSU, 1070, 162/74, 248/70, MO, ALT: transmission rate 1–3%	Werner <i>et al.,</i> 1977
Mouse: NMRI	Beverley	Cysts s.c.	Undefined number of cysts 5–8 days after mating	75% offspring with positive serology; offspring survival 40%; number of offspring approximately 20% compared to non- infected control; 30% mortality of infected mothers during gestation	Cotrimoxazole treatment during pregnancy almost normalized offspring number and survival	Nguyen and Stadtsbaeder, 1985
Mouse: BALB/c, BALB/k (inbred)	Beverley	Cysts orally	8 weeks before mating 5 cysts; day 12 of pregnancy 20 cysts	Transmission rate of approximately 50% (cysts for first time in pregnancy only)	Congenital infection occurred only if the mother was infected for the first time during pregnancy	Roberts and Alexander, 1992

Mouse: BALB/c (inbred)	Р	Cysts orally	2 cysts between days 6 and 15 of pregnancy	Fetal transmission rate 50–60%	Minocycline-treated mice showed transmission in only 3.6%	Fux <i>et al.</i> , 2000
Rat: Sprague- Dawley	RH	Tachyzoites i.p.	1×10^7 and 2×10^7 tachyzoites 6–8 weeks before mating		Virtually no fetal transmission (3 offspring from 140 in total – all in a single litter)	Remington et al., 1958
Rat: Sprague- Dawley, Osborne- Mendel, Black rat, Holtzman rat	RH, S-6, Beverley	Tachyzoites (RH, S-6) and cysts (Beverley) i.p.	1×10^4 to 1×10^7 tachyzoites and undefined number of cysts 2–8 months prior to gestation		No fetal transmission from chronically infected rats with RH or S-6; Beverley: 5% transmission	Remington et al., 1961
Rat: Sprague- Dawley	CT-1	Oocysts orally or s.c.; bradyzoites s.c.	1×10^4 oocysts at 7–15 days of pregnancy; 1×10^4 bradyzoites at 10–14 days of pregnancy	Transmission rate of 82.1% (oocysts orally), 90.9% (oocysts s.c.), 43.8% (bradyzoites s.c.)	No transmission in chronically infected rats	Dubey and Shen, 1991
Rat: Fischer	RH, 76K, Prugniaud	RH: tachyzoites i.p.; Prugniaud and 76K: cysts orally	Between 8th and 12th days of pregnancy: 8×10^6 RH tachyzoites; 1200 Prugniaud or 76K cysts	Transmission rates of 58% (RH), 63% (Prugniaud), 35% (76K)	No transmission to fetuses of chronically infected rats, even if rats were reinfected during pregnancy	Zenner <i>et al.</i> , 1993, 1999a
Rat: Sprague- Dawley	VEG	Oocysts orally	1×10^4 oocysts on day 6, 9, 12, or 15 of gestation	Transmission rates: 33% (day 6), 55% (day 9), 83% (day 12) and 57% (day 15)	Virtually no transmission to the next generation	Dubey <i>et al.</i> , 1997

Continued

Species and strain of animal used*	<i>Toxoplasma</i> strain administered*	<i>Toxoplasma</i> stage and route of infection	Administration time point and <i>Toxoplasma</i> dose in relation to pregnancy	Outcome of animal infection I (with regard to fetal transmission in acutely infected animals)	Outcome of animal infection II (with regard to transmission in chronically infected animals)	Publication**
Rat: Wistar and Long Evans	12 different strains of low to high pathogenicity for mice (e.g. M7741, Beverley, M49)	Cysts orally	2 × 10 ² to 3.4 × 10 ³ cysts at 6–8 and 15 days of pregnancy	Overall transmission of 44% with a range of 0–90% attributed to genetically based susceptibility of outbred Wistar rats; transmission not affected by the strain or dose of <i>Toxoplasma</i> nor by the time point of infection	Transmission more frequent in Long Evans than in Wistar rats	Freyre <i>et al.,</i> 2001a
Rat: Wistar	6 different strains of low to high pathogenicity for mice	Oocysts orally	1 × 10 ⁴ oocysts at 15 days of pregnancy	Transmission rates of 10–80%; higher transmission with strains more pathogenic for the mice	No statistically significant differences in rate of transmission in rats fed with cysts (previous work)	Freyre <i>et al.,</i> 2003a
Guinea pig: not specified	Beverley	Cysts i.p.	60 or 100 cysts at 4–54 days of pregnancy; 100–200 cysts 2–6 months before mating	Transmission rate of 100% if infection during pregnancy	Transmission rate of 30% if infection before pregnancy; overall high number of stillborn or severely ill pups	Wright, 1972

TABLE 7.2 Congenital models of toxoplasmosis—cont'd

TOXOPLASMA ANIMAL MODELS AND THERAPEUTICS

Guinea pig: Dunkin- Hartley	C56	Tachyzoites intradermally	5×10^5 tachyzoites after 7 weeks of gestation	Transmission in more than 80%	SAG1-immunized animals with lower transmission rates	Haumont <i>et al.</i> , 2000
Guinea pig: Dunkin- Hartley	RH, 76K, Prugniaud	Tachyzoites (RH) i.p.; cysts (76K, Prugniaud) i.p. or orally	Various time points from 90 days before pregnancy to day 40 of pregnancy: 100 tachyzoites (i.p.); 100 cysts (i.p. or orally)	Transmission rates if infection during pregnancy: 54% (RH); 84% (Prugniaud); 86% (76K)	Transmission rate of 17% if infection 30–90 days before mating; overall high number of stillborn and non-viable fetuses	Flori <i>et al.,</i> 2002
Guinea pig: Dunkin- Hartley	76K	Cysts orally	100 cysts at day 20 or 40 of pregnancy	Transmission rates of 84.6% and 100% after inoculation on days 20 and 40, respectively	Infection assessed by real-time PCR	Flori <i>et al.</i> , 2003
Rabbit: not specified	Witting/ ALT-	Cysts i.p. prior to pregnancy; cysts orally during pregnancy	200 cysts –19 days before mating; 200 cysts during the first or second trimenon to pre- infected or non- pre-infected animals	Fetal transmission rate up to 79%	No congenital transmission when first infection was placed at least 35 days prior to mating	Werner <i>et al.</i> , 1977
Primate: <i>Macaca mulatta</i> (rhesus)	RH	Tachyzoites i.v.	5 × 10 ⁶ tachyzoites on day 90 or 130 of pregnancy	Overall transmission rate of 61%, which is comparable to that found in humans		Schoondermark- van de Ven <i>et</i> <i>al.</i> , 1993

*For definition of animal or *Toxoplasma* strains, please see respective articles. **Due to numerous publications on this topic, only few were exemplarily chosen for this table.

The rhesus monkey model is theoretically perhaps the best animal model to prove the efficacy of a drug against human congenital toxoplasmosis, especially with regard to placental transmission. However, the housing and handling of monkeys requires special facilities and trained employees. It is also time-consuming and expensive, which may limit the numbers of animals used for the studies. Considering this fact, and bearing in mind that the outcome of the congenital infection (as in humans) often seems to be subclinical or at least shows a high degree of variation (Schoondermarkvan de Ven et al., 1993), no direct drug evaluation concerned with its influence on the clinical course of a congenital toxoplasmosis seems practical so far. Thus, drug efficacy must indirectly be extrapolated from the demonstration of the parasite or its DNA in the amniotic fluid or fetal tissue, and by pharmacokinetic data of the drug that may also be obtained from the monkey fetus. In summary, the rhesus monkey model might have its place as a last evaluation step for a new drug before it is admitted to clinical trials.

7.2.5 Rabbit

Surprisingly, rabbit congenital toxoplasmosis has not been extensively studied, although rabbits are widely used laboratory animals and transmission from the mother to the fetus has been demonstrated (Uhlikova and Hubner, 1973). In addition, fetuses from chronically infected mothers have been protected, thus a rabbit model would share common features with the infection in man (Werner et al., 1977). A rabbit model may be of particular interest when the small size of other common laboratory animals (such as mice and rats) hampers experiments - for instance, when larger volumes or subsequent blood samples are needed for examination of an antibody response (Araujo and Remington, 1975). To our knowledge, no pharmaceutical studies on congenital toxoplasmosis have been conducted using a rabbit model.

7.2.6 Other animals

Various other animals have been suggested as models for congenital toxoplasmosis, but have never been broadly used. As an example, although pigs are well known to acquire toxoplasmosis and to play a decisive role in transmitting the disease to humans via their meat (for review, see Tenter et al., 2000 and Montoya et al., 2004), their use as animal models has never been thoroughly investigated. However, when infecting pregnant minipigs with strains of different virulence, even a strain that was considered apathogenic to both pigs and mice resulted in significant numbers of congenitally infected piglets (Jungersen et al., 2001). Such a pig model of congenital toxoplasmosis may therefore be of value for situations where bigger animals are needed, and they may be relevant animal models for certain Toxoplasma strains that despite low virulence are obviously transmitted to the fetus (Jungersen et al., 2001).

Congenital disease due to T. gondii is a major cause of abortion and neonatal mortality in sheep. In addition, undercooked meat from infected sheep is an important source of infection for man. Although congenital transmission is well described and in fact is known as a major cause of abortion in this species (Dubey and Rommel, 1992; Anderson et al., 1994), an animal model mimicking congenital infection in humans has, to our knowledge, not been established. Recent studies with sheep indicate that reactivation of chronic infection during pregnancy may be a major cause of the congenital infection (Duncanson et al., 2001; Williams et al., 2005). In this respect, congenital toxoplasmosis in sheep seems more to resemble the usual mode of congenital transmission in mice than that in man.

Just recently, Que *et al.* reported a novel chicken embryo model which had been adopted from a chicken model that had been developed for the study of metastatic diseases (Que *et al.*, 2004). Basically, tachyzoites were injected directly into the chorioallantoic veins of 12-day-old chicks, and after an incubation period of 3 to 6 days the degree of infection was assessed by histopathological examination and quantitative real-time PCR on the *Toxoplasma* DNA. As this model also provides the possibility of injecting drugs via the chorioalloantioic vein, it may prove useful for an initial drug screening set-up with a course of infection that is shorter than that in mice (Que *et al.*, 2004).

7.3 OCULAR TOXOPLASMOSIS

Ocular toxoplasmosis may be acquired by infection after birth, but in a substantial percentage of cases the etiology of human ocular toxoplasmosis seems to be connected with in utero infection of the fetus via a mother whose primary infection was acquired during gestation (for review, see Holland, 2003). Consequently, ocular toxoplasmosis may be considered to be on the one hand a postnatally acquired disease, and on the other a late manifestation of congenital toxoplasmosis. However, it seems to be practical for a description of animal models to produce an ocular disease mainly by a primary infection of adults, although there have been efforts which have successfully established an ocular disease as a consequence of a transplacental Toxoplasma transmission (Hutchison et al., 1982; Lee et al., 1983; Hay et al., 1984; Dutton et al., 1986).

In patients with underlying immunosuppression or immune defects, such as with bonemarrow transplantation or HIV, toxoplasmic chorioretinitis is often associated with concurrent toxoplasmic encephalitis (TE) or disseminated infection (for review, see Montoya et al., 2004). However, even in AIDS patients toxoplasmic chorioretinitis is encountered relatively infrequently (Holland et al., 1988a), so most of the human cases of ocular toxoplasmosis are found in immunocompetent patients. Indeed, T. gondii is one of the most frequently identified causes of uveitis and the most commonly identitified pathogen infecting the retina of otherwise immunocompetent individuals (Holland, 1999) and, regardless of whether ocular toxoplasmosis is due to a reactivated congenital infection or to an infection that is acquired after birth, it usually presents in the immunocompetent host as a more-or-less localized eye disease (Montoya et al., 2004). Therefore, an animal model, particularly one that may be suited to the evaluation of the efficacy of a given drug, should ideally be characterized by a localized eye infection (or at least predominantly by a localized eye infection) rather than by a generalized or CNS infection where the eye is just one disease location among others - an experimental challenge that it is not easy to fulfill. However, current models are either based on primary local inoculation of *T. gondii* into the animal's eye, on a semilocal infection via the carotid artery, or on a primary systemic infection which then affects the eyes as the predominant organ of manifestation (see Tables 7.3 and 7.4).

7.3.1 Models based on local eye infection

In order to meet the above-mentioned requirements, the localized infection of animal eyes was the first choice - which, for technical reasons, required the use of larger animals. Thus, until 1982 the rabbit served as the most important existing experimental model for ocular toxoplasmosis, and morphological lesions of acute experimental Toxoplasma chorioretinitis were produced by injection of parasites intravitreally (Kaufman, 1960) into the anterior chamber (Beverley et al., 1954; Beverley, 1958, 1961; Jacobs et al., 1964) or by trans-scleral inoculation into the suprachoroidal space (Nozik and O'Connor, 1968; Tabbara et al., 1974; Rollins et al., 1982). In fact, the anterior chamber model was used to show efficacy of pyrimethamine and sulfadiazine (Jacobs et al., 1964), whereas the latter model was used to demonstrate the efficacy of clindamycin and minocycline on toxoplasmic chorioretinitis (Tabbara et al., 1974; Rollins et al., 1982).

In 1982, a primate model was established that reliably produced acute toxoplasmic chorioretinitis by injection of viable RH strain Toxoplasma organisms (Culbertson et al., 1982; Newman et al., 1982). Whereas in the rabbit model the injections were made trans-sclerally into the suprachoroidal space at the posterior pole, in monkeys it was not possible to expose the posterior part of the sclera for direct injection. Therefore, the retinal injections were made through the pars plana across the vitreous cavity, directly into the superficial part of the retina at the posterior pole (Culbertson et al., 1982). Non-human primates as well as rabbits infected with a transvitreal approach were later also used in other studies (Webb et al., 1984; Garweg et al., 1998; Holland et al., 1988b). These transvitreal inoculation models have the principal drawback that the integrity of the vitreous cavity is disrupted

and they produce some mechanical damage to the retina (Culbertson et al., 1982); on the other hand, the blood-retinal barrier may be better maintained than by using the suprachoroidal approach, at least during the initial phase of the infection (Garweg et al., 1998). Hence the disease profile in the suprachoroidal model involves an initial local infection followed by a very early systemic one, and as such the situation is not immunologically comparable with that evinced in humans (Friedrich and Müller, 1989). In any case, apart from the shorter time course, the transvitreal inoculation primate model in particular represents clinical and histopathological conditions resembling those of the natural disease in man (Culbertson et al., 1982). It may also circumvent a disadvantage of the rabbit model, which is the anatomic dissimilarity of the retina compared to humans (O'Connor, 1984). However, to our knowledge this model has not yet been used for assessing drugs but rather to elucidate the pathogenesis of ocular toxoplasmosis.

In addition to the local eye infection in larger animals, a more recent model uses B6 as well as MRL mice and injection of T. gondii (PLK strain, a clone of Me49) into the anterior eye chamber (Hu et al., 1999a, 1999b). Pathological and histopathological features of this model resemble in part acute ocular toxoplasmosis in humans, particularly when mice have been primed (pre-infected per-orally) (Hu et al., 1999a). As local infection models with larger animals and especially primates are difficult and costly, this mouse model may offer a rational alternative, at least for larger-scaled controlled studies with therapeutics to be screened. In addition, the disadvantage of the small infection area that has to be investigated may partly be overcome by the use of current highly sensitive detection methods such as PCR or quantitative real-time PCR. The other disadvantage of potentially extensive needle damage, especially when small animals such as mice are used, may perhaps be circumvented when local instillation of the parasite is used instead of intraocular injection. A very recent investigation has shown that this, in principle, leads to infection of the retinal vessels with glial reactions (Tedescos et al., 2005). However, to our knowledge local eve infection models in mice have not yet been used for pharmacological drug testing but rather for immunological and pathogenetical studies (Hu *et al.*, 2001; Lu *et al.*, 2004, 2005).

Local infection of guinea pig eyes was also reliably achieved as early as in 1956 by injecting the RH strain into the vitreous humor (Hogan et al., 1956). This animal was selected because of its relative resistance to toxoplasmic infection, and because the size of the eye did not prohibit ophthalmoscopic examination. In fact, as early as in 1964 a guinea pig model with posterior chamber inoculation of a low virulent T. gondii strain together with a rabbit model mentioned above was successfully used to show a therapeutic effect of sulfadiazine and pyrimethamine in the treatment of ocular disease (Jacobs et al., 1964). However, to our knowledge, local infection models using guinea pigs have not been used for pharmacological studies during the last few decades.

7.3.2 Models based on infection via the carotid artery

An intermediate model, situated between the localized eye infection and the eye infection as a consequence of a generalized challenge, was established by Davidson et al. in cats. These authors used intracarotid inoculation to concentrate the parasites in ocular tissues to gain more predictable experimental ocular lesions with fewer systemic side effects (Davidson et al., 1993). Indeed, all eight cats infected with a relatively small number of the Me49 strain developed the ocular disease but showed no signs or only mild signs of a generalized infection (three cats developed an increase in temperature). The multifocal areas of choroidal and retinal inflammation exhibited many similarities to ocular toxoplasmosis in humans; however, it differed from human ocular toxoplasmosis in its primary choroidal versus retinal nature (Davidson et al., 1993).

The cat model has been used once to examine the effect of clindamycin in the treatment of ocular toxoplasmosis (Davidson *et al.*, 1996). Paradoxically, clindamycin administration was associated with increased morbidity and mortality from hepatitis and interstitial pneumonia, which are characteristic of generalized toxoplasmosis. The reasons for

this outcome were unclear, and may have been due to various aspects of the experimental setting (Davidson *et al.*, 1996). However, as the definitive natural host, the cat may also differ from humans in some undefined manner with regard to its immunologic response to the parasite. This leads us to believe that it is not the ideal laboratory animal for drug testing.

7.3.3 Models based on systemic infection

As can be seen in some 20 publications, meanwhile, the most frequently used animal for systemic infection models is the mouse. Basically, two different methods have been employed to establish the disease:

- Infection of pregnant mice to induce the development of ocular lesions in the pups (Hay *et al.*, 1981, 1984; Hutchison *et al.*, 1982; Lee *et al.*, 1983; Dutton *et al.*, 1986)
- Systemic infection of mice which then predominantly develop ocular manifestations (Gazzinelli *et al.*, 1994; Olle *et al.*, 1994, 1996; Lyons *et al.*, 2001; Shen *et al.*, 2001; Norose *et al.*, 2003, 2005).

In the congenital model, the infection is not established via direct inoculation of vital parasites into the eye but via infection of gestating female mice with, for example, the Beverley strain (Hay et al., 1981; Hay and Kerrigan, 1982; Hutchison et al., 1982). This model has the advantage that its etiology is probably analogous, to a substantial extent, with human ocular toxoplasmosis in that the fetus becomes infected in utero via a mother whose primary infection is acquired during pregnancy (Hay et al., 1984). Interestingly, it was found that the ocular lesions in this model resemble the features that have been described in experimental allergic uveitis (EAU) (Lee et al., 1983; Hay et al., 1984; Dutton et al., 1986), and in fact a mouse model (the adult, not the congenital) was thereafter used to further clarify the pathogenesis and particularly the nature and influence of the immune response involved in the ocular disease (Gazzinelli et al., 1994; Olle et al., 1996). However, for pharmacological

screening studies the congenital model as described does not seem to be appropriate because, in spite of low postnatal mortality, it has the disadvantage that ocular morbidity, discovered by cataract manifestation, is only present in 5 percent of the pups (Hutchison *et al.*, 1982). Nevertheless, the use of different mouse and *Toxoplasma* strains, as well as new sensitive screening methods, may further develop such a model to be suitable for drug testing.

Gazzinelli reported that C57BL/6 (B6) mice developed mild intraocular inflammation commonly observed 15 days after intraperitoneal injection of cysts of the Me49 strain, demonstrating the possible usefulness of adult mice for an eye model (Gazzinelli et al., 1994). In most of the ocular lesions the presence of the parasite could not be demonstrated even with the PCR technique, but the parasite load did increase after treatment of mice with antibodies directed against lymphocytes or cytokines (IFN- γ or TNF- α) (Gazzinelli et al., 1994). Treatment with anti-IFN-y also ended with clinical eye lesions, including single foci of chorioretinitis, and multifocal lesions of diffuse areas of retinal necrosis in an experimental model of chronically infected Swiss Webster mice (avirulent Beverley strain) (Olle et al., 1996). Using also the T. gondii Beverley strain for i.p. infection, Lyons et al. found retinal inflammation most marked in the inner retinal layers of wild type 129/SVJ mice and more severe in corresponding IL-6 knockout mice (Lyons et al., 2001).

Recently, Norose *et al.* established a mouse model for the ocular disease that followed the natural per-oral route of infection with five cysts of a *T. gondii* avirulent strain (Norose *et al.*, 2003). The model was established for the immunocompetent host with resistant BALB/c or susceptible C57BL/6, and for the immunodeficient host with corresponding IFN- γ knockout (GKO) mice. Whereas all GKO mice died after 11–12 days showing disseminated infection, both strains of WT mice survived for more than 1 month. In contrast to the GKO mice, there was no histopathological evidence for inflammation in the eyes and brains of wild type mice, and no characteristic findings using fluorescein angiography and documentation with a fundus camera (Norose *et al.*, 2003). Electroretinograms, as shown later, were also only changed in GKO mice (Norose *et al.*, 2005). However, the authors were able to show differential parasite distribution in the eyes of WT mice using a quantitative competitive polymerase chain reaction (QC-PCR).

In summary, adult mouse models may provide reasonable tools for investigation of various pathological or pathogenetical aspects, particularly in the immunocompromised host; they may also provide a system for screening drugs for systemic toxoplasmosis in the immunocompromised host when using various knockout mice such as, IFN- γ GKOs (Belal et al., 2004). Because of the mild effects on eyes in WT mice using avirulent T. gondii strains or overwhelming systemic infection using virulent strains, they have not yet been used and seem not to be well suited for drug testing in particular respect to ocular toxoplasmosis. At any rate, QC-PCR (Kobayashi et al., 1999) combined with DNA extraction of different eve parts (cornea, iris/ciliary body, lens, posterior retina, peripheral retina, choroids, sclera, optical nerve, and brain) (Norose et al., 2003) allows measurement of the parasite load in the eyes of small animals such as mice, and may also prove valuable in other models of ocular toxoplasmosis.

Based on the observations of Frenkel, who found frequent but sporadic ocular disease in Syrian Golden hamsters several months after inoculation with the RH or CJ strains of Toxoplasma (Frenkel, 1953, 1955), reliable models with this animal and the Me49 strain were developed that show ocular disease following the i.p. or oral infection route (Pavesio et al., 1995; Gormley et al., 1999). The main advantage of these models is that they consistently produce ocular disease with a short incubation time but without artificial breaching of the ocular barrier and also without causing any clinical signs of systemic disease. In addition, hamster eyes are large enough to allow fundus photography to document the progression of the infection, and, as usually encountered in humans when immunity is not impaired, the disease resolves spontaneously with time, without treatment (Pavesio et al., 1995; Gormley et al., 1999). However, there are marked differences from the human disease - for example, vitritis was not usually significant in hamsters (Pavesio et al., 1995). In conclusion, the hamster model may be an option as a drug-screening model mainly because of its good reproducibility and monitoring possibilities. However, one study compared conventional therapies with atovaquone and did not show

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Infection model	Specific comments	Animal species	Pharmacological studies (literature examples)
Localized eye infection models	Best for confining infection to eyes but with the principal drawback of tissue needle damage	Rabbit	Beverley, 1958; Kaufman, 1960; Jacobs <i>et al.</i> , 1964; Tabbara <i>et al.</i> , 1974; Rollins <i>et al.</i> , 1982
		Primate Mouse Guinea pig	– – Jacobs <i>et al.</i> , 1964
Infection via the carotid artery	Cat is definitive natural host which may influence, for example, immunoreactions	Cat	Davidson <i>et al.</i> , 1996
Systemic infection models	Particularly hamsters produce consistent ocular disease following i.p. or oral infection	Mouse Hamster	Olle <i>et al.</i> , 1996 Gormley <i>et al.</i> , 1998

TABLE 7.3 Pharmacological studies on ocular toxoplasmosis

TABLE 7.4 Ocular models of toxoplasmosis

Species and strain of animal used*	<i>Toxoplasma</i> strain administered*	<i>Toxoplasma</i> dose, stage and route of infection	Outcome of animal infection	Remarks	Publication**
Mouse: strain A albino	Beverley	10 cysts s.c. on day 12 of pregnancy	Approximately 50% of offspring infected, 5.3% of these developed cataract; acute uveitis in a small proportion of eyes	Model of congenital ocular toxoplasmosis	Hutchison <i>et al.</i> , 1982; Dutton <i>et al.</i> , 1986
Mouse: C57BL/6	Me49	10–20 cysts i.p.	Mild uveitis and retinal vasculitis in all infected animals at 15 days post-infection	In most ocular lesions the parasite could not be demonstrated even with PCR	Gazzinelli <i>et al.</i> , 1994
Mouse: Swiss Webster	Beverley	Cysts (undefined number) i.p.; immunosuppression by injecting polyclonal antibodies against γ-interferon	26% of mice developed chorioretinitis on days 13–15	<i>T. gondii</i> was revealed by routine cellular cultures in all immunosuppressed mice with ocular lesions	Olle <i>et al.</i> , 1994
Mouse: C57BL/6, MRL-MpJ	PLK (derived from Me49)	50 to 5×10^4 tachyzoites injected into the anterior chamber	Dose-dependent intraocular inflammation: 50 (none), 5×10^2 and 5×10^3 (moderate to severe), 5×10^4 tachyzoites (severe, early mortality)	Some protection if mice were pre- infected before challenge	Hu <i>et al.</i> , 1999a
Mouse: 129/SVJ (WT), IL-6- deficient strain with same genetic background	Beverley	10 cysts i.p.	Regular mild to moderate retinochoroiditis 4 weeks after challenge, severe inflammation in IL-6-deficient mice	Cytokine expression study	Lyons <i>et al.,</i> 2001
Mouse: C57BL/6, B6MRL/Ipr and B6MRL/gld (defective Fas or FasL expression, respectively)	Me49	20–30 cysts i.p.	Regular mild retinochoroiditis and moderate encephalitis after 14 days, becoming worse after 28 days	No significant difference in the degree of ocular inflammation between wild type and Fas or FasL mutant mice	Shen <i>et al.,</i> 2001

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Continued

 TABLE 7.4 Ocular models of toxoplasmosis—cont'd

	Toxoplasma				
Species and strain of animal used*	strain administered*	<i>Toxoplasma</i> dose, stage and route of infection	Outcome of animal infection	Remarks	Publication**
Mouse: C57BL/6, BALB/c and IFN-γ knockout mice (GKO) of both wild type backgrounds	Fukaya	5 cysts per-orally	Evidence of eye inflammation in GKO mice only; assessment via PCR, histopathology and fluorescein angiography	Toxoplasmic eye vasculitis model for GKO mice; GKO mice died 11–12 days after infection	Norose <i>et al.</i> , 2003
Mouse: C57BL/6	ME49	5 × 10 ³ bradyzoites injected intravitreally or via conjunctival instillation	Regular retinochoroiditis with both infection routes 7 days after infection	Additional eye damage caused by the intravitreal injection; instillation route preferable	Tedescos <i>et al.,</i> 2005
Mouse: C57BL/6, BALB/c, CBA/J	RH, PLK, SAG1 (P30)-deficient RH derived mutant strain	100 tachyzoites injected into the anterior chamber	C57BL/6: severe eye inflammation and 100% mortality with all <i>T. gondii</i> strains; BALB/c and CBA/J: mild to medium eye inflammation most pronounced with RH (all mice survived)	All strains of mice were protected after i.p. vaccination with temperature- sensitive mutant tachyzoites	Lu <i>et al.</i> , 2005
Rabbit: not specified	113-CE	5×10^3 or 1×10^4 tachyzoites injected into the anterior chamber	Uveitis developed after a few days	Pyrimethamine- sulfadiazine treatment	Jacobs <i>et al.</i> , 1964
Rabbit: pigmented Dutch rabbits, New Zealand white rabbits, pigmented California rabbits	RH, Beverley	Trans-scleral inoculation of 1000–2000 tachyzoites into the suprachoroidal space	RH: animal death from encephalitis; Beverley: retinochoroiditis in most animals after 7 days, resolving after 3 weeks	California rabbits best for technical handling	Nozik and O'Connor, 1968
Rabbit: pigmented California rabbits	Beverley	Trans-scleral inoculation of 400 tachyzoites into the suprachoroidal space	Constant induction of retinochoroiditis	Clinical improvement of retinochoroiditis in clindamycin- treated rabbits	Tabbara <i>et al.,</i> 1974
Rabbit: pigmented California rabbits	RH	Trans-scleral inoculation of 400 tachyzoites into the suprachoroidal space	Non-treated animals developed retinitis but all died from toxoplasmic encephalitis	Minocycline prevented death from toxoplasmic encephalitis in 75% of animals	Rollins <i>et al.</i> , 1982

Rabbit: Burgundy	ВК	Injection of 5×10^3 tachyzoites across the vitreous cavity in to the superficial part of the retina	All animals developed retinochoroiditis after 7 days; 22% of naïve rabbits died from generalized infection	Primed animals also with high incidence of retinochoroiditis (>90%)	Garweg <i>et al.</i> , 1998
Syrian Golden Hamster	ME49	10–25 cysts i.p.	All animals developed bilateral eye disease peaking after 4–5 weeks	No animal developed signs of systemic disease	Pavesio <i>et al.,</i> 1995
Syrian Golden Hamster	ME49	100 cysts orally	All animals developed unilateral or bilateral eye disease 4–8 weeks after infection	No animal developed signs of systemic disease	Gormley <i>et al.</i> , 1999
Guinea pig: not specified	RH	5×10^3 tachyzoites injected into the posterior chamber	Most animals developed acute chorioretinitis within 1–3 weeks	Recovery of <i>Toxoplasma</i> from eyes even after 10 months	Hogan <i>et al.,</i> 1956
Guinea pig: not specified	RH	5×10^3 tachyzoites injected into the posterior chamber	Not specified	Pyrimethamine- sulfadiazine treatment	Jacobs <i>et al.,</i> 1964
<i>Calomys callosus</i> : Canabrava strain	ME49	20 cysts orally at day 5–7 of pregnant or non- pregnant animals	40% of fetuses presented ocular lesions; 50–75% of adult animals presented unilateral ocular cysts	Model for congenital as well as for acquired ocular toxoplasmosis	Pereira Mde <i>et al.</i> , 1999
Domestic cat	ME49	5×10^3 tachyzoites inoculated into the common carotid artery	Progressive, multifocal retinal, and choroidal inflammatory foci (mostly bilateral) beginning 5–8 days post-inoculation in all cats tested	Minimal to no clinical signs of generalized toxoplasmosis; resolution of lesions 21–70 days post-inoculation	Davidson <i>et al.,</i> 1993
Primate: Macaca fascicularis, Cercopithecus aethiops, Macaca mulatta (rhesus)	RH	Injection of 5×10^3 to 1×10^5 tachyzoites across the vitreous cavity into the superficial part of the retina	Retinitis was reliably produced in all monkeys' eyes injected with 1×10^4 or more living tachyzoites	After 20 days the lesions began to resolve	Culbertson et al., 1982

*For definition of animal or *Toxoplasma* strains, please see respective articles. **Due to numerous publications on this topic, only few were exemplarily chosen for this table.

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any drug effects on the acute disease but only on the number of cerebral *Toxoplasma* cysts (Gormley *et al.*, 1998).

In addition to conventional laboratory animals, an acquired as well as a congenital model of ocular toxoplasmosis has been established in *Calomys callosus*, a wild rodent found in Central Brazil (Pereira Mde *et al.*, 1999). Following oral infection with the Me45 strain, 40 percent of fetuses presented ocular lesions (examined after laparatomic removal of the fetus) while the majority of adults presented ocular lesions in the acquired study setting. Adult animals survived the infection for several months without treatment, and demonstrated no clinical signs of systemic disease (Pereira Mde *et al.*, 1999). Whether this animal model is suited for drug studies might be shown by future investigations.

7.4 CEREBRAL TOXOPLASMOSIS

This section discusses animal models available for toxoplasmic encephalitis (TE) as the predominant manifestation of the disease in the immunocompromised host. It also includes acute systemic models where disseminated infection is prominent and where the brain is usually involved as a part of this. The by far most often used animal for acute or cerebral toxoplasmosis studies, particularly with respect to pharmacological testing, as indicated by numerous publications, is the mouse (for examples, see Perea and Daza, 1976; Grossman and Remington, 1979; Chang and Pechere, 1987; Hofflin and Remington, 1987a, 1987b; Chang et al., 1988, 1991, 1994; Israelski and Remington, 1990; Derouin et al., 1991, 1992; Araujo et al., 1991a, 1992a, 1992b, 1996, 1998; Weiss et al., 1992; Rodriguez-Diaz et al., 1993; Romand et al., 1993, 1995, 1996; Alder et al., 1994; Dumas et al., 1994, 1999; Olliaro et al., 1994; Khan et al., 1996, 2000; Martinez et al., 1996; Aguirre-Cruz and Sotelo, 1998; Aguirre-Cruz et al., 1998; Sordet et al., 1998; Djurkovic-Djakovic et al., 1999, 2002, 2005; Moshkani and Dalimi, 2000; Schöler et al., 2001; Ferreira et al., 2002; Degerli et al., 2003; Belal et al., 2004; Dunay et al., 2004; Lescano et al., 2004; Grujic et al., 2005; Ling et al., 2005).

For some purposes hamsters (Frenkel et al., 1975; Gormley et al., 1998) or rats (Foulet et al., 1994; Dubey, 1996; De Champs et al., 1997; Kempf et al., 1999; Zenner et al., 1999b; Freyre et al., 2001b, 2003b, 2004) have been used, but usually not for drug evaluation. Models are based on a primary acute infection of the animals, on direct inoculation of the parasite into the animal brain with or without immunosuppression, or on a chronic infection of the animal that may be immunosuppressed (for example with immunosuppressive drugs or radiation, antibodies directed against lymphocytes or cytokines, or concomitant infections with viruses that modulate the immunosystem) (see Tables 7.5 and 7.6). For certain applications, the use of genetically modified mice with various defects in their immune system may also be an option. In general, the acute and strictly localized models have most often been used to evaluate the treatment efficacy of antiparasitic drugs, whereas the chronic infection models have been used to study their influence on cyst formation and/or prevention of a relapsing disease.

7.4.1 Acute infection models

Acute infection models are usually associated with a 100 percent mortality of laboratory animals within 8 to 10 days, and survival in particular treatment groups is estimated by the Kaplan-Meier product limit survival analysis. So far these have been the overall preferred models for drug testing, mainly because of their consistent reproducibility. Most often, between 2×10^2 and 2×10^4 tachyzoites of the virulent RH strain are injected i.p. into female Swiss Webster mice (for examples, see Khan et al., 1996, 1998; Araujo et al., 1997; Djurkovic-Djakovic et al., 1999), but sometimes animals are infected orally with, for example, 10 cysts of T. gondii C56 (Araujo et al., 1997; Khan et al., 1998; Yardley et al., 2002). Mice are then usually observed for 30 days from the date of infection. Surviving mice are examined for residual infection by microscopy of brain tissue for the presence of T. gondii cysts, or by i.p. subinoculation of suspensions of portions of various organs into healthy mice. Unfortunately these models are not very

close to most Toxoplasma-induced human diseases of the immunocompromised host, where the infection clinically takes a more localized course not always (though often) confined to the brain. In contrast, acute primary toxoplasmosis in animal models is a generalized infection, substantially involving organs other than the brain - for example, the lungs or the liver. Thus, there are inherent difficulties in these models for the projection of drug efficacy deduced from a Kaplan-Meier diagram which ultimately is dependent not only on direct drug action but also on organ-specific pharmacokinetics and drug metabolism. This means, as a consequence, that a drug successfully tested in acute infection models may fail in the treatment of localized brain infection, and vice versa. At any rate, this model is the standard model for the first in vivo screening of a new drug. In addition to survival curves, the count or titration of cysts of succumbed and/or surviving animal brains as well as subinoculation into fresh mice may be performed. Alternatively, tissue-culture methods or modern detection methods such as PCR from organs of survivors may be used to assess the residual parasite load (Weiss et al., 1991; Miedouge et al., 1997; Djurkovic-Djakovic et al., 1999).

In addition to primary evaluation of drug efficacy based on a fatal outcome of acute toxoplasmosis, a mouse model has been developed that does not have the major disadvantage of the possibly severe suffering of mice before they die from overwhelming T. gondii infection (Samuel et al., 2003; Mui et al., 2005). In this model, mice are also inoculated i.p. with the RH strain, but on the fourth day after infection 1.5 ml of phosphatebuffered saline (PBS) is injected i.p. and then withdrawn together with all peritoneal fluid. Total numbers of parasites and concentrations of parasites are quantitated microscopically as the basis for subsequent statistical analysis (Samuel et al., 2003). In fact, this model has been proven by the addition of sulfadiazine to the drinking water of infected mice, which significantly reduces the parasite burden (Samuel et al., 2003) and thus may well be suited to replacing the survival-based acute infection models in the future.

7.4.2 Localized brain infection models

The direct inoculation of tachyzoites into the frontal lobe of mice may deliver the most reproducible results, with lesions histologically resembling those that could be observed in immunocompromised man (Hofflin et al., 1987). The model has been successfully used, for example, to demonstrate the efficacy of clindamycin, roxithromycine, and gamma interferon on Toxoplasma encephalitis (Hofflin and Remington, 1987a, 1987b). However, later, in another model also with localized brain infection with the highly virulent RH strain, clindamycin showed no detectable effect; instead, pyrimethamine, sulfadiazine, and their combination were useful in terms of mortality and histopathology (Arribas et al., 1995). At any rate, the procedure of local brain inoculation requires high technical expertise, and thus may well not be suitable for investigations on a larger scale in average laboratories. In addition, the main disadvantage of this model is that TE does not follow the natural history of T. gondii infection, as it is not a consequence of recrudescence of a previously established infection. It may therefore not be ideal for pharmacological trials related to these aspects (for example, for investigating drug interference with mechanisms of recrudescence), but rather for studying certain features of pathogenesis.

7.4.3 Chronic cystogenic infection models

Chronic infection models may be suited to evaluating the efficacy of drugs against the cyst form of *T. gondii*. Indeed, the cyst form is considered to be the most important of the life cycle of the parasite with regard to pathogenesis of toxoplasmosis in immunocompromised individuals, especially with regard to development of toxoplasmic encephalitis (Frenkel *et al.*, 1975; Frenkel and Escajadillo, 1987; Hofflin *et al.*, 1987; Ferguson *et al.*, 1989).

In corresponding models, mice are usually infected i.p. or orally with a mildly virulent typical type II cystogenic *Toxoplasma* strain such as, for example, ME49. They then develop a chronic progressive toxoplasmic encephalitis (type II strains such as the ME49 strain tend continuously to give rise to new cyst formation, presumably preceded by cyst rupture and proliferation of tachyzoites that are then converted into bradyzoites) and, unless treated, begin to die within the following weeks or months with various mortality rates depending on the mouse and Toxoplasma strains used as well as on the doses used for infection (Araujo et al., 1991b; Dumas et al., 1994; Djurkovic-Djakovic et al., 2002). Treated mice and untreated controls are usually sacrificed at determined time points, and the activity of drugs is assessed by light-microscopic examination and counting of cysts from brains previously ground with a pestle and mortar and suspended in a defined volume of PBS (Araujo et al., 1991b; Sarciron et al., 1997; Djurkovic-Djakovic et al., 2002; Lescano et al., 2004). Alternatively, brains of mice may be examined histopathologically by scoring the severity of inflammatory lesions (Araujo et al., 1996), and/or Kaplan-Meier curves may be obtained (Dumas et al., 1999; Djurkovic-Djakovic et al., 2002).

7.4.4 Chronic relapsing infection models (reactivated toxoplasmosis)

In order to describe and mimic the natural course of reactivation of *T. gondii* infection in man,

attempts have been made to develop animal models based on reactivation of a chronic infection. It was first observed in 1966 that when infecting splenectomized mice, or mice treated with cortisone or 6-mercaptopurine (6-MP), with the T. gondii Beverley strain, the course of the disease was greatly altered in the experimental animals, distinguished by signs of severe neurological involvement and meningoencephalitis (Stahl et al., 1966). Reactivation of an otherwise chronic infection had succeeded in hamsters infected with the RH strain by the administration of cortisone, cyclophosphamide, or whole body irradiation (Frenkel et al., 1975). On the other hand, in mice only little reactivation using cortisol acetate, azathioprine or cyclosporine was observed (Sumyuen et al., 1996), but reactivation was obtained with dexamethasone (DMX) (Nicoll et al., 1997). However, brain cysts were only demonstrated in a minority of the animals.

Perhaps the most promising mouse model that is based on immunosuppressive drugs is that of Djurkovic-Djakovic *et al.*, who used a low virulent type II *T. gondii* strain (Me49) to assess the efficacy of atovaquone combined with clindamycin treatment (Djurkovic-Djakovic *et al.*, 2002). Indeed, type II strains are also responsible for most cases of human TE (Howe and Sibley, 1995). Reactivation was achieved in those animals that previously had been *Text continued on page 177*

Pharmacological studies Infection model (mouse) Specific comments (literature examples) Standard models for drug tests; i.p. Acute infection models Khan et al., 1996, 1998; Araujo et al., infection usually with 100% mortality 1997; Mui et al., 2005 within 8-10 days (type I strains) Localized brain infection Direct inoculation of parasites into the Hofflin and Remington, 1987a, 1987b; models brain; does not follow the natural Arribas et al., 1995 route of infection Chronic cystogenic Intraperitonea, or oral infection with Araujo et al., 1991b, 1996; Dumas infection models type II strains; assessment e.g. via et al., 1994, 1999 brain cysts counting Chronic relapsing Infection with type II strains; drug-Schöler et al., 2001; Djurkovicinfection models induced immunosuppression of Djakovic et al., 2002; Dunay et al., mice or use of genetically altered 2004 (reactivated immunodeficient mice toxoplasmosis)

 TABLE 7.5 Pharmacological studies on cerebral and acute toxoplasmosis

 TABLE 7.6 Relapsing TE models of toxoplasmosis

Species and strain of mouse used*	<i>Toxoplasma</i> strain administered*	<i>Toxoplasma</i> dose, stage and route of infection	Means for reactivation	Outcome of animal infection	Remarks	Publication**
Porton	M3	30 cysts i.p.	Dexamethasone 6 weeks after infection	40% of mice developed clinical signs of toxoplasmosis, most of them with brain inflammation	Brain cysts observed in only 30–40% of mice with suspected toxoplasmosis	Nicoll <i>et al.</i> , 1997
Swiss Webster	ME49	10 cysts orally	Dexamethasone (DXM) alone or combined with cortisone acetate (CA) 6 weeks after infection	Mortality after 7 weeks with immunosuppres- sion: untreated 0%, DXM 61.1%, DXM + CA 85%, uninfected + DXM 33%	Mean cyst number 2–9-fold increased compared to untreated control; 14.2% developed clinical TE (both treatment regimens)	Djurkovic- Djakovic and Milenkovic, 2001
B6	C56 (or ME49)	1×10^5 tachyzoites i.p. of C56 followed by 2 weeks of treatment with sulfadiazine; (20 cysts ME49 i.p. for mice pre-infected with virus)	Co-infection with LP-BM5 murine leukemia virus 8 weeks after challenge with C56; (co-infection 12, 8, 4, or 2 weeks before ME49 challenge)	Chronic infection with C56: 30–40% mortality by 80 days following viral co-infection; mice with encephalitic lesions	No effects if challenge (ME49) 4 or 2 weeks after viral infection; all mice died if challenge 12 or 8 weeks after viral infection (pneumonitis, only occasional necrotic areas in brain)	Gazzinelli <i>et al.</i> , 1992
C57BL/6	Fukaya	10 cysts i.p.	Co-infection with LP-BM5 murine leukemia virus 6 weeks after <i>Toxoplasma</i> infection	All mice infected with both <i>T. gondii</i> and LP-BM5 MuLV died from 9 to 14 weeks after the virus infection due to severe encephalitis	In contrast to other studies with the LP-BM5 virus, in this study other organs than the brain seem to be less affected	Watanabe <i>et al.</i> , 1993
C57BL/6	C-strain	10 cysts orally	Co-infection with LP-BM5 murine leukemia virus 30 days before or 20, 30, and 60 days after challenge	None of the animals developed <i>Toxoplasma</i> encephalitis	Increase in <i>Toxoplasma</i> lung counts	Lacroix <i>et al.</i> , 1994

Continued

CEREBRAL TOXOPLASMOSIS

Species and strain of mouse used*	<i>Toxoplasma</i> strain administered*	<i>Toxoplasma</i> dose, stage and route of infection	Means for reactivation	Outcome of animal infection	Remarks	Publication**
C57BL/6	ME49	15 cysts orally	Co-infection with LP-BM5 2 weeks after challenge	70–80% of animals succumbed to disseminated infection (including brain but also lung, spleen and liver) by 12 weeks after LP- BM5 challenge	Transfer of immune CD8+T cells prevented reactivation	Khan <i>et al.</i> , 1999
SCID (on C.B-17/ Smn background	ME49 I)	20 cysts i.p.	Sulfadiazine treatment started 10 days after infection for 18 days, then discontinuation of therapy	All SCID mice died with TE 6–9 days after sulfadiazine treatment was stopped	No other organs except the brain with cysts, tachyzoites, or inflammation foci	Johnson, 1992
SCID	ME49	10 cysts orally	Sulfadiazine treatment started 2 days after infection for 3 weeks, then discontinuation of therapy	Mortality 100% within 2 weeks (TE); splenocyte transfer from immune syngenic donors prevented reactivation	Reactivation started from liver spreading into other organs (including the brain)	Beaman <i>et al.</i> , 1994
Inbred interferon- $(IFN-\gamma)^{-/-}$ (BALB/c mouse deficient for IFN- γ)	ME49	10 cysts i.p or orally	Sulfadiazine treatment started 4 days after infection for 3 weeks, then discontinuation of therapy	Mortality due to TE 100% within 1 week independent from infection modus; control WT BALB/c mice survived for more than 3 months	Treatment with recombinant IFN-γ prevented TE	Suzuki <i>et al.</i> , 2000
Inbred ICSBP/ IRF-8 ^{-/-} (C57BL/6 mouse deficient for interferon regulatory factor 8)	ME49	10 cysts orally	Sulfadiazine treatment started 2 days after infection for 3 weeks, then discontinuation of therapy	Mortality 100% within 2 weeks; synchronized development of TE	Treatment as well as maintenance therapy studies possible	Schöler <i>et al.</i> , 2001; Dunay <i>et al.</i> , 2004

 TABLE 7.6 Relapsing TE models of toxoplasmosis—cont'd

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TOXOPLASMA ANIMAL MODELS AND THERAPEUTICS

Species and strain of mouse used*	<i>Toxoplasma</i> strain administered*	<i>Toxoplasma</i> dose, stage and route of infection	Outcome of animal infection	Remarks	Publication**
Swiss Webster	C56	1 × 10 ⁴ tachyzoites injected intra- cerebrally	Normal mice survived but immunosup- pressed died from progressive disease (no rates given); immunosuppression with cortisone, cyclophosphamide, or cyclosporine	Cerebral lesions: inflammation intensity, tachyzoite and cyst number dependent on type of immunosup- pression	Hofflin <i>et al.</i> , 1987
Swiss Webster	C56	1 × 10 ⁴ tachyzoites injected intra- cerebrally	Mortality of 40% in normal mice and 100% in cortisone- treated animals	Drug evaluation study (efficacy of clindamycin was shown)	Hofflin and Remington, 1987a
Swiss Ico	RH	1 × 10 ³ tachyzoites injected intracerebrally	100% of animals died within 5 days after infection due to necrotizing <i>Toxoplasma</i> meningoencephalitis	Pharmaceutical study: evaluation of highly active drugs possible	Arribas <i>et al.,</i> 1995

Models based on localized brain infection instead of reactivation

Continued

CEREBRAL TOXOPLASMOSIS

Species and strain of mouse used*	<i>Toxoplasma</i> strain administered*	<i>Toxoplasma</i> dose, stage and route of infection	Outcome of animal infection	Remarks	Publication**
CBA/Ca (inbred)	ME49	20 cysts i.p. or orally	Development of a chronic progressive encephalitis and; mice begin to die approximately 6 weeks after infection	Assessment by cyst counting (light microscopy) or brain histopathology scoring	Araujo <i>et al.,</i> 1996
C57BL/6J	PRU	10 cysts i.p.	Development of a chronic progressive encephalitis characterized by brain cysts and inflammation with mortality of 60–80% within the following months	Assessment by brain histopathology scoring	Dumas <i>et al.</i> , 1999

TABLE 7.6 Relapsing TE models of toxoplasmosis—cont'd

*For definition of animal or *Toxoplasma* strains, please see respective articles. **Due to numerous publications on this topic, only few were exemplarily chosen for this table.

Models based on chronic progressive brain infection instead of reactivation (Cystogenic models)

orally infected with 10 tissue cysts of the Me49 strain, by immunosuppression with DXM alone and, more efficiently, by combined hydrocortisone-21-acetate (CA) treatment (Djurkovic-Djakovic and Milenkovic, 2001; Djurkovic-Djakovic *et al.*, 2002).

In addition to the above-mentioned models, reactivation may be induced in dual-infection models with *T. gondii* and viruses such as CMV (Pomeroy *et al.*, 1989) and LP-BM5 murine leukemia virus, which is responsible for murine AIDS (Gazzinelli *et al.*, 1992; Watanabe *et al.*, 1993; Lacroix *et al.*, 1994; Khan *et al.*, 1999). Such models are most useful for investigation of the immunopathogenesis of analogous disease in humans; however, due to their complexity and obstacles in reproducibility they may not be well suited to pharmacological investigations (Lacroix *et al.*, 1994).

Another strategy rather than suppressing the host immune system is based on the use of heavily immunocompromised mice, such as mice with severe combined immunodeficiency (SCID mice), lacking T and B lymphocytes, or athymic (nude mice), which lack functional T cells. An otherwise fatal infection in such mice with Toxoplasma exhibiting low virulence for immunocompetent non-SCID mice may be brought to chronicity by sulfadiazine treatment, while its withdrawal leads to relapse (Johnson, 1992; Beaman et al., 1994). However, so far such models have been mostly used to investigate immunopathogenesis of relapsing or acute toxoplasmosis, rather than to assess drug regimens for treatment or prevention of the reactivated disease (for review, see Denkers and Gazzinelli, 1998). In addition, heavily immunocompromised animals, such as SCID or athymic mice, are difficult to work with because of their severely impaired immune systems and the requirement for them to be kept in sterile conditions to prevent opportunistic infections.

A promising alternative to SCID mouse models has been presented by Suzuki *et al.* with a mouse deficient for interferon- γ (Suzuki *et al.*, 2000): in BALB/c mice – i.e. in a strain genetically relatively resistant to *T. gondii* infection (Suzuki *et al.*, 1991, 1994; Brown *et al.*, 1995) – INF- γ but not TNF- α or iNOS was crucial for resistance against the development of TE, and mice deficient for INF- γ died after an infection with the ME49 *T. gondii* strain

when treatment with sulfadiazine was discontinued (Suzuki et al., 2000). Based on these findings, reactivated TE in mice deficient for the interferon regulatory factor 8 (interferon consensus sequence binding protein ICSBP/IRF-8-/- on a C57BL/6 background) was achieved after oral infection with cysts of the ME49 strain, by withdrawal of sulfadiazine treatment (Schöler et al., 2001). This model, which relies on an impairment of the IL-12 dependent IFN-γ production (Holtschke et al., 1996; Scharton-Kersten et al., 1997), was then used to show the efficacy of atovaquone in the treatment of TE (Schöler et al., 2001) and thereafter also to prevent the disease from relapsing by an atovaquone maintenance therapy (Dunay et al., 2004). The results obtained with this new murine model of reactivated toxoplasmosis mimicked the signs of reactivated toxoplasmosis in immunocompromised patients, including the presence of parasiteassociated focal necrotic lesions in the brain parenchyma, and meningeal inflammation (Schöler et al., 2001; Dunay et al., 2004). Mice with impairment in INF- γ production, in contrast to SCID or nude mice, may not be as prone to common infection; however, they have to be kept under specificpathogen-free conditions, reducing the number of facilities where such experiments can be performed. Advantages in comparison to the induction of reactivation by administration of immunosuppressive drugs include the easiness of reactivation simply by discontinuation of sulfadiazine, and a relatively synchronized development of TE within days (Schöler et al., 2001).

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Biochemistry and Metabolism of *Toxoplasma gondii*

T. Asai and S. Tomavo

8.1 Introduction8.2 Carbohydrate metabolism8.3 Glycolipid anchors8.4 Nucleotide biosynthesis

8.5 Nucleoside triphosphate hydrolase (NTPase) *References*

8.1 INTRODUCTION

That Toxoplasma gondii and all other parasites of the phylum Apicomplexa reside and replicate exclusively within eukaryotic cells suggests that these parasites depend on the metabolism of their hosts and that they have evolved metabolic pathways reflecting their intracellular lifestyle. In addition, these parasites may display specific or alternate metabolism as documented by the recent discovery of novel metabolic pathways in the remnant and vestigial plastid (apicoplast) found in many apicomplexan parasites. Evidence for unique parasite metabolic pathways has direct implications for understanding the parasite's requirements for intracellular growth. Further, these pathways may provide unique targets for compounds designed to inhibit and eradicate infection caused by these important human and animal pathogens.

While targeting unique parasite pathways is an attractive strategy, in practice the procurement of enough pure parasites for biochemical study or purification of parasite components such as enzymes is extremely difficult. Biochemical and metabolic studies of *T. gondii* have also been difficult to execute because of continual problems with contamination of parasite preparations with host-cell components. Accordingly, only a few studies of biochemistry and metabolism of *T. gondii* have been reported.

Recent advances in gene engineering technology and molecular biology, including the genome database projects, are clearing many obstacles. For instance, it is now easy to obtain a predicted aminoacid sequence of an enzyme of *T. gondii* from the genome database (http://www.ToxoDB.org/). These data can be used to clone or synthesize the gene, and to produce a recombinant enzyme to characterize the enzyme. Bioinformatic schemes for several metabolic pathways of *T. gondii* have been compiled and released to the public at the genome database.

This chapter discusses aspects of metabolism, including carbohydrate metabolism, glucose metabolism during tachyzoite-bradyzoite differentiation, glycolipid anchors, nucleotide biosynthetic pathways, and NTPase, a peculiar enzyme unique to T. gondii and the closely related parasite Neospora caninum. It will also focus on new insights into distinct metabolic pathways, their evolutionary roots, and their contribution to Toxoplasma gondii survival during intracellular development and differentiation. In addition to this chapter, metabolism of the apicoplast and mitochondrion is discussed in Chapter 9. Chapter 19 encompasses a comprehensive discussion of amino-acid and nucleotide pathways with comparison to other Apicomplexa.

8.2 CARBOHYDRATE METABOLISM

8.2.1 Developmentally regulated expression of amylopectin in *T. gondii*

One evolutionary hallmark of the close relationship between the photosynthetic micro-organisms and apicomplexan parasites is defined by the existence of a storage polysaccharide named amylopectin in the cytoplasm of some apicomplexans. Another reflection of this relationship is the presence of a vestigial plastid 'apicoplast', discussed in Chapter 9. In contrast to plants, which contain starch defined as a branched amylopectin and amylose in the chloroplasts, the encysted bradyzoite and sporozoite forms of apicomplexans such as T. gondii (Coppin et al., 2005), Eimeria (Karkhanis et al., 1993), and Cryptosporidium (Harris et al., 2004) accumulate amylopectin, a polymer of linear glucose that is not present in Plasmodium and Babesia species. It has been speculated that the disappearance of amylopectin in Eimeria sporozoites resulted in the inability of the parasite to establish successful infection in mice (Augustine, 1980; Nakai and Ogimoto, 1983). In *T. gondii*, the bradyzoites accumulate abundant amylopectin granules and numerous micronemes. In contrast, the tachyzoites lack amylopectin, and fewer micronemes are present (Figure 8.1). Amylopectin is probably consumed when the encysted and dormant bradyzoites switch into the rapidly replicating tachyzoites. It is postulated that amylopectin provides an energy source, as its degradation to glucose can provide metabolic intermediates or substrates for glycolysis or mitochondrial oxidative phosphorylation.

8.2.2 *T. gondii* displays a uniquely simple pathway for amylopectin synthesis

Using bioinformatic searches, several gene candidates encoding enzymes that are probably involved in amylopectin biosynthesis were identified (Coppin *et al.*, 2005). These putative enzymes can be grouped in two classes:

- 1. Enzymes that are involved in amylopectin synthesis, such as amylopectin synthase, branching enzymes, UDP-glucose pyrophosphorylase, isoamylase, indirect debranching enzyme, α -1, 4-glucanotransferase, and glycogenin
- 2. Enzymes for amylopectin degradation, like α -amylase, dikinase or R1 protein, phosphorylase, and α -glucosidase. Based on the presence of these enzymes, metabolic pathways and enzymes involved in amylopectin synthesis in *T. gondii* are probably similar to those of starch synthesis in the unicellular green algae *Chlamydomonas reinhardtii* (Figure 8.2).

Surprisingly, all of these genes are present in the *Toxoplasma* genome as a unique copy, suggesting that redundant genes are not required for the synthesis of a genuine crystalline amylopectin in this protozoan parasite (Ball and Morell, 2003; Coppin *et al.*, 2005). This is in violation of the current dogma that suggests that redundancy of genes is required to build a crystalline starch in plants. Even in the simplest unicellular picophytoplanktonic green alga, *Ostreococcus tauri*, there is multiplicity



FIGURE 8.1 Transmission electron micrographs of bradyzoites (Br) within a tissue cyst (Panel A). Note the presence of the cyst wall (CW) and numerous amylopectin granules (AG) in the cytoplasm of the bradyzoites. Panels B and C shows a higher magnification of ultrastructural morphology of bradyzoite and tachyzoite which lacks amylopectin granules. Rh, rhoptry; DG, dense granules; Mi, micronemes and M, mitochondrion; N, nucleus; CW, cyst wall; PV, parasitophorous vacuole.

of genes and redundancy of isoenzymes involved in starch synthesis (Ral *et al.*, 2004).

Only UDP-glucose pyrophosphorylase and UDP-glucose utilizing amylopectin synthase are found in T. gondii. Comparative genomic analyses involving the unicellular red alga Cyanidioschyzon merolae (Matsuzaki et al., 2004), the unicellular green alga Chlamydomonas reinhardtii, the yeast Saccharomyces cerevisiae, and the bacterium Escherichia coli revealed that both C. merolae and T. gondii contain a UDP-glucose utilizing glycogen (starch) synthase-like sequences and glycogenins. These enzymes are specific for the eukaryote UDP-glucose based pathway. In addition, UDP-glucose utilizing glycogen synthase activity has been detected in the crude extract from T. gondii while only ADP-glucose dependent activity is present in Chlamydomonas lysates (Coppin et al., 2005). T. gondii also contains an indirect debranching enzyme, a bifunctional enzyme that carries both α -1,4-glucanotransferase and amylo-1,6-glucosidase activities in fungi and animals (Figure 8.2).

However, the characteristic most typifying the amylopectin biosynthetic pathway in *T. gondii* is the presence of genes that are of plant origin. Among the genes that distinguish plant starch metabolism from those of the animal, fungal, and bacterial glycogen pathways are the isoamylase and R1 (glucan water dikinase activity)-like sequences in *T. gondii*. This suggests that both plant- and animal-like amylopectin biosynthetic pathways are required for the synthesis of crystalline amylopectin in the parasite (Figure 8.2).

8.2.3 Evolutionary origins of enzymes of amylopectin biosynthesis

Both plant- and animal-like metabolisms are probably involved in amylopectin biosynthesis in *T. gondii*. This is likely a signature of the evolutionary origin of apicomplexan parasites. These parasites contain a vestigial plastid 'apicoplast' that is derived from a secondary endosymbiosis with the engulfment of a unicellular alga (McFadden *et al.*,



FIGURE 8.2 Proposed metabolic pathways involved in the biosynthesis of semi-crystalline polysaccharide storage amylopectin of *Toxoplasma gondii*. The biosynthetic pathway and enzymes involved therein (glycogenin, UDP-glucose pyrophosphorylase, amylopectin synthase, branching enzymes, indirect debranching enzymes) are shown in black and red arrows, respectively (see color plate). The enzymes (α -amylase, R1 protein, phosphorylase and α -glucosidase) involved in the degradation pathway (green arrow) are indicated in brown. The putative genes encoding these enzymes have been identified in the genome sequence of *T. gondii* (http://www.ToxoDB.org/). The presence of active amylopectin synthase using UDP-glucose as a substrate has been assayed in tachyzoite and bradyzoite crude extracts. This figure is reproduced in color in the color plate section.

1996; Köhler *et al.*, 1997; Cai, *et al.*, 2003; Waller *et al.*, 2003). Phylogenetic analyses performed with two key enzymes (amylopectin synthase and the R1 protein) demonstrate that the *T. gondii* amylopectin synthesis pathway has evolved from the red algal starch synthesizing machinery through a secondary endosymbiotic event (Coppin *et al.*, 2005). These phylogenetic data, together with the presence of the enzymes and the enzymatic activities described above, establish that apicomplexans and red algae such as *C. merolae* use a UDP-glucose pathway to build water-insoluble amylopectin. This is the

pathway used by all floridean starch-accumulating organisms (Nyvall *et al.*, 1999).

It is equally apparent that apicomplexans also contain plant-like genes that are not found in yeast and mammals. These consist of genes that are required in plants and green algae and encode enzymes (water dikinase or R1 protein, α -1, 4-glucanotransferase or D-enzyme, and isoamylase) that are involved in the breakdown and synthesis of starch. The presence of these plantlike enzymes could be useful for the discovery of inhibitors that can interfere with the synthesis or

degradation of amylopectin in apicomplexan parasites.

8.2.4 Stage-specific expression of genes involved in glucose catabolism

It is noteworthy that some genes coding for enzymes involved in the biosynthesis of amylopectin and others in the glycolytic pathway are developmentally regulated during tachyzoite to bradyzoite stage conversion (see Chapter 13 for a discussion of bradyzoite differentiation).

8.2.4.1 Stage-specific expression of genes involved in amylopectin degradation

The expression pattern of the genes involved in amylopectin biosynthesis in tachyzoites and bradyzoites isolated from mouse brain cysts has been investigated by RT-PCR. Transcripts coding for enzymes known to be involved in the catabolic functions, such as the R1 protein, α -glucan phosphorylase, α -glucosidase, and α -amylase, are preferentially expressed in bradyzoites (Coppin *et al.*, 2005). In constrast, transcripts coding for enzymes known to be involved in amylopectin synthesis (glycogenin, amylopectin synthase, branching enzyme) are preferentially expressed in tachyzoites but can also be detected at lower expression levels in bradyzoites.

This pattern is consistent with the production of amylopectin during differentiation of tachyzoites into bradyzoites, and with the mobilization of the glucose stores during bradyzoite to tachyzoite interconversion (Tomavo, 2001). Even though there is evidence for transcription of these genes, it remains to be determined whether the transcripts detected are translated into functional proteins and enzymes. Therefore, specific antibodies or enzymatic activities need to be tested in order to demonstrate protein synthesis. Even if the enzymatic activity of amylopectin synthase has been demonstrated in both tachyzoites and bradyzoites (Coppin et al., 2005), post-transcriptional regulation may also occur on some of the stage-specific transcripts detected.

8.2.4.2 Stage-specific expression of genes involved in glycolysis

Glucose 6-phosphate isomerases and lactate dehydrogenases The glycolytic enzyme lactate dehydrogenase (LDH, EC 1.1.1.27) is a glycolytic enzyme that catalyses the interconversion of pyruvate to lactate using NAD+ as a co-enzyme (Figure 8.3). Two stage-specific LDH genes have been identified; the tachyzoite-specific LDH1, and the bradyzoite-specific LDH2 (Yang and Parmley, 1995, 1997). The transcript of LDH2 was only detected in the bradyzoite stage, while mRNA of LDH1 was found in both bradyzoite and tachyzoite stages. The absence of LDH2 mRNA in the tachyzoite suggests that the transcription of LDH2 is suppressed during transition from the bradyzoite to the tachyzoite stage. Conversely, the data indicate that LDH1 is the only isoenzyme produced by the tachyzoites. The level of LDH2 mRNA increased markedly in vitro during bradyzoite induction, suggesting that transcription activation and/or mRNA stability may explain the stage-specific expression of LDH2 gene in T. gondii (Yang and Parmley, 1997). Because the predicted isoelectric points of the two LDHs are different, two-dimensional electrophoresis has been used to demonstrate that only one LDH protein is expressed in each developmental parasitic stage.

It has also been demonstrated that LDH1 and LDH2 share a unique structural feature with LDH from the malarial parasite *Plasmodium falciparum* (pLDH), namely a five-amino acid insertion into the substrate specificity loop. This insertion has only been observed in pLDH, LDH1 and LDH2 (Bzik *et al.*, 1993; Yang and Parmley, 1997). All other LDH enzymes described so far do not contain this insertion. The insertion in LDH2 is identical to the insertion in pLDH (KSDKE), but differs slightly from the insertion in LDH1 (KPDSE).

Comparative studies on the kinetic properties of *T. gondii* LDH1 and LDH2 and *P. falciparum* LDH showed that LDH1 and LDH2 exhibit broader substrate specificity than pLDH. For both LDH1 and LDH2, 3-phenylpyruvate is an excellent substrate – even better than pyruvate when LDH2 was tested



FIGURE 8.3 Schematic representation of the link between glycolysis and amylopectin biosynthesis. The classical pathway of glycolysis is shown in the right panel. Phosphoglucose isomerase, enolase, and lactate dehydrogenase (shown in red; see color plate) are found as two iso-enzymes; each enzyme is stage-specifically expressed in the tachyzoites or in the bradyzoites (see more details in the text). This figure is reproduced in color in the color plate section.

with both substrates. In contrast, pLDH does not utilize 3-phenylpyruvate (Dando *et al.*, 2001). In addition, both LDH1 and LDH2 can utilize the NAD analog 3-acetylpyridine adenine dinucleotide (APAD) efficiently, similarly to pLDH. A range of inhibitors including gossypol and derivatives inhibit LDH1, LDH2, and pLDH, but in general LDH2 is more sensitive than LDH1. LDH1 also shows substrate inhibition despite the substitution in both LDH1 and LDH2 of a methionine for serine-163, a residue that is thought to be critical for production of substrate inhibition (Dando *et al.*, 2001). Most importantly, gossypol and gossylic iminolactone have been shown to display inhibition of *T. gondii* tachyzoite growth in fibroblast cultures.

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The differences in sensitivities to inhibitors between LDH1 and LDH2 further illustrate how these enzymes may have evolved to serve separate roles during stage development. It should be noted that these studies have been performed with recombinant enzymes. These observations remain to be confirmed with native purified enzymes, since other co-factors may also be involved in the modulation of enzymatic properties. Nevertheless, it can be speculated that the apparent greater sensitivity of recombinant LDH2 to gossypol and derivatives may be leads for the design of inhibitors that could be exploited as chemotherapeutic agents to eliminate cysts from chronically infected hosts. For this purpose, the crystal structure of LDH may be required. Kavanagh *et al.* (2004) have crystallized LDH1 in its apoform and in its ternary complexes containing NAD+ or the NAD+ analog 3-acetylpyridine adenine dinucleotide (APAD(+)) and sulfate or the inhibitor oxalate. Superimposition of LDH1 with human muscleand heart-specific LDH isoforms reveals differences in residues that line the active site. This increases LDH1's hydrophobicity. It was concluded that these differences would aid in designing inhibitors specific for LDH1 that may be useful in treating toxoplasmic encephalitis and other complications that arise in immunocompromised patients.

Another glycolytic enzyme that is stage-specific is glucose 6-phosphate isomerase (G6-PI, EC 5.3.1.9), which catalyses the interconversion of glucose 6-phosphate to fructose 6-phosphate (Figure 8.3). A cDNA fragment encoding G6-PI was isolated from a bradyzoite-specific subtractive library and the full-length cDNA was used to complement an E. coli mutant lacking G6-PI (Dzierszinski et al., 1999; Yahiaoui et al., 1999). RT-PCR data have demonstrated that the transcript coding G6-PI is preferentially present in bradyzoites, while a minute amount can be detected in tachyzoites. Western blot analysis performed with specific polyclonal antibodies revealed G6-PI only in encysted bradyzoites, demonstrating the stagespecific expression of G6-PI in T. gondii. It remains to be determined, however, whether the other putative G6-PI coding genes presently described in the Toxoplasma genome correspond to the tachyzoite-specific G6-PI. Here, only the T. gondii cDNA coding G6-PI has been tested in E. coli complementation; its enzymatic activity with the purified enzyme has not been directly assayed.

Enolases Within the glycolytic pathway, enolase or ENO (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) catalyzes the conversion of 2-phospho-glycerate to phosphoenolpyruvate (Figure 8.3). As for LDH, two stage-specific enolase-coding genes have been described (Dzierszinski *et al.*, 1999). The two genes are located on the same chromosome and separated only by an intergenic

sequence of 1.6 kilobases. Both transcript and protein corresponding to the product of the gene named ENO1 are only detected in bradyzoites while ENO2's transcript and protein are found in tachyzoites. The amino-acid identity between ENO1 and ENO2 was found to be 73 percent.

Interestingly, when compared with human and other mammalians' enolases, both enolases contain a pentapeptide insertion: EWGYC in ENO2, and the almost identical EWGWS motif in ENO1 (Dzierszinski et al., 1999) and enolase of Plasmodium falciparum (Read et al., 1994), respectively. In addition, another dipeptide EK/DK insertion was also found in ENO1 and ENO2 of T. gondii and in P. falciparum enolase. Superimposition of the model tridimensional structure of ENO1 or ENO2 with that of human enolase revealed a perfect match between their 3D-models except for the presence of two extra loops corresponding to the pentapeptide EWGWC and the dipeptide EK insertion, respectively. The presence of these two loops was also evident in P. falciparum and, surprisingly, in plant enolases (Dzierszinski et al., 1999).

The functions of these two loops were investigated by site-directed mutagenesis of the pentapeptide, the dipeptide, and both loops in the ENO1 recombinant enzymes (Dzierszinski *et al.*, 2001). The enzymatic properties of these mutated enzymes and of the wild-type enzyme demonstrated that the deletion of a single EK loop does not affect the K_m of the enzyme, but the deletion of both loops causes a 13-fold increase of the enzyme K_m . Deletion of the pentapeptide EWGWC gave a five-fold increase of the K_m compared to the values of the wild-type enzyme.

In addition, the K_m , V_{max} , and temperature stability of pure recombinant ENO1 and ENO2 enzymes were also compared. While the K_m values are identical, ENO1 and ENO2 display distinct V_{max} with a value three-fold higher for ENO2 than for ENO1, suggesting that the two isoenzymes have the same affinity for the substrate 2-PGA but exhibit different rates of substrate consumption. The denaturation temperature of ENO1 was also found to be higher than that of ENO2, indicating that the tachyzoite ENO2 is more thermolabile than the bradyzoite ENO1. The enzymatic properties of the two stagespecific enolases seem to be in good agreement with the metabolic and physiological adaptation required during *T. gondii* differentiation and encystation. It can be postulated that these enzymes play discrete biological functions that most probably involve profound carbohydrate metabolism modifications such as the biosynthesis or degradation of amylopectin that occurs during the stage conversion of *T. gondii*.

8.2.5 Parasite glycolytic enzymes involved in other biological functions

In eukaryotic cells, many kinds of multifunctional regulatory proteins have been identified that perform distinct biochemical functions in the nucleus, the cytoplasm, or both. Recent studies establish that metabolic enzymes display biological roles distinct from their cognate functions. Perhaps the best-studied examples are enzymes that double as eye-lens proteins essential for normal vision: lactate dehydrogenase (crystalline in ducks and crocodiles), α -enolase (crystalline in lamprey and turtles), and argininosuccinate lyase (crystalline in birds and reptiles) (Piatigorsky, 2003).

Another example of a multifunctional protein is glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which interacts with a wide variety of RNAs, including ribosomal RNA, tRNA, hammerhead ribozymes, and the 3' untranslated region of many mRNAs. Proposed functions for GAPDH include regulation of helicase activity, tRNA and mRNA export, RNA unfolding, translational regulation, regulation of viral gene expression, and regulation of mRNA stability (Sirover, 2005). In Plasmodium falciparum, it has been shown that the N-terminal domain of GAPDH mediates GTPase Rab2-dependent recruitment to membranes, suggesting that GAPDH exerts non-glycolytic function(s) in the parasite, possibly including a role in vesicular transport and biogenesis of apical organelles (Daubenberger et al., 2003).

In *T. gondii*, a number of studies have described several glycolytic enzymes with functions distinct from their primary functions. Using pull-down

assays, Jewett and Sibley (2003) reported that the parasite aldolase is the molecular link between micronemal thrombospondin-anonymous related proteins (TRAP family) and the acto-myosin motor involved in apicomplexan parasite gliding and host-cell invasion. PRP1 (parafusin-related protein 1) is an ortholog of phosphoglucomutase that is associated with micronemes (Matheissen *et al.*, 2001, 2003).

To investigate the significance of T. gondii lactate dehydrogenases, LDH1 and LDH2, in the control of a metabolic flux during parasite differentiation, the expression of these two isoenzymes was knocked down in a stage-specific manner (Al-Anouti et al., 2004). These LDH knockdown parasites exhibited variable growth rates in either the tachyzoite or the bradyzoite stage when compared with the wild-type parasites. Their differentiation processes were impaired in vitro, and they were unable to form tissue cysts in a murine model system. In addition, all mice infected with the knockdown of LDH1 and LDH2 expression gave rise to virulence-attenuated parasites, and survived a subsequent challenge with parental parasites at a dose that usually causes 100 percent mortality. It has been concluded that LDH expression is important for the cell cycle and differentiation of T. gondii. However, the precise mechanisms by which LDH knockdown impairs parasite growth and differentiation remain to be elucidated.

8.2.5.1 T. gondii enolases are involved in nuclear functions

Extensive studies performed on the stage-specific expression of enolases of *T. gondii* using the polyclonal antibodies specific to ENO1 and ENO2 revealed that both enolase isoenzymes can be detected in the nucleus of the parasite (Ferguson *et al.*, 2002). The accumulation of enolase signal in the nucleus is observed in both tachyzoites and bradyzoites, but only in dividing zoites. The nuclear signal of ENO1 in the brain of 12-day-infected mice is detected early in tachyzoites that are differentiating into bradyzoites. The expression of ENO1 in these intermediate zoites appears earlier than that of the classical bradyzoite surface

marker, P36 or SAG4, and these ENO1-expressing intermediate zoites are still expressing the tachyzoite SAG1 surface marker. In addition, it has been found that only the tachyzoite-specific ENO2 is expressed in the dividing sexual forms of *T. gondii* examined in infected cells derived from the cat. Taken together, these data suggest that this novel subcellular localization can be ascribed to novel nuclear activity displayed by ENO1 and ENO2.

It should be noted that the nuclear localization of enolase was first described in human cells where the enzyme binds to the c-Myc promoter and acts as transcriptional repressor in cancer cells (Feo et al., 2000). The binding to DNA target and the domain in enolase that is involved in the transcriptional regulation has been identified (Subramanian and Miller, 2000). Interestingly, the factor isolated from cold-resistant mutants of Arabidopsis thaliana using genetic approaches was identified as A. thaliana enolase, which binds to a DNA motif similar to that described in human cells (Lee et al., 2002). Thus, it could be postulated that T. gondii enolases might display similar transcriptional or other nuclear functions involved in the intracellular growth of the parasite. However, further experimental support needs to be provided for the precise nuclear activity of enolases in T. gondii.

8.3 GLYCOLIPID ANCHORS

8.3.1 Structure of *Toxoplasma* glycosylphosphatidylinositol and its role in membrane anchoring

Extensive studies have demonstrated that many proteins are attached to the eukaryote cell membranes via inositol-containing glycophos-pholipids (GPIs). In *T. gondii*, the major surface proteins are anchored to the parasite surface by a GPI-membrane anchor (Nagel and Boothroyd, 1989; Tomavo *et al.*, 1989). This type of anchor seems to be more frequently used in *Toxoplasma* and other protozoa than in higher eukaryotes.

When it became clear that numerous surface antigens of *T. gondii* are GPI-anchored, several

studies established the structure and biosynthesis of these membrane anchors, and in particular of their putative precursors. The structure of GPI anchors of these proteins has been determined by combining metabolic labeling (tritiated glucosamine, mannose, galactose, palmitic and myristic acids, and inositol) and their sensitivity to a lipase named phosphatidylinositol phospholipase C (PI-PLC) (Tomavo *et al.*, 1989). The release of these proteins from the surface of live parasites, which causes a cross-reacting determinant (CRD) of the soluble forms to be accessible to anti-CRD serum of trypanosomes, also confirms that the major surface proteins (SAGs) of *Toxoplasma* possess GPI anchors.

Further investigations led to the determination of the *Toxoplasma* GPI anchor structure that is composed of the evolutionarily conserved linear GPI core, ethanolamine-PO₄-6Man α 1-2Man α 1-6Man α 1-4GlcNac α 1-6-inositol on SAG1 (P30) and P23 (Figure 8.3, Tomavo *et al.*, 1992a, 1993). Candidate glycolipid precursors that are probably transferred *in bloc* to the nascent membrane proteins of *T. gondii* have been identified and isolated either in living parasites or in parasite extracts.

Four mature glycolipids and other intermediate forms have been characterized (Tomavo *et al.*, 1992b). These four major glycolipids have the same GPI core structure and can serve as preassembled precursors of GPI anchors linked to these proteins. In addition, a lipophilic 'low molecular weight' antigen (4.6 kDa), identified using human patient sera and monoclonal antibodies (Sharma *et al.*, 1983; Tomavo *et al.*, 1994), was shown to be glycophosphoinositols that are not linked to proteins (free GPIs) and localize in the plasma membrane of the parasites. These free GPIs or low molecular weight antigens were shown to elicit an early immunoglobulin M response in humans.

The detailed structures of these free GPIs have been determined using metabolic labeling and enzymatic digestion, followed by classical chromatographic analysis, nuclear magnetic resonance imaging, and fast-atom bombardment-mass spectroscopy (Striepen *et al.*, 1997). The following two GPI structures were elucidated: the classical structure
(ethanolamine-PO₄)-Man α 1-2Man α 1-6(GalNAc β 1-4) Man α 1-4GlcNAC-inositol-PO₄-lipid and a novel structure (ethanolamine-PO₄)-Man α -2Man α 1-6 (Glc α 1-4GalNAc β 1-4)Man α 1-4GlcNAC-inositol-PO₄lipid both with and without terminal ethanolamine phosphate. Only *T. gondii* GPIs bearing the unique glucose-N-acetylgalactosamine side branch are immunogenic in humans (Striepen *et al.*, 1997).

8.3.2 Role of GPIs in cell signaling and host immune response

Except for their role in membrane insertion of surface proteins in *T. gondii*, the biological functions of GPIs are presently unknown. In other eukaryotic systems, GPIs can display functions involved in signal transduction. One possible function of the GPI anchor might be to allow a closer association of the proteins with themselves and other surface proteins in the membrane (Tomavo, 1996). Consistent with this idea, genetically engineered transmembrane-anchored SAG1 does not show the usual observed association of GPI-anchored SAG1 with itself and/or other proteins (Seeber *et al.*, 1998).

As stated, Toxoplasma free-GPIs elucidate strong and early immunogenic responses during host infection. Data from other protozoa suggest that other functions of GPIs in host immune response are possible. In Plasmodium falciparum, the GPI moiety, free or associated with protein, induces tumor necrosis factor and interleukin-1 production by macrophages, and regulates metabolism in adipocytes (Schofield and Hackett, 1993). Deacylation with specific phospholipases abolishes cytokine induction. When administered to mice in vivo the malaria parasite GPI induces cytokine release, a transient pyrexia and hypoglycemia, and profound and lethal cachexia, in the presence of sensitizing agents. The data suggest that the GPI of *Plasmodium* is a potent glycolipid toxin that may be responsible for a novel pathogenic process. It has been further demonstrated that Plasmodium GPI directly and specifically increases cell adhesion molecule expression in HUVECs, and parasite cytoadherence (Schofield et al., 1996). These parasites' GPIs induce rapid activation of a tyrosine kinase in macrophages.

The minimal structure requirement for tyrosine kinase activation is the evolutionarily conserved core glycan sequence Man α 1,2Man α 1,6Man α 1-4GlcN1-6myo-inositol. The GPI alone appears sufficient to mimic the activities of malaria parasite extracts in the signaling pathway leading to TNF expression (Tachado *et al.*, 1997).

Thus, GPIs of intraerythrocytic Plasmodium falciparum induce pro-inflammatory cytokine responses. It was also reported that adults who have resistance to clinical malaria contain high levels of pertinent anti-GPI antibodies, whereas susceptible children lack or have low levels of short-lived antibody response. Individuals who were not exposed to P. falciparum completely lack anti-GPI antibodies. Absence of a pertinent anti-GPI antibody response correlated with malariaspecific anemia and fever, suggesting that anti-GPI antibodies provide protection against clinical malaria (Naik et al., 2000). These results could be evaluated in studies aimed at the defining the activity of chemically defined structures for toxicity, and results would have implications for the development of GPI-based therapies or vaccines.

The *P. falciparum* GPI glycan consisting of the sequence NH₂-CH₂-CH₂-PO₄-(Manα1-2) 6Manα1-2Manα1-6Manα-4GlcNH(2)α1-6myo-inositol-1, 2-cyclic-phosphate was chemically synthesized, conjugated to carriers, and used to immunize mice infected with P. berghei, a rodent model of severe malaria. The recipients were substantially protected against malarial acidosis, pulmonary edema, cerebral syndrome, and fatality (Schofield et al., 2002). Altogether, the above data suggest that GPI is a significant pro-inflammatory endotoxin of parasitic origin and it may contribute to pathogenesis and fatalities in humans. In addition, GPI may also be used as a prototype carbohydrate anti-toxin vaccine against malaria. It remains to be seen whether GPI has a similar role in clinical toxoplasmosis.

8.4 NUCLEOTIDE BIOSYNTHESIS

The most extensively studied metabolic pathways in *T. gondii* are those of pyrimidine and purine nucleotide biosynthesis. These pathways provide the substrates for DNA/RNA biosynthesis, and are commonly targeted for chemotherapy. They are logical areas to study in the rapidly multiplying tachyzoite form. Due to limited material, nearly all studies have been performed exclusively in tachyzoites. Illustrations of the present overall knowledge of nucleotide biosynthesis in the tachyzoite form of *T. gondii* are shown in Figures 8.4–8.7. Comparative analyses of genome sequences for several apicomplexans have revealed surprising differences among the Apicomplexa in nucleotide metabolism, which are discussed further in Chapter 19.

Early works determined that T. gondii has both de novo (Hill et al., 1981; O'Sullivan et al., 1981; Schwartzman and Pfefferkorn, 1981; Asai et al., 1983a) and salvage (Pfefferkorn and Pfefferkorn, 1977, 1980; Pfefferkorn, 1978; O'Sullivan et al., 1981; Iltzsch, 1993) pyrimidine nucleotide biosynthetic pathways. The *de novo* pyrimidine biosynthetic pathway is more important than the salvage pathway, and is essential for T. gondii growth and virulence. Pyrimidine auxotrophic mutants of T. gondii with disruption of the single copy carbamyl phosphate synthetase II gene (CPSII, E.C. 6.3.5.5) are avirulent in mice (Fox and Bzik, 2002). CPSII catalyzes the first step of the de novo pyrimidine biosynthetic pathway. Furthermore, pyrimidine starvation is one of the conditions that cause stage conversion from the tachyzoite to the bradyzoite (Bohne and Roos, 1997). The de novo pyrimidine biosynthetic pathway is shown in Figure 8.4.

8.4.1 Pyrimidine *de novo* biosynthetic pathway

The preliminary characterization of all six enzymes of the *de novo* pyrimidine biosynthetic pathway (Asai *et al.*, 1983a) indicated some distinctions between *T. gondii*-enzyme and host-enzyme activities. The mammalian host CPSII is part of a large multifunctional protein (CAD) composed of three enzymes: CPSII, aspartate carbamyltransferase (ATCase, E.C. 2.1.3.2), and dihydroorotase (DHOase, E.C. 3.5.2.3) (Mori and Tatibana, 1978; Davidson



FIGURE 8.4 T. gondii pyrimidine de novo biosynthetic pathways. Solid lines represent activities that were detected in T. gondii, the short dashed line represents activities of uridylate kinase and nucleoside diphosphate kinase that are not considered to be *de novo* enzymes, and the long dashed line represents an inhibitory effect on CPSII by UTP. Abbreviations used are as follows: CP, carbamyl phosphate; CAsp, carbamyl asparatate: DHO, dihvdroorotate: OA, orotic acid; OMP, orotidine 5'-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; Mt, mitochondrion. Enzyme activities are numbered as follows; (1) carbamyl phosphate synthetase II (CPSII), (2)asparatate carbamyltransferase (ATCase), (3) dihydroorotase (DHOase), (4) dihydroorotate dehydrogenase (DHO-DHase), (5) orotate phosphoribosyltransferase (OPRTase), (6) orotidylate decarboxylase (ODCase). *DHO-DHase activity is present in membranous fraction and may link to the respiratory chain of mitochondrion.

et al., 1993). These three enzymes comprise the first three enzymes in the pathway. In contrast to mammalian CAD, *T. gondii* CPSII is a cytosolic protein that is an independent enzyme with approximate molecular mass of 540 kDa (Asai *et al.*, 1983a). *T. gondii* has a single CPSII gene which is interrupted by 36 introns. The predicted protein encoded by the 37 CPSII exons is a 1687 amino-acid polypeptide with approximate molecular mass of 186 kDa (Fox and Bzik, 2003). Consequently, the native *T. gondii* CPSII appears to be a trimer of identical subunits.

This large CPSII is a common characteristic of other apicomplexan parasites (Flores *et al.*, 1994;

Chansiri and Bagnara, 1995). Bacteria and plants also possess the independent CPS proteins (Jones, 1980; Zhou *et al.*, 2000), however, the structural organization of these enzymes is different from those of mammalian and parasitic protozoan CPSII, including *T. gondii* CPSII.

The enzyme reaction of glutamine-dependent CPSII consists of two reactions; the reaction of glutamine amidotransferase (GATase, E.C. 2.4.2.14), and the reaction of CPS. *T. gondii* and other parasitic protozoa, including *Trypanosoma, Babesia*, and *Plasmodium*, express a bifunctional glutamine-dependent CPSII composed of an N-terminal GATase domain fused with C-terminal CPS domains (Aoki *et al.*, 1994; Flores *et al.*, 1994; Chansiri and Bagnara, 1995; Nara *et al.*, 1998; Gao *et al.*, 1999; Fox and Bzik, 2003). Bacteria and plants express a monofunctional GATase as well as a monofunctional CPS (Jones, 1980; Zhou *et al.*, 2000).

The mammalian CPSII is characteristically an allosterically controlled enzyme. Forward activation is by 5-phosphoribosyl-1-pyrophosphate (PRPP), and backward inhibition is by UTP (Jones, 1980). PRPP is a substrate of nucleotide biosynthesis, and provides the sugar phosphate moiety of nucleotides. The T. gondii CPSII activity is inhibited by UTP, but no activation by PRPP is reported (Asai et al., 1983a). The regulatory domain for the allosteric control of mammalian CPSII is the C-terminal ~150 amino-acid domain (Liu et al., 1994). The T. gondii CPSII contains significant amino-acid insertions in this expected regulatory domain (Fox and Bzik, 2003). Enlarged C-terminal regulatory domains are also reported for other apicomplexan parasites (Flores et al., 1994; Chansiri and Bagnara, 1995). The lack of activation of T. gondii CPSII by PRPP may be due to this enlarged C-terminal domain.

The *T. gondii* ATCase is a cytosolic monofunctional enzyme protein with approximate molecular mass of 140 kDa (Asai *et al.*, 1983a). The open reading frame of cloned *T. gondii* ATCase cDNA encodes a putative 423 amino-acid polypeptide with a predicted molecular mass of 46.8 kDa. Recombinant *T. gondii* ATCase with catalytic activity exhibits a molecular mass of 144 kDa (Mejias-Torres and Zimmermann, 2002). Native *T. gondii* ATCase is likely to be trimer of identical subunits. Plant ATCase (Khan *et al.*, 1999) is inhibited by UMP, and *E. coli* ATCase (Wales *et al.*, 1999) is inhibited by CTP and UTP (*E. coli*). However, no significant effect by any nucleotide has been reported on the activity of *T. gondii* ATCase (Asai *et al.*, 1983a; Mejias-Torres and Zimmermann, 2002).

The *T. gondii* DHOase is a cytosolic monofunctional enzyme with approximate molecular mass of 70 kDa whose activity is not regulated by nucleotides (Asai *et al.*, 1983a). No characterization of the *T. gondii* DHOase gene has been reported.

Dihydroorotate dehydrogenase (DHO-DHase, E.C. 1.3.3.1) is the fourth enzyme of the *de novo* pyrimidine biosynthetic pathway. DHO-DHases of living organisms are classified into two families. Found in some bacteria and some lower eukaryotes, family-1 enzymes are cytosolic proteins. On the other hand, family-2 enzymes are membrane-associated and link to the respiratory chain in mitochondria for their catalytic redox force (Bjornberg et al., 1997). The T. gondii DHO-DHase is exclusively recovered in the particulate fraction of tachyzoite extract and inhibited by respiratory chain inhibitors (Asai et al., 1983a). The predicted amino-acid sequence, with approximate molecular mass of 65 kDa from cloned cDNA, is most similar to family-2 DHO-DHases (Sierra-Pagan and Zimmermann, 2003).

Orotate phosphoribosyltranferase (OPRTase, E.C. 2.4.2.10) is the fifth enzyme of the pathway, and orotidylate decarboxylase (ODCase, E.C. 4.1.1.23) is the sixth. These are cytosolic enzymes, and co-sediment by sucrose gradient centrifugation at a position corresponding to a molecular mass of approximately 70 kDa (Asai *et al.*, 1983a). In some higher eukaryotes, these two enzymes are on the same polypeptide (Jones, 1980). In the apicomplexan parasite *Plasmodium falciparum*, the two enzymes exist as a multienzyme complex containing two subunits each of 33-kDa OPRTase and 38-kDa ODCase (Krungkrai *et al.*, 2005). It is not clear, but *T. gondii* OPRTase and ODCase are probably similar to the *P. falciparum* type.

8.4.2 Pyrimidine salvage biosynthetic pathway

The *T. gondii* tachyzoite pyrimidine salvage pathway is shown in Figure 8.5. *T. gondii* has five enzyme activities that are involved in the salvage of pyrimidine nucleosides and nucleobases: cytidine deaminase (E.C. 3.5.4.5), deoxycytidine deaminase (E.C. 3.5.4.14), uridine phosphorylase (E.C. 2.4.2.3), deoxyuridine phosphorylase (E.C. 2.4.2.23), and uracil phosphoribosyltransferase (UPRTase, E.C. 2.4.2.19) (Iltzsch, 1993). No detailed studies for the properties of pyrimidine nucleoside deaminases have been reported. Uridine phosphorylase has been characterized, and utilizes thymidine poorly (Chaudhary, Ting, Roos and Kim, personal communication).

It appears that all pyrimidine salvage in *T. gondii* proceeds through uracil, which is salvaged to the nucleotide level by UPRTase. However, the salvage pathway is not essential for tachyzoite viability and growth, since UPRTase-deficient mutants exhibit the same growth rate as normal tachyzoites (Donald and Roos, 1995). Although UPRTase may



FIGURE 8.5 *T. gondii* pyrimidine salvage biosynthetic pathways. Solid lines represent activities that were detected in *T. gondii*. Abbreviations used are as follows: Cyd, cytidine; dCyd, deoxycytidine; Urd, uridine; dUrd, deoxyuridine; Ura, uracil. Enzyme activities are numbered as follows; (1) cytidine deaminase, (2) deoxycytidine deaminase, (3) uridine phosphorylase (4) deoxyuridine phosphorylase, (5) uracil phosphoribosyltransferase (UPRTase). It may be highly possible that deaminase activities are catalyzed by one enzyme and that phosphorylase activities are also catalyzed by one enzyme.

not be important for tachyzoite growth, UPRTase is thought to be a possible therapeutic target and is one of the most extensively studied enzymes in T. gondii at molecular level, including crystal structure analysis (Carter et al., 1997; Schumacher et al., 1998, 2002). The T. gondii UPRTase recognizes uracil only, and no other naturally occurring pyrimidine and purine bases (Carter et al., 1997). Without substrates or its activator GTP, recombinant T. gondii UPRTase behaves as a homodimer composed of two identical subunits (27 kDa). The physiologically active UPRTase is tetramer, and this tetrameric structure is stabilized by binding of GTP leading to a more active state (Schumacher et al., 2002). The other native protozoan UPRTases from Crithidia luciliae (Asai et al., 1990) and Giardia intestinalis (Dai et al., 1995) also behave as homodimers.

8.4.3 Purine salvage biosynthetic pathway

Like all parasitic protozoa and many intracellular pathogens, T. gondii is incapable of de novo purine nucleotide biosynthesis. It relies on salvage pathways for purine nucleotides that are essential for parasite growth and survival (Perrotto et al., 1971; Schwartzman and Pfefferkorn, 1982; Krug et al., 1989; Ullman and Carter, 1995). An illustration of the T. gondii tachyzoite purine-salvage pathway is shown in Figure 8.6. T. gondii is thought to have six enzyme activities that are involved in the salvage of purine nucleosides and nucleobases: adenine deaminase (E.C. 3.5.4.2), adenosine deaminase (E.C. 3.5.4.4), inosine phosphorylase (E.C. 2.4.2.1), guanosine phosphorylase (E.C. 2.4.2.15), adenosine kinase (AK, E.C. 2.7.1.20), and hypoxanthine-xanthineguanine phosphoribosyltransferase (HXGPRTase, E.C. 2.4.2.8) (Chaudhary et al., 2004). No detailed studies for the properties of purine nucleoside deaminase, purine base deaminase have been reported. A gene for adenine deaminase but not for adenosine deaminase has been identified in the T. gondii genome (Chaudhary et al., 2004). Purine nucleoside phosphorylase has been characterized as having activity against inosine and guanosine, but not adenosine (Chaudhary et al., 2006).



FIGURE 8.6 *T. gondii* purine salvage biosynthetic pathways. Solid lines represent activities or enzyme genomes that were detected in *T. gondii*; the short dashed line represents activity of adenosine deaminase, the existence of which is highly suspected. Abbreviations used are as follows: Ade, adenine; Ado, adenosine; Ino, inosine; Guo, guanosine; Gua, guanine; Hyp, hypoxanthine; Xan, xanthine. Enzyme activities are numbered as follows; (1) adenine deaminase, (2) adenosine deaminase, (3) inosine phosphorylase, (4) guanosine phosphorylase, (5) adenosine kinase (AK), (6) hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRTase).

AK and HXGPRTase have been extensively studied at the molecular level, including crystal structure analyses (Donald et al., 1996; Schumacher et al., 1996, 2000; Darling et al., 1999; Heroux et al., 1999a, 1999b; White et al., 2000). Neither AK nor HXGPRTase is essential for T. gondii survival, since tachyzoites can survive elimination of either activity alone (Donald et al., 1996; Sullivan et al., 1999; Chaudhary et al., 2004). The generation of a double knockout mutant of both AK and HXGPRTase is impossible, suggesting that T. gondii accommodates only these two routes of purine salvage (Chaudhary et al., 2004). This situation is not common to all apicomplexan parasites, as discussed in Chapter 19. For instance, Cryptosporidium has AK but lacks HXGPRTase (Striepen et al., 2004), and Plasmodium has HXGPRTase (Vasanthakumar et al., 1990) but lacks AK (Chaudhary et al., 2004).

The *T. gondii* AK is a monomeric protein with molecular mass of 39.3 kDa, and shares less than 30 percent sequence identity with the AKs of other organisms (Sullivan *et al.*, 1999). The *T. gondii* AK

shows a strict specificity for adenosine among naturally occurring nucleosides, such as inosine and guanosine (Darling *et al.*, 1999).

Two isozymes of T. gondii HXGPRTase have been identified as the predicted translation products of differentially spliced mRNAs transcribed from a single gene (Donald et al., 1996). The crystal structure of isozyme-I is tetramer, composed of four identical subunits (26.4 kDa) (Schumacher et al., 1996; Heroux et al., 1999a). The subunit of isozyme II possesses an extra 49 amino acids that are inserted 7 amino acids downstream of the N-terminus, and the other sequence of isozyme II is identical to that of isozyme I (Donald et al., 1996). The isozyme I is predominantly cytosolic, whereas the isozyme II localizes to the tachyzoite inner membrane complex, and the extra 49 amino-acid sequence of isozyme II contains a membrane-targeting signal (Chaudhary et al., 2005). The two isozymes form heterotetramers when co-expressed in E. coli (White et al., 2000) or in tachyzoite in vivo (Chaudhary et al., 2005). For the membrane association of enzyme, at least two isozyme-II subunits in the tetramer are necessary (Chaudhary et al., 2005). There is no significant difference in kinetic properties between isozymes, and it is not understood why T. gondii possesses two HXGPRTase isozymes.

The adenosine transporter in T. gondii has been identified, and the presence of additional permeation pathways for other purine nucleosides, purine bases of T. gondii has been suggested (Schwab et al., 1995; De Kong et al., 2003). The gene of T. gondii adenosine transporter is cloned and expressed in Xenopus laevis oocytes (Chiang et al., 1999). Adenosine uptake by the expressed adenosine transporter is inhibited by various nucleosides, nucleoside analogs, hypoxanthine, guanine, and dipyridamole (Chiang et al., 1999), suggesting that one transporter may play a role in all the transportations of nucleosides and nucleobases into the tachyzoite. Recent studies using nitrobenzylthioinosine (an inhibitor of nucleoside transporter in mammalian cells) and various non-physiological beta-L-enantiomers of purine nucleosides demonstrate that the T. gondii adenosine/purine nucleoside transporter(s) lacks stereospecificity and substrate specificity in the transportation of purine nucleosides (Al Safarialani *et al.*, 2003). There is still no consensus regarding how many transport systems for purine nucleosides/nucleobases are present in the tachyzoite.

8.5 NUCLEOSIDE TRIPHOSPHATE HYDROLASE (NTPase)

8.5.1 Distinctive features and uniqueness

The tachyzoite form of *T. gondii* has a novel nucleoside triphosphate hydrolase (NTPase, EC 3.6.1.3), described for the first time in 1983 (Asai *et al.*, 1983b). The properties of the *T. gondii* NTPase, such as substrate specificity and divalent cation requirements, are most similar to those of E(ecto)-type ATPases (Plesner, 1995). E-type ATPases are ubiquitous in eukaryotic cells, and a number of parasitic protozoan E-type ATPases has been reported (see reviews in Plesner, 1995; Meyer-Fernandes, 2002). In the past half-century, there have been several suggestions regarding the function of E-type ATPase.

The physiological function of *T. gondii* NTPase is undoubtedly different from those of ubiquitous common E-type ATPases, for the reasons discussed below. Although its function is not yet understood, *T. gondii* NTPase must play an extraordinary and unique role for tachyzoite replication and survival.

The most striking feature of *T. gondii* NTPase is its great abundance in the tachyzoite cell. The *T. gondii* NTPase is one of the main proteins, and the calculated amount of NTPase protein is about 2–8 percent of the total tachyzoite proteins (Asai *et al.*, 1983b, 1987; Nakaar *et al.*, 1998). In contrast, the common E-type ATPases are in extremely low abundance. Even the most highly purified E-type ATPases ever reported would not be visible as a band in electrophoretic gels (Plesner, 1995).

The next striking feature of *T. gondii* NTPase is that the enzyme seems to be a dormant enzyme under ordinary conditions. Dithiol compounds,

such as dithiothreitol (DTT), are essential for activation of NTPase activity *in vitro* (Asai *et al.*, 1983b). No common E-type ATPases that require activation by DTT have been reported. This feature, with the great abundance of NTPase in the tachyzoite cell, leads to the surprising conclusion that *T. gondii* conceals an enormous potency for ATP hydrolysis. In fact, the specific activity of ATP hydrolysis (U/mg protein), assayed in the presence of DTT, in the whole tachyzoite cell is about 5000 times higher than that of mouse spleen cell (Asai *et al.*, 1986).

The presence of DTT-activated NTPase in the closely related apicomplexan parasite *Neospora caninum* has also been reported (Asai *et al.*, 1998). However, DTT-activated NTPase enzymes have not been identified in other protozoa, including the apicomplexan parasites *Plasmodium berghei* (Asai *et al.*, 1986) and *Eimeria tenella* (Asai *et al.*, 1998). DTT-activated NTPase enzymes are thought to be restricted to *T. gondii* and *N. caninum*.

Another critical difference between the T. gondii NTPase and ubiquitous common E-type ATPases is that T. gondii NTPase seems to fulfill its function from outside of the tachyzoite cell. Tachyzoite replication occurs in the host cell within the parasitophorous vacuole. During formation of the parasitophorous vacuole, the tachyzoite secretes NTPase into the vacuole from the dense granule one of the secretory organelles of the tachyzoite (Bermudes et al., 1994; Sibley et al., 1994). The secreted soluble NTPase gradually associates with the intravacuolar network which is constructed within the vacuole, and it changes to a membraneassociated insoluble enzyme (Carruthers and Sibley, 1997). The ubiquitous common E-type ATPases, including other protozoan E-type enzymes, are not secreted from the cells (Plesner, 1995; Meyer-Fernandes, 2002). An illustration of NTPase in the tachyzoite-infected cell is shown in Figure 8.7.

8.5.2 NTPase isoforms and their molecular properties

The type I strains of *T. gondii*, which are acutely virulent in mice (Sibley and Boothroyd, 1992), contain two isoforms of NTPase (Bermudes *et al.*,



FIGURE 8.7 Behavior of NTPase in the tachyzoite-infected cell. NTPase is secreted from the dense granule of tachyzoite into the parasitophorous vacuolar space and associates to the intravacuolar network. Then, NTPase activity may be regulated by oxido-reduction change in its molecule affected by dithiol compound or unknown dithiol-disulfide oxidoreductase within the parasitophorous vacuole. Abbreviations used are as follows: DG, dense granule; PVM, parasitophorous vacuole membrane; IVN, intravacuolar network; N, nucleus.

1994; Asai et al., 1995). One of the isoforms (NTPase-I in Asai et al., 1995; NTP3 in Bermudes et al., 1994) preferentially hydrolyzes triphosphate nucleosides, while another isoform (NTPase-II in Asai et al., 1995; NTP1 in Bermudes et al., 1994) hydrolyzes tri- and diphosphate nucleosides at approximately equal rates. The NTPase-I isoform appears to be present only in the type I virulent strains, while NTPase-II is universally present in all T. gondii strains (Asai et al., 1995). Although the presence of NTPase-I seems to be one of the primary factors of virulence in mice, no direct evidence for a role in virulence has been proven. Neospora caninum, which is avirulent in mice, has only NTPase-I type enzyme, and no NTPase-II type enzyme has been detected (Asai et al., 1998).

The complete cDNAs for NTPase-I and NTPase-II encode predicted open reading frames of identical size that differ in 16 of 628 amino acids between the two isoforms (Asai *et al.*, 1995). The molecular mass of native NTPases are approximately 260 kDa, composed of four identical subunits with predicted molecular mass of 67 kDa (Asai *et al.*, 1995). Both isoforms of the NTPase contain an N-terminal hydrophobic signal peptide (25 amino acids) that is absent in native NTPase purified from the tachyzoites (Asai *et al.*, 1995). It is supposed that this signal peptide serves as a signal for transport of NTPase to the dense granule. Other dense-granule proteins have similar N-terminal hydrophobic signal peptides (Cesbron-Delauw *et al.*, 1996).

As mentioned above, the primary difference between these NTPase isoforms lies in their ability to hydrolyze nucleoside triphosphate versus diphosphate substrates. While NTPase-II hydrolyzes ATP to ADP and ADP to AMP at almost the same rate, both native and recombinant NTPase-Is hydrolyze ADP to AMP at a much slower rate – less than 1 percent of the rate for ATP (Asai *et al.*, 1995, 1998). This suggests that *T. gondii* NTPase should

be classified as NTP diphosphohydrolase (apyrase, EC 3.6.1.5), a new gene family of E-type ATPases. *T. gondii* NTPase has homology to apyrase, and an antibody against *T. gondii* NTPase recognizes apyrases of potato and *Schistosoma mansoni* (Vasconcelos *et al.*, 1996), and *Trypanosoma cruzi* (Fietto *et al.*, 2004). The abundant DTT-activated NTPase in *N. caninum* has no apyrase activity, and its substrate specificity is restricted to nucleoside triphosphate (Asai *et al.*, 1998).

8.5.3 Possible physiological function for NTPase

Protozoan parasites are purine auxotrophs (Berens et al., 1995). It has been speculated that the NTPases of T. gondii are involved in the salvage of purine nucleosides from the host cell (Bermudes et al., 1994). The T. gondii NTPase is secreted from the tachyzoite into the parasitophorous vacuole; therefore it contains a higher potency of hydrolyzing ATP to ADP and AMP (Figure 8.7). As mentioned earlier, purines are salvaged through adenosine kinase or HXGPRTase. However, the tachyzoite lacks an ecto-5'-nucleotidase activity that would dephosphorylate AMP to adenosine, the substrate of adenosine kinase (Ngo et al., 2000). Furthermore, it is clear that the primary role of the NTPase of N. caninum tachyzoites is not likely to involve purine acquisition, since that parasite lacks enzymatic activities for progressively cleaving nucleotides to their monophosphate form (Asai et al., 1998). It is therefore doubtful whether the T. gondii NTPases are involved in purine salvage.

Exposure of the tachyzoites to DTT can activate egress of previously non-motile intravacuolar tachyzoites within 60 seconds with a concurrent Ca²⁺ flux (Stommel *et al.*, 1997). Furthermore, *T. gondii* NTPase is found largely in an inactive oxidized form in the parasitophorous vacuole, and is readily activated by DTT. This leads to subsequent rapid depletion of host ATP and egress of tachyzoites (Silverman *et al.*, 1998). Whether or not the *T. gondii* NTPase is involved in naturally occurring tachyzoite egress from host cells, these phenomena are quite suggestive for the physiological function of *T. gondii* NTPase.

Since DTT is not present in nature, thioredoxin, the most abundant cellular-reducing dithiol catalyst, was tested as an activator. The reduced form of thioredoxin can activate NTPase, and the oxidized form of thioredoxin has a reversible effect on NTPase activity in vitro (Asai and Kim, 1987). This suggests that thioredoxin may regulate NTPase activity in vivo. However, host-cell thioredoxin may be excluded from the parasitophorous vacuole by the parasitophorous vacuolar membrane (Schwab et al., 1994). Although glutathione itself has no activation effect on NTPase in vitro (Asai et al., 1983b), it is reported that glutathione promoters activate a Ca2+ flux and decrease ATP levels in tachyzoite-infected fibroblasts (Stommel et al., 2001). These investigators further postulate that the NTPase is activated by glutaredoxin, a small protein with an active center disulfide. Glutaredoxin may be reduced by glutathione, leading ultimately to tachyzoite egress. Finally, the tachyzoites control their egress by secreting glutaredoxin from their bodies into the parasitophorous vacuole for activation of NTPase (Stommel et al., 2001). This model requires further testing, but it is possible that NTPase may be activated by unknown dithiol compound derived from the host cell or tachyzoites. Alternatively, NTPase activity may be regulated by an unknown protein disulfide oxidoreductase within the vacuole.

There is still no consensus for the physiological function of *T. gondii* NTPase. Despite this, it is thought that the *T. gondii* NTPase plays an extremely important function in tachyzoite replication and survival, since the enzyme is expressed mainly in tachyzoite form (Ferguson *et al.*, 1999). Attempts to disrupt the genes have failed, and antisense depletion studies suggest that NTPase is essential for parasite replication (Nakaar *et al.*, 1999).

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The Apicoplast and Mitochondrion of *Toxoplasma gondii*

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9.1 Introduction9.2 The apicoplast9.3 The mitochondrion

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9.1 INTRODUCTION

The hallmark of a eukaryotic cell is the division of the cell contents into specialized membranebounded compartments. These organelles provide multiple benefits to the cell, including protecting the rest of the cell from dangerous reaction products, generating gradients that can be exploited for biological processes, and separating potentially interfering pathways.

The partitioning of the eukaryotic cell led Wallin to suggest, in the 1920s, that it is a collection of symbiotic microorganisms (Wallin, 1927). Of the numerous organelles in eukaryotic cells, two provide evidence of endosymbiosis since they contain small genomes and are bounded by double instead of single membranes: the mitochondrion and the chloroplast (or, more generically, plastids). Their genomes encode key proteins needed for the specialized function of these organelles, including some components of a separate translation system, and a variable phalanx of other genes. By 1970, these oddities spurred Margulis to propose the endosymbiont theory (Margulis, 1970), which postulates that the presentday eukaryote originated from multiple interacting organisms and, more specifically, that these organelles are the remnants of engulfed prokaryotic cells. Initially viewed as outlandish, today this idea is firmly entrenched in biological doctrine. Along with the endosymbiont theory, students learn that the mitochondrion is the powerhouse of the cell and that photosynthesis occurs in chloroplasts, but generally know little regarding the other vital metabolic activities performed by these organelles.

In the origins of these organelles lie new possibilities for intervention in diseases caused by apicomplexan parasites, including birth defects, blindness, and encephalitis due to *T. gondii*, malaria due to *Plasmodium* species, and numerous veterinary diseases. The intense interest in the endosymbiont organelles of apicomplexans lies in the realization that these parasites have not one but two extrachromosomal DNAs, each residing in its own organelle. The single mitochondrion has a minimal genome with unique rRNA genes and presumably unique ribosomes. The second organelle is the apicoplast (apicomplexan plastid), a leftover from a photosynthetic past. There is only one apicoplast per cell, and the genome it contains is a remnant chloroplast DNA. This novel and totally unanticipated finding suggested the presence of plant-like metabolic pathways quite different from those in the vertebrate hosts. Such pathways would provide a host of new chemotherapeutic targets. The unique characteristics of apicomplexan mitochondria also present possibilities for intervention. Studying the origins and activities of the DNA-containing organelles of T. gondii and of the malaria parasite Plasmodium falciparum has paid big dividends already, and there are undoubtedly more to come.

Work on the apicoplast and mitochondrion of T. gondii is inextricably intertwined with studies of these organelles in Plasmodium. Plasmodium falciparum in particular has been intensively studied due to its major health relevance. While the following sections focus on T. gondii, work with other apicomplexans will be noted. For some topics, work on P. falciparum predominates and is described in greater detail to provide a framework for understanding the T. gondii organelles. Despite the many similarities, there are also some surprising differences between T. gondii and P. falciparum. Consequently, predictions of apicoplast or mitochondrial functions based on data from just one organism must be tempered with caution. Topics include a brief history of the identification and origins of the organelles, genome content and gene expression, replication, and trafficking of proteins to the organelles. We also discuss insights from antibiotic sensitivity studies, organelle metabolism, and the potential for further drug development. For those seeking additional detail, there have been several recent reviews on topics in this chapter, most focusing on the apicoplast (Foth and McFadden, 2003; Seeber, 2003; Ralph et al., 2004; Sato and Wilson, 2005; Waller and McFadden, 2005; Wiesner and Seeber, 2005; Wilson, 2005).

9.2 THE APICOPLAST

9.2.1 History

Electron micrographs provided the first indication of the variety of subcellular organelles in T. gondii (Figure 9.1). Some appear quite conventional, such as the ER, the Golgi, and a mitochondrion bounded by two membranes. Others are novel to apicomplexans. Prominent examples include rhoptries, micronemes, dense granules, and the conoid, comprising the apical complex from which the phylum takes its name (described in Chapter 11). The parasites also possess an unusual structure called the inner membrane complex, which lies under the plasma membrane in the apical half of the cell. Among the novel organelles described early in the study of apicomplexans was one surrounded by multiple membranes, but which had no obvious function. It did not even have the same name in different apicomplexans, being known as the spherical body in Plasmodium, the Lamellärer Körper, Hohlzylinder, and Golgi adjunct, and vésicule plurimembranaire in T. gondii, and the grosse Vakuole mit Kräftinger Wandung in Eimeria, to name a few (cited in Siddall, 1992). The multiple membranes provided the first clue to the organelle's unusual identity, but although secondary endosymbiosis had already been invoked as the origin of some chloroplasts (Greenwood et al., 1977; Lee, 1977; Gibbs, 1978; Whatley et al., 1979), the possibility that this might be relevant to the mystery organelle was missed for decades.

The next clue came from studies of parasite genomes. In 1984, Borst and colleagues reported 12-micron and 23-micron circular extrachromosomal DNAs from *T. gondii*, the latter being head-totail dimers of the former (Borst *et al.*, 1984). When spread for electron microscopy, many of the molecules adopted a cruciform structure. These data echoed earlier reports of similarly sized circular extrachromosomal DNAs in *Plasmodium lophurae*



(A) Electron micrograph of a section through intracellular tachyzoite showing the apicoplast (A), and two regions of the elongated mitochondrion (Mi), anterior to the nucleus (N). Bar = 500 nm.

(B) Enlargement of the boxed area from Figure 9.1A, rotated 90° left. Note the multiple membranes bounding the apicoplast, the fingerlike cristae of the mitochondrion, and the Golgi body (G). Bar = 100 nm. Image courtesy of David Ferguson, reproduced with permission from Ferguson *et al.* (2005), *Eukaryot. Cell* **4**, 818, with slight modification (rotation).



(Kilejian, 1975) and *Plasmodium berghei* (Dore *et al.*, 1983). These molecules matched the size range and conformation expected for mitochondrial genomes of unicellular eukaryotes, and so were immediately labeled as such. It was, of course, the logical conclusion. It was also wrong. Who would have suspected these were remnant chloroplast genomes? The only clue was the cruciform structure, typical of chloroplast but not mitochondrial genomes. Certainly no one connected the circular genomes with the multi-membraned organelle.

Research on organelle DNA in apicomplexans was initially pursued exclusively in *Plasmodium*. Williamson, Wilson, and colleagues identified three bands in isopycnic sucrose density gradients of *Plasmodium knowlesi* (Williamson *et al.*, 1985) and *P falciparum* (Gardner *et al.*, 1988) lysates. One was lighter than the main band of nuclear DNA, as is usually the case for mitochondrial genomes. It proved to be a ~35 kb circular DNA (Williamson *et al.*, 1985; Gardner *et al.*, 1988), and when spread for electron microscopy it demonstrated a cruciform structure (Williamson *et al.*, 1985). It thus displayed the characteristics of the previously reported 'mitochondrial' DNAs of apicomplexans.

The lowest band on the P. falciparum gradient, described as 'diffuse and weakly fluorescent' (Feagin et al., 1992), migrated just below the nuclear DNA band and proved to contain tandem repeats of a 6 kb DNA sequence. A similar repeated sequence had been identified in P. yoelii (Vaidya and Arasu, 1987; Suplick et al., 1988; Vaidya et al., 1989) and P. gallinaceum (Aldritt et al., 1989; Joseph, 1990). Upon sequencing, the '6 kb element' in all three species was found to encode classic mitochondrial proteins (apocytochrome b, cob; cytochrome c oxidase subunit I, cox1; and subunit III, cox3), and small, fragmented rRNAs (Aldritt et al., 1989; Vaidya et al., 1989; Joseph, 1990; Feagin, 1992; Feagin et al., 1992) (see section 9.3.2). Despite its minute size, this repeated element has the requisite minimum of genes invariably

expected in mitochondrial genomes (Gillham, 1994). But if the 6 kb element was the mitochondrial genome, what was the 35 kb DNA?

Analysis of the 35kb DNA revealed that it contains a large inverted repeat composed of two copies of a small subunit (SSU) rRNA and large subunit (LSU) rRNA, arranged tail to tail (Gardner et al., 1988, 1991a, 1993). The rRNAs are similar to those of prokaryotes, as expected for both mitochondrial and plastid rRNAs. However, mitochondrial genomes do not typically have duplicated rRNAs, while those of chloroplasts do (Gillham, 1994). Further sequencing showed that the 35 kb DNA also encodes subunits of a eubacterial-like RNA polymerase (Gardner et al., 1991b). This is unequivocally a plastid characteristic; all plastid genomes studied thus far encode and are transcribed by such RNA polymerases. Some plastids import one or more additional RNA polymerases from the cytoplasm. In contrast, almost all mitochondria employ a single subunit RNA polymerase most closely related to phage RNA polymerases (Gray and Lang, 1998). Further analysis from the P. falciparum 35 kb DNA identified components of an organelle translation system, but no photosynthesis-related genes.

Serendipitously, the chloroplast genomes of Epifagus virginiana, a non-green plant, and Astasia longis, a non-green alga, were under analysis at the same time. These are both much reduced in size compared to those of green plants. Plastidencoded genes related to photosynthesis were missing but those needed for expression of the organellar genome were retained, including rRNAs duplicated as an inverted repeat (reviewed in dePamphilis and Palmer, 1989). The parallels with the P. falciparum 35 kb DNA are striking (Figure 9.2). With the accumulating data, the formerly implausible explanation that the 35 kb DNA was derived from chloroplast DNA became increasingly believable (Wilson et al., 1991; Palmer, 1992). It is now well established that apicomplexans have ancestors in the plant kingdom, though many questions remain about the details (see section 9.2.2).

The *T. gondii* plastid genome was sequenced by 1997 (GenBank accession U87145, RefSeq NC-001799). It is strikingly similar to its *P. falciparum*



FIGURE 9.2 Plastid genome structure. Schematic depiction of the plastid genomes of *Nicotiana tabacum* (Nt), a green plant; *Epifagus virginiana* (Ev), a non-green plant, and *P. falciparum* (Pf). Genome sizes are indicated. The salient feature of each genome is an inverted repeat, producing two IR regions per genome (thickened lines). The IRs include rRNAs, tRNAs, and except for *P. falciparum*, some protein-coding genes. IR size for *N. tabacum, E. virginiana*, and *P. falciparum*: 25 kb, 23 kb, and 5 kb, respectively.

counterpart in gene content and organization (see section 9.2.3). Complete apicoplast genome sequences are currently available for three more apicomplexans - the chicken pathogen Eimeria tenella (Cai et al., 2003), and the bovine pathogens Theileria parva (Gardner et al., 2005) and Babesia bovis (O.T. Lau, E.H. Roalson, K.A. Brayton, V.M. Nene, D.P. Knowles, and T.F. McElwain, personal communication) - and partial sequences are available for numerous other species (Lang-Unnasch and Aiello, 1999; Obornik et al., 2002a). Genome sequencing projects by the Pathogen Sequencing Group at The Sanger Institute provide apicoplast gene sequences for additional apicomplexans, including Theileria annulata, Babesia bigemina, and multiple Plasmodium species (http://www.sanger.ac.uk/Projects/Protozoa/). Conservation of gene content and genome organization is strong; the principal difference is that the piroplasms *Babesia* and *Theileria* have only one copy of the rDNA transcription unit

(Gardner *et al.*, 2005; Lau *et al.*, personal communication). This high degree of genome similarity is matched by functional conservation that is largely dependent on the import of nuclearly encoded proteins (see sections 9.2.7 and 9.2.9). This makes *Toxoplasma* an excellent model for study of the apicomplexan plastid.

A lingering question was the subcellular location of the 35 kb genome. It did not co-localize with the mitochondrial genome in sucrose gradients of P. falciparum lysates (Wilson et al., 1992), so a mitochondrial location appeared unlikely. As a remnant chloroplast genome, it should reside in an organelle with more than one bounding membrane. The 35 kb DNA was a genome without a home, and the spherical body was an organelle without a role might they intersect? The well-defined subcellular structure of T. gondii makes it more amenable for cell biological studies than P. falciparum, so it is unsurprising that the localization question was first answered for T. gondii. In the mid-1990s, in situ hybridization studies using probes derived from the T. gondii 35kb DNA showed that this genome resides in an organelle located just apical to the nucleus, the hitherto mysterious multi-membraned organelle (McFadden et al., 1996; Köhler et al., 1997). The P. falciparum 35 kb DNA has also been demonstrated to reside in the corresponding organelle (Wilson et al., 1996). The plethora of names for the organelle have been replaced with a single term: the apicoplast, for apicomplexan plastid. Solving the initial mysteries of the homeless genome and the unexplained organelle has generated a number of fascinating questions: How does a group of obligate intracellular parasites get a plastid? Why has it been maintained in non-photosynthetic organisms? What role does it play for the cell? What possibilities for disease intervention result from the presence of 'plant' genes in protozoan pathogens?

9.2.2 Evolution

Evidence points to a single origin for mitochondria, with an α -proteobacterial ancestor (reviewed in Lang et al., 1999). Despite earlier suggestions that there might be more than one origin of chloroplasts, the most recent data now point to a single endosymbiotic event there as well, with the engulfed organism being a photosynthetic cyanobacterium (reviewed in Gray, 1993; McFadden and van Dooren, 2004). The characteristic double membranes of both mitochondria and chloroplasts are believed to reflect the endosymbiotic event that produced them. As the relationship evolved to symbiosis, many genes in the engulfed partner were transferred to the nucleus, and those gene products essential to organellar function were translated in the cytosol and subsequently imported across two membranes into the organelle. However, a number of organisms have not just two, but three or four bounding membranes around their chloroplasts. To explain this, the endosymbiont hypothesis was expanded to include secondary endosymbiosis (Figure 9.3), in



FIGURE 9.3 Secondary endosymbiosis entails engulfment of an algal cell by a eukaryote (A); loss of genes from the algal cell, with some transferred to the host nucleus (B); and finally loss of the algal nucleus, leaving behind an organelle bounded by four membranes (C). Further steps may reduce the number of bounding membranes to three. P, plastid; M, mitochondrion; N, nucleus. Blue, host cell; green algal cell. This figure is reproduced in color in the color plate section.

which both partners are eukaryotes (reviewed in Archibald and Keeling, 2002). All cases known to date involve engulfment of a photosynthetic alga which already bears a chloroplast. The two inner membranes are believed to be the original chloroplast membranes, the third membrane representing the outer membrane of the algal cell, and the outermost membrane deriving from the host endomembrane system. Loss of one membrane has occurred in some organisms. Euglenoids (three membranes), dinoflagellates (usually three membranes), chlorarachniophyte algae (four membranes), and cryptomonad algae (four membranes with the outermost appearing fused to the host rough ER) are prominent examples of organisms with secondary chloroplasts.

The plethora of other membranous structures in T. gondii and P. falciparum cells has made it challenging to determine the number of membranes surrounding the apicoplast. Numerous electron micrographs show four membranes surrounding the T. gondii apicoplast, and that number is generally accepted (Köhler et al., 1997; McFadden and Roos, 1999). In a recent paper Köhler (2005) has argued, based on transmission electron micrographs, that the T. gondii apicoplast has only two bounding membranes, and is in fact a primary rather than secondary plastid. However, this suggestion is inconsistent with the trafficking mechanism employed to target nuclearly encoded proteins into the apicoplast (see section 9.2.7). Reports for Plasmodium are mixed. Hopkins et al. (1999) described three bounding membranes and numerous adjacent membrane whorls, while McFadden and Roos (1999) reported four membranes, as in T. gondii and several other apicomplexans (reviewed in Waller and McFadden, 2005). Methods of sample preparation for electron microscopy can affect preservation of membrane structure and may explain the differences reported.

Cryptomonad and chlorarachniophyte algae are especially intriguing. Not only are their chloroplasts surrounded by four membranes, but a remnant nucleus, the nucleomorph, nestles between the second and third membranes. Nucleomorph genomes are much reduced, with just three small chromosomes totaling a few hundred kb of DNA (Gilson and McFadden, 1996; Douglas *et al.*, 2001). Despite their small size, these genomes encode components for their own perpetuation and expression plus a few other proteins, including some destined for import to the chloroplast (Gilson and McFadden, 1996, 1997; Zauner *et al.*, 2000). Most of the genes needed for photosynthesis have been shifted to the nucleus, and their protein products are trafficked across all four membranes. In contrast to these algae, apicomplexans have lost the endosymbiont nucleus and photosynthesis-related genes, which they no longer need as intracellular parasites. However, they continue to employ the apicoplast as a synthetic compartment (see section 9.2.9).

The movement of foreign genes into a host's nucleus is called lateral gene transfer (reviewed in Doolittle et al., 2003; Bapteste et al., 2004). As noted above, some transferred genes encode products that are trafficked back to the organelle. Others may provide the host with new synthetic capabilities in the cytosol or other compartments. Still others may be redundant, allowing for the evolution of new functions, while some may simply replace the corresponding endogenous genes. The rapidly increasing pool of genome sequences has greatly accelerated our understanding of lateral gene transfer, showing it to be widespread and of considerable scope. Secondary endosymbiosis permits additional lateral gene transfer, from both the nucleus and the chloroplast of the endosymbiont to the nucleus of the host. Importantly, that means that genes from the endosymbiont's nucleus, which may have no relationship to plastid function, may still provide insights useful for deciphering the history of secondary endosymbionts. Phylogenetic analyses of apicomplexans have considered genes encoded by the apicoplast genome, nuclear genes encoding apicoplast-targeted proteins, and genes not related to apicoplast function.

A recurring question in analyses of apicomplexan evolution is the identity of the secondary endosymbiont: whether it was a red or a green alga. This question has not been resolved, since data supporting both contentions have been gathered (Table 9.1). Initial analyses showed that *rpo*B (Gardner *et al.*, 1994b) and *rpo*C1 (Howe, 1992)

Organism	Gene(s) tested	Conclusion	Basis	Reference
P. falciparum	rpoB	Plant-like/not bacterial	Phylogenetic comparisons	Gardner <i>et al.,</i> 1991b
Sarcocystis, Theileria, and Crythecodinium	16S rRNA	Monophyly of apicomplexans and dinoflagellates	phylogenetic comparisons	Gajadhar <i>et al.,</i> 1991
Multiple	5S and 16S rRNAs	Monophyly of apicomplexans and dinoflagellates	Phylogenetic comparisons	Wolters, 1991
P. falciparum	гроС	Plant-like/not bacterial, probably green	Phylogenetic comparisons	Howe, 1992
P. falciparum	ORF470 (sufB)	Red	Similar gene in red algae	Williamson <i>et al.,</i> 1994
P. falciparum, B. bovis	Apicoplast rRNAs	Red	Phylogenetic comparisons	Egea and Lang- Unnasch, 1995
P. falciparum	Ribosomal proteins	Red	Ribosomal protein super cluster	Wilson and Williamson, 1997
P. falciparum	Multiple apicoplast- encoded genes	Not green	Phylogenetic comparisons; cladistic analysis of gene loss and rearrangement	Blanchard and Hicks, 1999
P. falciparum	Ribosomal proteins	Red	Ribosomal protein super cluster	Stoebe and Kowallik, 1999
P. falciparum, T. gondii	Plastid rRNAs	Red	Supports monophyly of apicomplexans and dinoflagellates	Zhang <i>et al</i> ., 2000
P. falciparum, T. gondii	GAPDH	Red	Loss of cyanobacterial GAPDH shared by apicomplexans and dinoflagellates	Fast <i>et al.</i> , 2001
Multiple	Plastid SSU rRNA	Green	Apicomplexans group closer to green algae	Obornik <i>et al.</i> , 2002b
T. parva, P. falciparum, T. gondii	cox2	Green	Split <i>cox</i> 2 gene by lateral transfer from green algal endosymbiont	Funes <i>et al.</i> , 2002
T. parva, P. falciparum, T. gondii	cox2	Not green	Split <i>cox</i> 2 gene may be independently derived	Waller <i>et al.</i> , 2003
E. tenella	rpoB, rpoC1, rpoC2	Green	Phylogeny	Cai <i>et al.</i> , 2003
T. gondii	Pathway enzymes	Red	Storage polysaccharide biosynthesis similar to that of red algae	Coppin <i>et al.,</i> 2005
B. bovis	Six plastid genes	Green	Phylogeny	Lau <i>et al.</i> , personal communication

TABLE 9.1	Analyses	of apico	plast origin
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were more like chloroplast counterparts than Phylogenetic analyses bacterial ones. of apicoplast-encoded genes, considered singly or in groups, have usually favored a green algal origin (Köhler et al., 1997; Cai et al., 2003; Lau et al., personal communication). SufB (then named ORF470), with >50 percent identity to a red algal chloroplast gene, is an exception (Williamson et al., 1994). A red algal origin is also favored by the arrangement of plastid-encoded ribosomal proteins, a characteristic often considered to discriminate red from green algae (Stoebe and Kowallik, 1999). While predictions from gene arrangement observations are tempered by evidence of significant rearrangement of plastid genomes in non-photosynthetic algae and plants when compared to their close photosynthetic relatives (Stoebe and Kowallik, 1999), gene loss comparisons also favor a red algal ancestry for apicoplasts. The genes rpl3, rpl6, rps5, rps17, clpC, and sufB (then called vcf24) are found in the apicoplast genome and the red algal lineage, but are missing in the green algal lineage (Blanchard and Hicks, 1999). To further complicate the picture, Obornik et al. (2002b) observed that the apicoplast-encoded rpsl2, rpl2, and rpsl4 are most similar to mitochondrial counterparts, arguing for a possible lateral transfer of mitochondrial genes to the plastid genome. Such organelle-toorganelle gene transfers have been previously reported (reviewed in Stern, 1987).

Studies involving a wide variety of genes and organisms have consistently shown that apicomplexans cluster most closely with dinoflagellates and ciliates (Gajadhar et al., 1991; Wolters, 1991); together, these comprise the alveolate clade. Since the secondary plastids of dinoflagellates are widely considered to derive from a red algal lineage, this relationship suggests a red algal lineage for apicomplexan plastids. The nuclearly encoded enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) provides a good example of this reasoning (Fast et al., 2001). Plants, euglenoids, and red and green algae encode two GAPDHs in the nucleus, one eukaryotic and one cyanobacterial in origin. Dinoflagellates and apicomplexans also have two GAPDH genes, but they derive from apparent duplication of the eukaryotic GAPDH. The cyanobacterial GAPDH has been lost. This similarity in gene loss argues for a common ancestry for dinoflagellates and apicomplexans and, by extension, a red algal ancestry for the apicoplast.

The type of storage polysaccharide made by cells and the pathway by which they make it are conserved within large taxonomic groupings, but vary between them. *T. gondii* bradyzoites contain numerous large storage granules containing amylopectin. Apicomplexans and dinoflagellates make their storage polysaccharides via a UDP-glucose pathway, like red algae, whereas plants and green algae use an ADP-glucose system (Coppin *et al.*, 2005). Once again, the evidence tilts toward a red algal ancestor.

In an interesting twist, a mitochondrial gene has been suggested to provide evidence for a green algal apicoplast ancestor (Funes et al., 2002). In apicomplexans and green algae, the mitochondrial cytochrome c oxidase II (cox2) gene has been split into two genes and transferred to the nucleus. Each gene encodes its own targeting sequence, so their protein products appear to be trafficked to the mitochondrion independently. Phylogenetic analysis of the split cox2 genes from T. gondii, P. falciparum, and T. parva showed them clustering most closely with green algae. In contrast, the mitochondrially encoded apocytochrome b gene of the same species clustered with a red alga. The authors favor the interpretation that the split cox2 gene was acquired from the nucleus of a secondarily endosymbiosed green alga, and thus that the plastid also derives from the green algal lineage.

This interpretation has been questioned (Waller *et al.*, 2003). Ciliate *cox2* genes have a 300 aminoacid insert at the disjunction point found in *cox2* of apicomplexans and green algae. Considerable variation is therefore tolerated in that part of the protein, and the split *cox2* sequences could be independently derived – a point bolstered by the lack of conservation of intron sites between the apicomplexan and green algal *cox2* genes. Since most mitochondrially encoded genes lack introns, these were probably acquired after transfer to the nucleus. Inclusion of ciliate *cox2* sequences in phylogenetic analysis puts ciliates between Apicomplexa and green algae. These data argue that the split *cox*2 gene in apicomplexans does not provide strong support for a green algal lineage (Waller *et al.*, 2003).

Claims for a red or green algal lineage for the apicomplexan endosymbiont will not be easily resolved, since multiple lines of evidence can be marshaled on both sides of the debate. Indeed, an exclusive claim for either is not a good fit for the data (Funes *et al.*, 2004). The truth, if it is ever to be attained, will likely require more extensive analyses of non-apicomplexan alveolates.

9.2.3 The apicoplast genome

The number of plastid genomes per cell is contested. Based on nucleic acid hybridization, Köhler et al. (1997) reported 5-6 copies per cell for the T. gondii plastid genome and a single plastid genome per cell for P. falciparum. This is consistent with a prior estimate of 1-2 copies for P. falciparum (Wilson et al., 1993). More recently, Matsuzaki et al. (2001) have re-examined genome copy number by measurement of apicoplastlocalized fluorescence following DAPI staining. Their analysis suggests 25 copies of the apicoplast genome in T. gondii and 15 in P. falciparum - a considerable increase in both cases. Multiple copies of a genome would facilitate repair of mutations by gene conversion, making the higher numbers attractive from a functional point of view, but the matter remains unresolved.

When photosynthetic capability is not needed, the loss of photosynthesis-related genes from the chloroplast genome is profound (reviewed in dePamphilis and Palmer, 1989). Chloroplast genomes average 150–200 kb in size, but those of non-photosynthetic plants are ~70 kb and the *T. gondii* plastid genome is ~35 kb (Figure 9.2). Apicoplast genomes are quite similar, suggesting that much of the reduction in coding capacity happened prior to splitting the apicomplexan lineages. The genomes of *P. falciparum* (Wilson *et al.*, 1996), *T. gondii* (ToxoDB), and *E. tenella* (Cai *et al.*, 2003) are all ~35 kb in size, with a large inverted repeat. The repeat unit consists of small subunit (SSU) and large subunit (LSU) rRNAs encoded head to head and separated by seven tRNA genes. A single tRNA gene is found at the 3' ends of both rRNAs. As noted above, this organization is highly reminiscent of chloroplast genomes. Curiously, the apicoplast genomes of the piroplasms *T. parva* (Gardner *et al.*, 2005) and *B. bovis* (Lau *et al.*, personal communication) lack the repeat structure and have only a single set of rRNAs with no intervening tRNAs. This reduces the genome size to ~22 kb.

The gene content of apicoplast genomes is highly conserved, although the relative location of genes can differ. The similarities of these genomes to each other are greatest within each taxonomic group, as anticipated. On the other hand, Cryptosporidium parvum, which had previously been considered a coccidian (like Toxoplasma and *Eimeria*), lacks an apicoplast genome as well as the organelle and its metabolic pathways (Riordan et al., 1999, 2003; Zhu et al., 2000a). Consideration of C. parvum's genome sequence further supports its considerable differences from other coccidians (Keithly et al., 1997; Zhu et al., 2000b). It is now considered more similar to the gregarines, apicomplexan parasites of insects and mollusks.

Most apicoplast genes encode components needed for expression of the apicoplast genome (Figure 9.4). All of the tRNAs needed for apicoplast protein synthesis are encoded by the apicoplast genome, although they are clustered differently in piroplasms than in coccideans and sporozoans (Wilson et al., 1996; Gardner et al., 2005; Lau et al., personal communication). The protein coding genes include three subunits of a multi-subunit, eubacterial-like RNA polymerase: rpoB, rpoC1, and rpoC2 (Wilson et al., 1996), as is the case for chloroplast genomes. Chloroplast genomes also generally encode rpoA, the remaining major subunit of the RNA polymerases (Gillham, 1994). In P. falciparum, that gene is found in the nuclear genome and has a predicted apicoplast targeting sequence (PF13_0040). Highly scoring sequences can be detected in T. gondii genome by BLAST query (Feagin, unpublished results). The other proteincoding genes include 17 ribosomal proteins, and the translation elongation factor Tu (Wilson et al., 1996).



FIGURE 9.4 Schematic map of the *T. gondii* apicoplast, with genes above or below the line depending on direction of transcription (left to right above the line). Protein coding genes are gray; those marked with asterisks contain internal TGA codons. ORFs of unknown significance are identified with a single lower case letter. Large and small subunit ribosomal protein genes (*rpl* and *rps*) are designated L and S. Other protein-coding genes are named as in the text. Non-coding RNAs are clear. tRNAs are identified by the single letter code above or below their location. LSU rRNA and SSU rRNA, large and small subunit rRNAs, respectively. Data derived from Genbank reference sequence NC_001799.

There are only two apicoplast-encoded genes that have predicted functions other than gene expression: *clp*C and *suf*B (see section 9.2.9). Finally, a handful of unidentified open reading frames are modestly conserved between *T. gondii* and *P. falciparum* (http://www.sas.upenn.edu/~jkissing/toxomap. html). They predict small basic proteins which may be additional ribosomal proteins, some of which are difficult to identify due to limited conservation. This possibility is particularly attractive given that the total number of plastid ribosomal proteins, including those predicted to be imported from the cytosol, is less than that needed for functional ribosomes (Gardner *et al.*, 2002).

9.2.4 Expression of the apicoplast genome

Apicoplast gene expression data derive primarily from *P. falciparum*. Individual transcripts exist for the rRNAs (Gardner *et al.*, 1988, 1991a, 1993) and tRNAs (Preiser *et al.*, 1995), but transcription appears to be polycistronic (Feagin and Drew, 1995; Wilson *et al.*, 1996). Although a precursor/mature RNA relationship has not been definitively shown, primer extension analysis of the plastid rRNAs, corroborated by RNase protection experiments, provided evidence for longer, less abundant RNAs (Gardner *et al.*, 1991a, 1993). Serendipitously, two RNase protection products at the 3' end of the SSU rRNA differed in size by 70 nt, and the larger product later was shown to encompass a tRNA (Gardner *et al.*, 1994a).

In contrast to the rapidly processed rRNA/tRNA transcripts, transcripts from rpoB and rpoC1 remain on long polycistronic transcripts, up to ~12 kb (Feagin and Drew, 1995). Wilson et al. (1996) detected long transcripts for additional P. falciparum apicoplast genes. Their attempts to map the 5' ends of individual genes were often unsuccessful. Together, these points suggest extensive polycistronic transcription, with little accompanying cleavage. Supporting evidence comes from RT-PCR studies showing that transcripts spanning multiple genes can be detected (Wilson et al., 1996). In P. falciparum, the abundance of many RNAs encoded by the apicoplast genome appears to be co-regulated during the erythrocytic cycle. They are most abundant in the schizont phase (Bozdech et al., 2003), which is consistent with earlier data assessing individual transcripts (Feagin and Drew, 1995). The near identity of the expression patterns for adjacent genes is likely strongly affected by polycistronic transcription.

A substantial proportion of the apicoplast genome is devoted to genes for translation, and several lines of indirect evidence point to functional protein synthesis in the organelle. The rRNAs and tRNAs encoded by the P. falciparum apicoplast genome are conventional in size and predicted structure, and have the expected conservation of sequence (Gardner et al., 1991a, 1993, 1994a). Those of T. gondii, while not analyzed in depth, appear similar. Electron micrographs of the P. falciparum (Hopkins et al., 1999) and T. gondii (McFadden et al., 1996) apicoplasts show granular structures of a size consistent with organelle ribosomes. Finally, a number of compounds known to inhibit protein synthesis on eubacterial and organelle ribosomes have negative effects on growth of T. gondii and P. falciparum in culture (see section 9.2.8). Direct evidence for apicoplast protein synthesis is scant. Roy et al. (1999) reported data consistent with apicoplast polysomes, but these have not been pursued. Chaubey et al. (2005) used antibodies raised against heterologously expressed apicoplast EF-Tu to detect its synthesis in P. falciparum. However, there are other elongation factors and related proteins in the P. falciparum genome, and the authors did not report the specificity of their antisera.

The T. gondii apicoplast genome sequence has 33 TGA stop codons embedded in 17 of the 28 predicted protein coding genes. E. tenella (Cai et al., 2003), Neospora caninum (Lang-Unnasch and Aiello, 1999), T. parva (Gardner et al., 2005), and B. bovis (Lau et al., personal communication) have since been found to have numerous TGA stop codons in different positions in apicoplast genes. It has long been known that many mitochondrial genomes decode UGA as a tryptophan codon rather than a stop codon (Gillham, 1994), although this alternate codon usage has not been reported for chloroplast genomes (M.W. Gray, personal communication). The apicoplast genomes appear to be the exception, since many of the TGA codons occur at sites of conserved tryptophans. Use of TGA for tryptophan means that it is unavailable as a stop codon, so T. gondii apicoplast genes end with TAA or TAG. Given the apparent ubiquity of TGA-use for tryptophan in apicoplast genomes, it

is interesting to note that there are no TGA codons in *Plasmodium* apicoplast genes, either internally or terminally. This suggests that *Plasmodium* lost the UGA decoding mechanism, or that it developed after *Plasmodium* branched from other apicomplexans. The tryptophan tRNA encoded by the *T. gondii* plastid genome has an anticodon (CCA) which is incapable of pairing with UGA. Other organisms have developed several mechanisms to overcome this difficulty (reviewed in Gray *et al.*, 2004), including RNA editing. The mechanism employed by *T. gondii* is not yet known.

Some apicoplast genes have internal TAA or TAG stop codons. *T. gondii rpo*C2 has one of each, both located toward the middle of the gene, as well as a TAA within *rps*8. It is unclear whether these are pseudogenes or whether a mechanism exists to allow translation through the internal stop codons. Both the *T. gondii* and *P. falciparum* apicoplast genomes have only a single gene with an intron: a conserved tRNA^{leu} (Wilson *et al.*, 1996; ToxoDB).

9.2.5 Apicoplast genome replication

Although the *T. gondii* and *P falciparum* apicoplast DNAs are strikingly similar in gene content and organization, they differ in an important element of physical structure: ~90 percent of the *P. falciparum* plastid genomes are circular, while the *T. gondii* DNA is in linear tandem arrays (Williamson *et al.*, 2001, 2002). This observation has immediate implications for replication of plastid DNA.

Several lines of evidence point to the linearity of *T. gondii* plastid DNA. The movement of circular molecules in gels is sensitive to electrophoresis conditions, revealed typically by comparing pulsed field gels electrophoresed using different pulse times and intensities. Williamson *et al.* (2001) found that Southern blots of such gels showed only a minor proportion of the *T. gondii* plastid DNA migrating as if circular. Instead, over 90 percent corresponded to a ladder consistent with differing numbers of the unit plastid genome, suggesting a tandem array. Restriction analysis of tandemly repeated sequences and circular molecules shows both can generate a circularly

permuted map, but the ends of a linear array produce additional restriction fragments. This is the result obtained for the *T. gondii* apicoplast genome. Further analysis confirmed that it is linearized in the middle of the inverted repeat and exists largely as tandemly repeated linear arrays. These data strongly suggest that the genome is replicated via a rolling circle mechanism. The progression of DNA polymerase around a circular genome can produce a displaced linear DNA. If it is not cleaved and recircularized, a linear concatamer of genomes is produced.

The proportion of linear to circular molecules is reversed in P. falciparum, with circular DNAs predominating. Using two-dimensional gel analysis to track branch points, Williamson and colleagues showed that most P. falciparum plastid DNA replication initiates at two sites mirrored in the inverted repeat and then proceeds via D-loop intermediates, eventually yielding a circular replicate (Williamson et al., 2002). The remaining DNA appears to replicate via a rolling circle mechanism, as has been predicted for T. gondii. By hybridization of nascent DNA to apicoplast sequences, another group initially identified two, then three, replication initiation sites in each copy of the P. falciparum inverted repeat (Singh et al., 2003, 2005). One is located at the tRNA cluster between the SSU and LSU rRNAs, consistent with prior data (Williamson et al., 2002), and appears to be substantially more active than the other two. Replication in other apicomplexans has not been studied, so it is unclear whether D-loop or rolling circle replication will be most common in the Apicomplexa.

9.2.6 Apicoplast division

To ensure perpetuation of their genomes, mitochondria and plastids must be present at all times, even if inactive. That means that mitochondria and plastids must divide and be partitioned during each cell cycle to provide organelles for the daughter cells. In unicellular eukaryotes, the events of organelle division tend to be coordinated with the cell cycle. This coordination is especially important since only a single mitochondrion and apicoplast are present in apicomplexaus, as opposed to the multiple chloroplasts and mitochondria found in each cell of multicellular eukaryotes.

The absence of a method to synchronize *T. gondii* complicates some aspects of analysis of coordination of events with the cell cycle. For example, it is unclear whether replication of plastid DNA is coordinated with nuclear DNA replication in *T. gondii*, as it is in *P. falciparum* (Shaw *et al.*, 2001; Williamson *et al.*, 2002). However, analysis of events in single *T. gondii* cells has produced insights into organelle division. The use of fluorescent reporters targeted to different cellular structures has expanded the tools available for cell-cycle analysis, permitting experiments to follow the partitioning of the apicoplast into daughter cells.

Division of the apicoplast is coordinated with the cell cycle. In an elegant study of apicoplast division in T. gondii (Striepen et al., 2000), a number of important observations were made (Figure 9.5). In apicomplexans, the centrosome is extranuclear and located close to the apicoplast, which is itself apical to the nucleus. During daughter cell formation, as the duplicated centrosomes move apart, the ends of the apicoplast follow. Apicoplast DNA is localized in a nucleoid and, as the organelle lengthens into a dumbbell and then a U-shape, two nucleoids can be seen, one at each end and each adjacent to a centrosome. Coordinately, the parasite nucleus is repositioned to the basal end of the cell and develops two arms that move toward the centrosomes in the forming daughter cells. Organelle division in many other organisms involves a division ring that constricts around the middle of an elongated organelle until it splits in two. However, genes for the responsible proteins, such as *ftsZ*, have not been detected in apicomplexan genomes. Striepen et al. (2000) instead have posed a controversial hypothesis that centrosome movement elongates the apicoplast and that the newly forming inner membrane complex is responsible for the division.

Matsuzaki *et al.* (2001) present a very different picture. This group analyzed plastid division employing DAPI staining to follow DNA and transmission electron microscopy to assess structural changes during apicoplast division. They describe



FIGURE 9.5 Apicoplast division during endodyogeny. A schematic depiction of endodyogeny is shown.

(A) Cell components are labeled: A, apicoplast; C, centrosome; Co, conoid; IMC, inner membrane complex; N, nucleus; Nu, nucleoid.

(B) Here, the nucleoid and centrosome have both been duplicated and the apicoplast lengthens as the centrosomes and nucleoids move outward.

(C) New conoids have formed and new IMC is developing. The nucleus and apicoplast are beginning to move into the forming daughter cells, both becoming U-shaped. The relative positions of the centrosomes and apicoplast have reversed.

(D) Daughter-cell formation is nearing completion. The organelles will return to the positions shown in (A) as cytokinesis is completed. Steps are as described in Striepen *et al.* (2000).

similar changes to apicoplast shape during division as well as other features: thickening at the ends of the elongating apicoplast, a 'scratched' appearance at its central constriction, and a darkstained structure associated with the constriction. They hypothesize that these may be, respectively, sites for attachments of centriole microtubules, a plastid division ring, and a structure involved in organelle scission. There are clearly many questions remaining to be answered before the mechanism of apicoplast division is resolved.

Apicoplast structure has also been examined in bradyzoites and in sexual-stage T. gondii (Dzierszinski et al., 2004; Ferguson et al., 2005). Tachyzoites are actively replicating stages that are principally responsible for the pathology of toxoplasmosis. Bradyzoites are encysted parasites that are only slowly replicative, but they can switch to the tachyzoite stage. It is bradyzoites that linger in the host and are responsible for recurrence of toxoplasmosis in previously infected individuals. Tachyzoites can be induced to switch to bradyzoites in culture; these share many characteristics with bradyzoites isolated from animals. Using fluorescent reporters localized to the apicoplast, Dzierszinski et al. (2004) found that 10-20 percent of in vitro-induced bradyzoites apparently lack plastids. Both mis-segregation and loss of signal without cell division were observed. In contrast, fluorescent tags showed no loss of mitochondria. Consistent with these observations, the authors noted parts of a mature in vivo bradyzoite cyst did not stain with antibodies to the apicoplast protein ACP. Complementing this observation are data from a serendipitous mis-segregating apicoplast mutant in tachyzoites (He et al., 2001a). Analysis of its phenotype demonstrated that T. gondii without apicoplasts are viable as long as they are within the original vacuole. They are, however, unable to replicate in a new vacuole. Thus, any bradyzoites lacking an apicoplast would not be able to initiate a productive infection upon reactivation.

Ferguson *et al.* (2005) performed a comprehensive examination of the apicoplast *in vivo*, including bradyzoites, and the asexual and sexual forms in cat small intestinal villi. Electron micrographs and immunofluorescence assays were used to examine each stage. For bradyzoites, they report that there is an apicoplast adjacent to each nucleus in mature cysts, in contrast to the findings of Dzierszinski *et al.* (2004). Ferguson *et al.* (2005) attribute this discrepancy to possible differences between *in vivo* and *in vitro* cysts. The life-cycle stages of *T. gondii* that occur in the feline host include the coccidean phase, which has some similarities to the asexual erythrocytic cycle of *Plasmodium*. When resident in villi of the cat small intestine, the plastid has the expected small ovoid shape in trophozoites, but in schizonts it has elongated and developed branches. This is quite similar to changes observed in *P. falciparum* apicoplast morphology in the corresponding stages (Waller *et al.*, 2000). The plastid in *T. gondii* microgametocytes, macrogametocytes, and macrogametes appears condensed and almost globular. In contrast, microgametes lack apicoplasts (Ferguson *et al.*, 2005). This finding implies that the apicoplast is maternally inherited in *T. gondii*, as it is in *P. falciparum* (Vaidya *et al.*, 1993; Creasey *et al.*, 1994).

9.2.7 Protein trafficking to the apicoplast

With the identification of the apicoplast genome, it became apparent that the transcription and translation of its resident genes (including many ribosomal proteins), as well as any apicoplastspecific functions, would require the collaboration of additional proteins. These proteins are encoded in the nucleus, and hence are called nucleus-encoded apicoplast-targeted (NEAT) proteins. A search of the T. gondii EST databases for proteins homologous to those found in chloroplasts led to the identification of several candidates for apicoplast ribosomal proteins, as well as some enzymes involved in fatty acid biosynthesis. Initially two such proteins, acyl carrier protein (ACP) and ribosomal protein S9 (S9), were confirmed to be localized to the apicoplast by microscopic analysis using specific antisera (Waller et al., 1998). These genes, in turn, provided tools for dissecting the manner in which proteins are targeted to the apicoplast.

Sequence analysis clearly showed that these proteins, all of which were predicted to reside within the lumen of the apicoplast, possessed N-terminal extensions as compared to their bacterial orthologs. In eukaryotic organisms, such extensions often contain topogenic information. For example, proteins localized to primary chloroplasts are usually targeted via an N-terminal transit peptide directly from the cytosol. Localization to the secretory system usually involves an N-terminal signal sequence. These presequences are rapidly removed by specific processing enzymes upon import (Bruce, 2001).

Interestingly, in those organisms with secondary chloroplasts, N-terminal extensions appear to contain a signal sequence followed by a transit sequence (Nassoury et al., 2005). This organization is exactly what is observed for both T. gondii and P. falciparum NEAT proteins predicted to reside in the apicoplast stroma (Waller et al., 1998). Using gene fusions, Waller et al. (1998) showed that the N-terminal extension of T. gondii ACP was able to target green fluorescent protein (GFP) to the apicoplast. Furthermore, presequences of P. falciparum predicted NEAT proteins were able to target GFP to the T. gondii apicoplast. These data suggest that at least some mechanisms of targeting are conserved across the Apicomplexa (Waller et al., 1998; Jomaa et al., 1999).

Both the signal and transit regions of the N-terminal extension of NEAT proteins are required to target a reporter to the apicoplast (Figure 9.6); deletion of either region results in mis-targeting. Without a transit sequence, the S9 signal sequence targets GFP for secretion (DeRocher et al., 2000; Yung et al., 2001), indicating that the reporter protein had entered the secretory system. Similar work in *P. falciparum* showed that the N-terminal region of ACP is able to target GFP for secretion (Waller et al., 2000). The signal sequences for these proteins do not appear any different from those of proteins targeted to other destinations in the secretory system. Indeed, in unpublished studies mentioned by Roos et al. (1999), replacing the endogenous signal sequence with one from a heterologous secretory protein did not alter targeting to the apicoplast. Without a signal sequence, the S9 transit peptide directs GFP to the mitochondrion (Figure 9.6), while a cytosolic localization is seen with GFP fusions to the transit peptide of ferredoxin-NADP+ reductase (FNR) in T. gondii (Harb et al., 2004). Similar findings were seen for ACP in malaria parasites (Waller et al., 2000). Taken together, these studies indicate that the first step in protein targeting to the apicoplast lumen is entry into the secretory system.

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FIGURE 9.6 Both domains of the N-terminal extension are required for targeting of NEAT proteins. GFP fusions containing the entire N-terminal extension of ribosomal protein S9 (aa 1-159, S+T-GFP), its signal sequence (aa 1-42, S-GFP) or its transit sequence (aa 33-159-159, T-GFP) were expressed in T. gondii. The left-hand panels show GFP fluorescence, while the righthand panels show DIC images of the same cells, which are residing within a parasitophorous vacuole in host fibroblasts. Co-localization with apicoplast markers (DNA or acetyl CoA carboxylase) demonstrated that the single dot observed upon expression of S+T-GFP corresponds to the apicoplast. Co-localization with mitochondrial markers HSP-60 and mitotracker showed that the T-GFP protein is found in the mitochondrion. S-GFP is found primarily in the parasitophorous vacuole, although some material can be seen within the endomembrane system of the parasite. Image courtesy of Dr Amy DeRocher.

The transit peptides of NEAT proteins vary in length, from about 50 to 200 amino acids (aa), and are very diverse in sequence. Like chloroplast transit peptides, these peptides have few acidic or hydrophobic residues (Foth *et al.*, 2003). In fact, the transit peptide of *T. gondii* S9, when fused to

GFP, allows GFP to be imported into isolated pea chloroplasts (DeRocher et al., 2004). Apicoplast transit peptides have a net positive charge. The T. gondii transit sequences are enriched for serine and threonine, amino acids shown to be important in plant transit peptides (Bruce, 2001). P. falciparum transit peptides are enriched for asparagine and lysine residues ((Foth et al., 2003), and mutational analysis showed that hydroxylated residues are not crucial for targeting (Waller et al., 2000). Sitedirected mutagenesis of the P. falciparum ACP transit peptide indicated that while basic residues at positions 2 and 6 were not essential, an acidic residue at position 2 prevented apicoplast targeting (Foth et al., 2003). A predicted HSP70 binding site in the transit peptide was also found to be important (Foth et al., 2003), suggesting that maintenance of the unfolded structure could be important in apicoplast targeting. Several studies have shown that T. gondii apicoplast transit peptides contain redundant information, since non-overlapping segments can still mediate targeting (DeRocher et al., 2000; Yung et al., 2001, 2003; Harb et al., 2004). Detailed mapping of transit peptide functions of T. gondii FNR suggests that release from the ER, localization to the apicoplast, binding to chaperones, and processing are specified by discrete domains (Harb et al., 2004).

The identification of NEAT proteins and characterization of the bipartite apicoplast targeting sequence has allowed development of bioinformatic models that predict which proteins may be localized to the apicoplast of malaria parasites. The genome of *P. falciparum* is completely assembled, and the essential features of the transit peptide are well-described, allowing the generation of neural network (PATS; Zuegge et al., 2001) and rule-based algorithms (PlasmoAP; Foth and McFadden, 2003) to identify candidate Plasmodium NEAT proteins. Both of these programs are available through a webbased interface at PlasmoDB.org, and PATS can also be found at the Modlab website at the Universität Frankfurt am Main (http://gecco.org.chemie.unifrankfurt.de/pats/pats-index.php). Algorithms for the identification of T. gondii NEAT proteins were not available when this review was prepared; however, with completion of annotation for the

T. gondii genome (ToxoDB 4.0, released July 2006), development of such algorithms will be a high priority.

Processing of chloroplast transit peptides is rapid, such that only the mature protein is seen under steady-state conditions (see, for example, Shanklin et al., 1995). In contrast, both the mature form and the precursor protein containing the transit peptide are observed for NEAT proteins, whether native proteins or artificial gene fusions (Waller et al., 1998; DeRocher et al., 2005). Pulsechase studies indicate that little processing is seen until 45-120 minutes after synthesis for T. gondii and P. falciparum ACP-GFP (van Dooren et al., 2002; DeRocher et al., 2005). Whether this delay (as compared to chloroplast protein processing) reflects the time required for complete import or the relative inefficiency of processing is not vet clear. A P. falciparum putative ortholog of the chloroplast stromal processing peptidase has been identified (van Dooren et al., 2002). The predicted protein includes a bipartite targeting sequence, suggesting that it lies within the apicoplast. A putative T. gondii ortholog can be detected in the gene models of ToxoDB (E values of $\sim 10^{-50}$). A functional apicoplast is required for processing of the transit peptide in T. gondii (He et al., 2001b). However, analysis of the S9 transit peptide indicates that multiple processing steps can occur, the first of which may take place before import is complete (Yung et al., 2001) - a possibility supported by studies of the transit peptide of FNR (Harb et al., 2004).

The path that NEAT proteins follow to go from the ER to the apicoplast stroma is not yet clear, although several models have been proposed (Figure 9.7). One hypothesis is that the apicoplast lies literally within the secretory system, with its outer membrane contiguous with the ER. All proteins would pass it on their way to other destinations and NEAT proteins would be grabbed by the apicoplast by virtue of their transit peptide. They would then be imported through the next three membranes. Another possibility is that proteins move by vesicular trafficking from the ER to apicoplast, either directly or indirectly. In this case,



FIGURE 9.7 Three models for protein targeting to the apicoplast. Model 1 shows direct import from the ER. Model 2 postulates vesicular trafficking through the Golgi. Model 3 proposes vesicular trafficking from the ER, bypassing the Golgi. Organelles indicated are: A, apicoplast; ER endoplasmic reticulum; G, Golgi; and N, nucleus.

the transit peptide would be responsible for packaging the proteins into appropriate vesicles, and specificity of vesicle trafficking would likely be conveyed by an additional component – possibly proteins such as SNAREs at the vesicle and apicoplast surfaces, as occurs for targeting to other destinations in the secretory system (Hong, 2005).

Recent studies have indicated that it is unlikely that T. gondii NEAT proteins that reside in the apicoplast lumen traffic through the Golgi. Exposure of T. gondii to brefeldin A (BFA) or low temperature block (15°C) both inhibit Golgi trafficking but are without effect on protein localization to the apicoplast as judged by deconvolution microscopy (DeRocher et al., 2005). These studies are complicated by the presence of pre-existing marker proteins in the apicoplast. To circumvent this difficulty, DeRocher et al. (2005) used a system that allows regulated exit of proteins from the ER (Figure 9.8). It is based on a tandem repeat of a conditional aggregation domain (CAD) fused to a reporter that bears the necessary topogenic information (Rollins et al., 2000). In the absence of a synthetic ligand, aggregation of the CAD domains blocks trafficking of the fusion protein. The addition of the ligand solubilizes the aggregated protein,



FIGURE 9.8 Apicoplast protein targeting studied by conditional aggregation. A GFP fusion protein bearing the bipartite extension of ribosomal protein S9, plus a tandem array of four CAD domains, was expressed in *T. gondii*. Removal of ligand causes aggregation of the CAD domains, while addition of ligand yields monomerization. When ligand is removed from the stable transfectants, the GFP is detected in the ER. When ligand is added for 4 hours the protein trafficks to the apicoplast. This tracking occurs even in the presence of BFA. GFP was detected using anti-GFP antibodies. DAPI staining reveals the DNA in the parasite nucleus and apicoplast, and in the upper and lower images, a portion of the host cell nucleus is visible (asterisk). Image courtesy of Dr Amy DeRocher.

This figure is reproduced in color in the color plate section.

releasing it for trafficking (Rivera et al., 2000). To study protein targeting to the T. gondii apicoplast, ligand was withdrawn for 2 days from parasites that were stably transfected with an apicoplast-targeted CAD-GFP. This blocked trafficking of the fusion protein, depleting it from the apicoplast during parasite division. Instead, the GFP reporter accumulated in the ER (Figure 9.8). Subsequent addition of ligand released the fusion protein, which rapidly localized to the plastid region (DeRocher et al., 2005). This localization was not blocked by BFA, or by 15°C block of ER to Golgi trafficking. However, a BFA-sensitive step is involved in protein targeting or maturation, since no transit peptide cleavage is observed in pulse-chase experiments in the presence of BFA (DeRocher et al., 2005).

In *P. falciparum*, expression of mRNAs for known NEAT proteins is temporally regulated during the erythrocytic cycle, peaking in the late trophozoite/early schizont stages (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003). When a *P falciparum* NEAT GFP fusion protein was expressed earlier than normal (by driving transcription using a promoter active earlier in the erythrocytic cycle), it trafficked to the parasitophorous vacuole in a BFA-dependent manner and also appeared in the apicoplast at the usual time in the cycle (Cheresh *et al.*, 2002). However, the general significance of this finding is not yet clear.

Little is known about the import apparatus of the apicoplast. The assumption, based on phylogenetic origins and similarity of targeting sequences, is that the apicoplast translocon resembles that of chloroplasts. This translocon is composed of multiple components in the inner (Tic) and outer (Toc) membranes (Jarvis and Soll, 2002; Nassoury *et al.*, 2005). Among the functions provided in the complex are specific binding to the transit sequence, channel formation, energy generation, and chaperone activity. However, other than a putative Tic22 and Toc34 (unpublished work cited in Waller and McFadden, 2005), database searches have not revealed promising candidates for orthologs of the chloroplast Tic and Toc complexes. Presumably, orthologous functions are present but the machinery is substantially diverged with respect to protein sequence. Isolated secondary plastids from the cryptomonad Guillardia theta, described by the authors as having two membranes, were capable of importing proteins bearing transit peptides from nucleomorph-encoded plastid proteins but not those bearing transit peptides from nuclearly encoded proteins (Wastl and Maier, 2000). These data raise the possibility of multiple mechanisms of protein import. Interestingly, the cryptomonad nucleomorph encodes at least two proteins recognizable as functioning in the import pathway for the inner membrane: Tic22 and Iap100 (Douglas et al., 2001).

The above studies concern the localization of soluble matrix proteins to the apicoplast. To date, proteins residing in the apicoplast membrane have not been conclusively identified, much less their route to the plastid. It can be presumed that a substantial membrane proteome is required for the import of proteins and substrates, and the export of products. As noted above, a candidate Tic22 has been identified, and it bears a bipartite targeting sequence. In chloroplasts, Tic22 lies in the intermembrane space (Kouranov et al., 1999). Thus it seems likely that at least some non-lumenal proteins reach the apicoplast via protein targeting sequences related to those already defined for soluble proteins. This mechanism is reminiscent of targeting of proteins to the inner membrane of the chloroplast, which is often mediated by a transit peptide (Silva-Filho et al., 1997; Roth et al., 2004). Other chloroplast proteins lack recognizable transit sequences (Funes et al., 2004), as do some potential apicoplast membrane proteins, such as the T. gondii sugar phosphate transporter (Karnataki, DeRocher, Feagin and Parsons, unpublished results). Certain proteins targeted to the outer membrane of the chloroplast, such as Toc75, have a transit sequence, while others have an N-terminal region resembling a signal sequence (Hofmann and Theg, 2005). It appears likely that a variety of mechanisms are employed for targeting to apicoplast membranes, possibly related to the final destination.

With four bounding membranes, the apicoplast offers multiple options for localization. Membrane composition may differ between them, and may convey some specificity to the targeting of membrane proteins. The lipid composition of chloroplast membranes is very distinct from that of other cellular membranes, with high levels of galactoglycerolipids (Block *et al.*, 1983). Although little is known about apicoplast membranes directly, galactolipids have been identified in *P. falciparum* and *T. gondii* (Marechal *et al.*, 2002; Bisanz *et al.*, 2006).

9.2.8 Drug sensitivities

The potential of the apicoplast as a drug target reflects its algal origin, with many proteins and pathways not shared by the human host. Furthermore, many apicoplast proteins are enzymes, which bind small molecules and hence have a higher possibility of being druggable (Hopkins and Groom, 2002). Indeed, several of the prokaryotic-like features of apicoplast functions can be inhibited by existing compounds. These have been important research tools, and some are important clinically as well. The ability of inhibitors of organellar DNA replication, transcription, and translation to kill T. gondii and P. falciparum indicate that organellar functions are essential. For example, the rifamycin S antibiotics, such as rifampicin and rifabutin, inhibit eubacterial RNA polymerases like those encoded by apicoplast genomes, and are active against both T. gondii and Plasmodium species (Alger et al., 1970; Divo et al., 1985; Strath et al., 1993; Araujo et al., 1994; Olliaro et al., 1994; Pukrittayakamee et al., 1994). Both the A and B subunits of P. falciparum DNA gyrase bear apicoplast targeting sequences (Khor et al., 2005), and inhibitors of DNA gyrase are toxic to T. gondii (Fichera and Roos, 1997).

T. gondii is sensitive to the macrolide antibiotic clindamycin, an inhibitor of prokaryotic-like translation, and a *T. gondii* mutant resistant to clindamycin was cross-resistant to the macrolide antibiotics azithromycin and spiramycin (Pfefferkorn and Borotz, 1994). Two other clindamycin-resistant *T. gondii* mutants were found to have a point mutation in the plastid large subunit rRNA that mapped

close to known clindamycin specificity determinants (Camps *et al.*, 2002). Macrolide interaction sites are known to be restricted to a small region of the ribosome's peptidyl transferase domain, and co-crystallizations of these drugs with ribosomes have shown that they all block the peptide exit tunnel (Hermann, 2005). Consequently, the observed cross-resistance is unsurprising.

Other inhibitors of prokaryotic-like translation include thiostrepton, which acts against P. falciparum (McConkey et al., 1997; Clough et al., 1997; Rogers et al., 1997) but not T. gondii. The differential sensitivity is likely due to an alternate nucleotide at the critical position in the T. gondii apicoplast large subunit rRNA (Clough et al., 1997). The antibiotic actinonin inhibits the peptide deformylase that removes the formyl group from the initiator methionine in eubacterial proteins. It shows some activity against P. falciparum in vitro, likely acting against either or both the apicoplast or mitochondrion (Rohrich et al., 2005). Tetracyclines, which inhibit translation by prokaryotic and prokaryoticlike ribosomes, decrease growth of both T. gondii (Tabbara et al., 1982; Chang et al., 1990, 1991) and

malaria parasites (Tabbara et al., 1982; Geary and Jensen, 1983; Divo et al., 1985). These antibiotics bind to the small subunit of the ribosome (Brodersen et al., 2000; Anokhina et al., 2004), and it has been suggested that, in apicomplexans, the mitochondrial ribosome is the main site of action, based on its effects on mitochondrial functions and pattern of protein synthesis inhibition in T. gondii and P. falciparum (Kiatfuengfoo et al., 1989; Beckers et al., 1995). However, Camps et al. (2002) reported that the kinetics of T. gondii growth inhibition by clindamycin and tetracycline were quite similar, including a delayed-death phenotype (see below). Thus tetracyclines appear to inhibit protein translation in the apicoplast in addition to or instead of the mitochondrion.

Although an effect on the mitochondrion often cannot be ruled out, many of the drugs discussed above clearly affect the apicoplast. They show an interesting effect, called delayed death (Fichera *et al.*, 1995). An example is the case of clindamycin (Figure 9.9A). After drug is added, parasite multiplication continues vigorously within the first vacuole. However, when establishing the second





(A) Kinetics of delayed death. *T. gondii* intracellular tachyzoites were treated with ciprofloxacin (25 μ M, squares) or clindamycin (1 μ M, circles) and their growth was compared to untreated parasites (crosses). The left-hand graph shows the growth within the first vacuole, where no significant effect was seen. After lysis and entry into the next host cell (right-hand graph), the drug-treated parasites die whether or not the drug ciprofloxacin is present (closed squares) or removed (open squares). Image courtesy of M.E. Fichera and D.S. Roos (1997) *Nature* **390**, 407, reprinted by permission from Macmillan Publishers Ltd.

(B) Replication without apicoplast division. *T. gondii* were transiently transfected with ACP-GFP-mROP1 and inoculated into an HFF cell monolayer. This image, taken 48 h after transfection, shows a vacuole with about 64 parasites, only 1 of which contains an apicoplast as shown by the fluorescence of the GFP reporter. Bar = $5 \mu m$. Image courtesy of C.Y. He and D.S. Roos (2001), *EMBO J* **20**, 330, reprinted by permission from *EMBO J*. This figure is reproduced in color in the color plate section.

vacuole, the parasites fail. This effect occurs even if the drug is removed at the second cycle. The delayed-death phenomenon was further explored using an unusual system for generating apicoplastdeficient cells (He et al., 2001a). Transient transfection of construct containing ACP-GFP fused to the rhoptry-targeting sequences of ROP1 was found to interfere with division of the apicoplast. After a series of divisions, the parasitophorous vacuole contained many parasites, but only one with an apicoplast. Within the original vacuole the cells remained healthy whether they contained an apicoplast or not (Figure 9.9B), and all were capable of invading a new host cell. However, only those with an apicoplast could proliferate in the new host. Hence, it appears that some molecules produced directly or indirectly by the apicoplast in the preceding cycle are required in the next round of infection. At least one molecular defect related to delayed death has been identified. The level of apicoplast DNA is dramatically reduced upon clindamycin treatment (Fichera et al., 1995; Fichera and Roos, 1997). Evidently the inability to translate certain proteins encoded by the apicoplast genome prevents the proper replication of the DNA, either directly or, more likely, indirectly.

Several of the antibiotics that target apicoplast functions are in use clinically. The antibiotic clindamycin is used as a second-line drug for toxoplasmosis, while spiramycin is chosen for toxoplasmosis in pregnancy. However, some of the agents, while effective *in vitro*, are not sufficiently antiparasitic for use in treatment of disease. A recent review has extensively discussed the apicoplast as a drug target in apicomplexan diseases (Wiesner and Seeber, 2005), and the topic is also covered in Chapter 19 of this volume.

9.2.9 Apicoplast metabolism

What functions are potentially localized to the apicoplast? As noted above, the apicoplast genome does not encode any proteins that provide clues to its metabolic role. Most of the genes encode proteins required for transcription or translation. A potential ortholog of ClpC (a chaperone component of the Tic complex) is encoded by the 35-kb element.

One gene (originally named ORF470, then ycf24, and now sufB) was first thought to encode an ABC transporter, but has more recently been suggested to be involved in iron metabolism (Rangachari et al., 2002; Wilson et al., 2003). Hence, most of the clues to apicoplast function come from the predicted NEAT proteins. Using PlasmoAP, Ralph et al. (2004) identified more than 500 P. falciparum proteins as potentially targeted to the apicoplast. The identification of the constellation of potential apicoplast proteins provides clues as to why the organelle is essential. Nonetheless, about 70 percent of the predicted NEAT proteins are of unknown function, being annotated as hypothetical proteins (Ralph et al., 2004). Further analysis will be required to determine which of these predicted proteins are expressed and are targeted to the apicoplast.

As yet, the *P. falciparum* data have not been systematically compared with the *T. gondii* predicted proteins. Nonetheless, like the genetic systems in the apicoplast, most NEAT proteins are likely to be cyanobacterial in origin, with an evolutionary history that does not overlap with that of the human host. Therefore, many of these pathways would, if essential, provide excellent potential drug targets. Some of the functions mapped to the apicoplast have already been experimentally demonstrated to be essential through the use of inhibitors, or have a high probability of being essential given their biological importance. A summary of metabolic pathways predicted or demonstrated to reside in the apicoplast is given in Figure 9.10.

Acyl carrier protein, part of the type II fatty acid biosynthesis pathway, was one of the first proteins recognized to be targeted to the apicoplast (Waller *et al.*, 1998). The type II pathway, typical of plants and algae, is mediated by a series of individual enzymes, all of which have been identified in the *P. falciparum* and *T. gondii* genomes. In contrast, the type I pathway used by mammals is mediated by a single, large multidomain molecule. Scans of the *T. gondii* genome database indicate that the parasites also contain a gene encoding a protein related to this multidomain molecule (cited as Crawford and Roos, unpublished, in Wiesner and Seeber, 2005). While it is clear that *T. gondii* can take up fatty acids from the host cell and perhaps



FIGURE 9.10 Metabolism of the apicoplast and mitochondrion. Several metabolic pathways have been associated with the apicoplast (depicted with four membranes) and the mitochondrion (depicted with two membranes), based on predicted targeting sequences. For some, localization of the relevant enzymes to the organelle has been experimentally confirmed. Two distinct pathways for synthesis of Fe–S cluster biosynthesis appear to be present. In contrast, heme biosynthesis requires the collaboration of enzymes in the two organelles as well as the cytosol.

utilize the type I pathway to synthesize additional fatty acids, new data indicate that the type II pathway is essential (Bisanz et al., 2006). Thiolactomycin, which inhibits the type II pathway for fatty acid synthesis in bacteria, can inhibit growth of both T. gondii and P. falciparum (Waller et al., 1998; unpublished data cited in McFadden and Roos, 1999). Similarly, triclosan, which inhibits enoyl-ACP-CoA reductase (which mediates the last step in the fatty acid synthesis cycle) kills both T. gondii and P. falciparum (McLeod et al., 2001; Perozzo et al., 2002). Interestingly, P. falciparum lacks the type I pathway. Conversely, Cryptosporidium parvum has only the type I pathway (Zhu et al., 2004). This observation correlates with the apparent lack of an apicoplast in C. parvum (Riordan et al., 1999; Zhu et al., 2000a; Keithly et al., 2005), and the absence

of apicoplast-encoded and NEAT genes in *C. parvum* (Abrahamsen *et al.*, 2004) and *Cryptosporidium hominis* (Xu *et al.*, 2004), as well as the lack of *Cryptosporidium* genes corresponding to known apicoplast pathways.

Acetyl CoA is required for fatty acid synthesis, and is generated from pyruvate by the action of the multienzyme complex pyruvate dehydrogenase. Recent studies show that T. gondii and P. falciparum possess genes encoding the subunits for an apicoplast pyruvate dehydrogenase, even though they lack a mitochondrial form (Foth et al., 2005; McMillan et al., 2005). The first step in production of fatty acids from acetyl CoA is catalyzed by acetyl CoA carboxylase (ACC). Plastid ACCs are inhibited by aryloxyphenoxypropionate herbicides, and T. gondii growth is inhibited by these compounds (Zuther et al., 1999). There are two T. gondii ACCs; one is cytosolic and the other has been identified and localized to the apicoplast (Jelenska et al., 2001). Recombinant ACC corresponding to the apicoplastlocalized protein has been confirmed as a target of aryloxyphenoxypropionates (Jelenska et al., 2002).

Isoprenoid synthesis is another pathway localized to the apicoplast. The enzymes mediating this pathway are distinct from those of the mevalonate pathway for isoprenoid synthesis found in the human host. The parasite pathway, called the DOXP pathway for its early intermediate 1-deoxy-D-xylulose 5' phosphate, has been more thoroughly studied in *P. falciparum*. Fosmidomycin, an antibiotic which inhibits DOXP reductase, is toxic to the malaria parasite (Jomaa *et al.*, 1999). However, it shows little activity against *T. gondii* despite presence of a *T. gondii* DOXP reductase gene. Resistance could result from specific changes in the target or lack of bioavailability.

How do the substrates for these pathways enter the apicoplast? By analogy with the chloroplast, it is likely that specific carriers are required. The malaria parasite possesses two sugar phosphate transporters, one of which carries an apicoplast targeting sequence, which could mediate the uptake of substrates (such as glyceraldehyde-3-phosphate or phosphoenolpyruvate) into the plastid (Mullin *et al.*, 2006). Our analysis of the *T. gondii* genome at $10\times$ coverage suggests a single sugar phosphate transporter is present (Karnataki *et al.*, unpublished results). It is challenging to predict the specificity of this transporter based on sequence alone, and in some cases plastid sugar phosphate translocators transport multiple substrates, albeit with differing affinities (Knappe *et al.*, 2003).

Accessory systems required for the functions of the above biosynthetic processes also must reside in the apicoplast. A redox system is present, comprised of ferredoxin and its partner ferredoxin-NADP+ reductase (Vollmer et al., 2001). The ferredoxin reductase is most similar to those of non-photosynthetic tissues of plants, which provide reducing equivalents for biosynthetic processes. In the apicoplast, NADPH is a required co-factor for DOXP reductase in isoprenoid synthesis and for the KAR (FabG) enzyme involved in apicoplast fatty acid biosynthesis. Lipovlation is needed for activity of the pyruvate dehydrogenase complex, and T. gondii does possess an apicoplasttargeted lipoic acid synthase (Thomsen-Zieger et al., 2003). Fatty acid biosynthesis requires the action of an acetyl CoA carboxylase, which contains a covalently attached biotin prosthetic group. The biotin group can be visualized with FITC-labeled streptavidin and co-localizes with apicoplast markers, confirming the predicted localization (Jelenska et al., 2001). An enzyme required for biotin ligation is also expected to be apicoplast-localized, and indeed P. falciparum possesses a gene encoding such an enzyme with a predicted apicoplast targeting sequence (Gardner et al., 2002).

Iron–sulfur clusters fulfill a variety of functions in proteins, most notably facilitating electron transfer. Several of the proteins that reside in the apicoplast contain Fe–S clusters, including ferredoxin and the enzymes GcpE (IspG) and LytB (IspH) involved in the last steps of isoprenoid synthesis. Lipoic acid synthase is also an Fe–S protein. Iron– sulfur clusters do not form spontaneously – they require the action of multiple proteins for their efficient formation. Seeber (2002) has recently summarized the data concerning the generation of Fe–S clusters in apicomplexans and contrasted it with those in other species. The series of reactions are highly conserved. The generation of elemental sulfur from cysteine is mediated by the

action of cysteine desulfurase, and its reduction is coupled to the oxidation of iron (brought in by a siderophore) on a specific protein scaffold. Additional proteins are required for the release of Fe-S clusters from the scaffold, and their coordination with other proteins. Ferredoxin is needed for reductive steps, and several additional proteins are also important. The compartmentation of these reactions is likely beneficial due to the generation of reactive intermediates. In this way, damage to other cellular components is avoided. In most eukaryotes, Fe-S cluster synthesis occurs in the mitochondrion. Recent work indicates that the apicoplast is likely to be a site for the synthesis of Fe-S clusters. The apicoplast encoded ORF470 is homologous, if not orthologous, to sufB (Wilson et al., 2003), which lies in the same bacterial operon as other genes involved Fe-S biosynthesis in bacteria (Nachin et al., 2001, 2003). Genes encoding other proteins likely to be involved in Fe-S cluster synthesis have been identified in P. falciparum, and several have been used to predict apicoplast targeting sequences (Ellis et al., 2001).

Heme biosynthesis is another organellar process in eukaryotes, occurring in the mitochondrion of the human host. Once again, compartmentation may serve to protect the cell from potentially damaging intermediates or byproducts. In apicomplexaus, heme biosynthesis is accomplished through a metabolic cooperation between the apicoplast and mitochondrion. All eight enzymes required for the de novo synthesis of heme are present in the genome of P. falciparum (Gardner et al., 2002). The enzyme mediating the first step in the pathway is δ -aminolevulinic acid synthase. It contains a predicted mitochondrial targeting sequence, and, in malaria parasites, GFP fusions with this sequence are targeted to the mitochondrion (Sato and Wilson, 2002; Sato et al., 2004). The next two enzymes in the pathway, porphobilinogen synthase and hydroxymethylbilane synthase, have bipartite leader sequences, and GFP fusions are targeted to the plastid (Sato et al., 2004). Porphobilinogen synthase belongs to the cyanobacterial/plastid clade (Obornik and Green, 2005), as expected for a plastid-targeted protein. However, the same authors note that two enzymes

functioning later in the pathway, porphobilinogen deaminase and uroporphyrinogen decarboxylase, do not cluster within this clade (Obornik and Green, 2005). Nonetheless, the latter has a predicted apicoplast targeting sequence (Sato *et al.*, 2004). Phylogenetic inference indicates that apicomplexan ferrochelatase, the last enzyme in the pathway, evolutionarily clusters with red algal and proteobacterial sequences. Its location has not been experimentally determined, but is proposed to be mitochondrial (Sato *et al.*, 2004). The variety in evolutionary origins of these

9.3 THE MITOCHONDRION

enzymes illustrates the mosaic nature of the

In surprising contradiction to the apicoplast, comparatively little is known about the *Toxoplasma* mitochondrion. This relative neglect in part reflects the novelty effect of the apicoplast, perhaps drawing attention away from the more mundane endosymbiotic organelle in the parasite. An even larger damper on mitochondrial research in *T. gondii* has been the unexpected difficulty of defining the mitochondrial genome in this parasite. However, ultrastructurally, *T. gondii* has a conventional protozoan mitochondrion: a single, double-membraned organelle per cell, with tubular rather than plate-like cristae (Figure 9.1).

9.3.1 Evolution

apicomplexan genome.

Phylogenetic analyses of the apicomplexan mitochondrial genomes have focused on relationships within genera and the association of parasite and host speciation (Rathore *et al.*, 2001; Joy *et al.*, 2003; Jongwutiwes *et al.*, 2005; Mu *et al.*, 2005). Most of these studies have compared *Plasmodium* species, but the increasing number of apicomplexan mitochondrial genome sequences may expand these activities. Still, the limited gene content of these genomes – only three protein genes and fragmented ribosomal RNAs – restricts what questions can be addressed and inferences drawn. Consequently, the deep evolutionary roots of the apicomplexan mitochondrion have been relatively neglected.

9.3.2 The mitochondrial genome

Despite considerable variation in size and gene content among species, all mitochondrial genomes known to date encode apocytochrome b (cob) and cytochrome c oxidase I (cox1). Cytochrome coxidase III (cox3) is nearly invariant, missing from the mitochondrial genome only in some green algae. Mitochondrial genomes also invariably encode eubacterial-like large and small subunit rRNAs (reviewed in Gillham, 1994; Lang et al., 1999; Gray et al., 2004). The T. gondii mitochondrial genome has proven very difficult to identify, due to the presence of *cob* and *cox*1 pseudogenes scattered throughout the nuclear genome (Ossorio et al., 1991). These pseudogene sequences and the neighboring nuclear DNA in T. gondii are flanked by inverted repeats, suggesting a retroposon-like mechanism for insertion and intragenomic proliferation of the mitochondrial sequence (Ossorio et al., 1991). Attempts to differentiate the bona fide mitochondrial genes from nuclear gene copies by first isolating the mitochondrion, then its DNA, have thus far been unsuccessful (S. Tomavo, personal communication). However, other apicomplexan mitochondrial DNAs provided clues for the likely characteristics of the T. gondii mitochondrial DNA.

Apicomplexan mitochondrial genomes are the smallest ever reported, having gone to extremes in reduction of coding capacity. Like the plastid genomes, they are conserved in gene content but not in organization or topology. Only 3 protein coding genes remain in the ~6-kb mitochondrial genomes, compared to 13 in the 16-kb mammalian mitochondrial genomes (reviewed in Feagin, 2000). However, those three are the hallmark genes: *cob*, cox1, and cox3. Apicomplexan mitochondrial genomes also contain short sequences similar to highly conserved portions of eubacterial-like large and small subunit rRNAs (reviewed in Feagin, 2000). Unexpectedly, these sequences are not contiguous but are fragmented and scattered out of order across the mitochondrial genome. Fragmented rRNAs have been identified in a number of organisms
(reviewed in Gray and Schnare, 1990), but the fragments encoded by apicomplexan mtDNAs are by far the smallest and their cumulative size is far less than conventional rRNAs. The *P. falciparum* mitochondrial rRNA genes and the corresponding transcripts have been studied in greatest detail, and comprise a set that is consistent with formation of a functioning minimal rRNA (Feagin *et al.*, 1992, 1997). Small RNAs corresponding to the rRNA genes have also been reported for *P. yoelii* (Suplick *et al.*, 1990) and *T. parva* (Kairo *et al.*, 1994). Dinoflagellates, considered the closest relatives of apicomplexans, also have fragmented mitochondrial rRNAs (Norman, 2001), so this characteristic appears to be quite ancient.

Gene loss to the nucleus is a classic feature of endosymbiont organelles, and recent data show that the mitochondrion can survive loss of its genome, most typically in modified form. The Trichomonas hydrogenosome has long been suggested to be a remnant mitochondrion (reviewed in Dyall and Johnson, 2000). More recently, other 'amitochondriate' protozoa, including Entamoeba histolytica, Giardia intestinalis, and Encephalitozoon cuniculi, have been shown to contain a membranebound compartment that contains mitochondrial markers such as mtHSP70, cpn60, and proteins needed for mitochondrial Fe-S cluster biosynthesis (Tovar et al., 1999; Williams et al., 2002; Regoes et al., 2005; Vavra, 2005). The apicomplexan Cryptosporidium parvum provides another example of organelle retention following organelle genome loss. It lacks both extrachromosomal genomes, but has a multi-membraned organelle. That it is a relict mitochondrion and not an apicoplast became clear when the markers noted above were shown to localize to the organelle (LaGier et al., 2003; Riordan et al., 2003; Slapeta and Keithly, 2004). Gregarines also appear to lack mitochondrial genomes and phylogenetic analyses now place C. parvum near them (Zhu et al., 2000b). Might the peppering of mitochondrial pseudogenes in the T. gondii nucleus reflect a next step in gene loss from an already tiny mitochondrial genome?

The *T. gondii* mitochondrion is clearly functional (see section 9.3.6), but it is also clear that at least some functions do not depend on having a mitochondrial genome. So is there a genome in the *T. gondii* mitochondrion? The answer is likely ves. Matsuzaki et al. (2001) described DAPIstained nucleoids in the T. gondii mitochondrion, and Williamson et al. (2001) noted unspecified mitochondrial sequences in a band from an isopycnic CsCl gradient of T. gondii DNA. As this chapter was being prepared, a preliminary T. gondii mitochondrial genome sequence was assembled as an outgrowth of the parasite genome project, and confirmed by amplification and mapping studies (D. Shanmugam, L. Peixoto, and D.S Roos, personal communication). Its characteristics are quite similar to other apicomplexan mitochondrial genomes, as shown in Figure 9.11. The draft sequence is ~6 kb in length, and includes the three expected protein coding genes, although all are encoded on the same DNA strand (in contrast to the situation in other apicomplexans). The T. gondii mitochondrial genome also includes sequences similar to highly conserved regions of rRNAs (Feagin, unpublished results). The mitochondrial genome is flanked by repeated sequences, and has some internal repeats. This is reminiscent of Theileria mitochondrial DNAs, which are single units with terminal inverted repeats (Hall et al., 1990; Kairo et al., 1994), but contrasts with the tandemly repeated mitochondrial DNAs of Plasmodium species (Vaidya and Arasu, 1987; Joseph et al., 1989). Verification that this DNA resides within the mitochondrion awaits further analysis.

9.3.3 Mitochondrial gene expression

Few studies have addressed expression of mitochondrial genes in *Toxoplasma*. The *cob* gene is the principal exception. COB resides in complex III of the electron transport chain, which is the site of action of atovaquone. This drug is effective against *T. gondii* and *P. falciparum*, but resistance develops rapidly in both parasites (Chiodini *et al.*, 1995; McFadden *et al.*, 2000). To evaluate the mechanism of resistance in *T. gondii*, McFadden *et al.* (2000) cloned and sequenced *cob* cDNA from atovaquone-sensitive and -resistant *T. gondii*.



FIGURE 9.11 Comparisons of apicomplexan mitochondrial genomes. Schematic maps of the mitochondrial genomes of P. falciparum, T. parva, and T. gondii are shown with genes above or below the line depending on direction of transcription (left to right above the line). Protein-coding genes are gray; fragments of large and small subunit rRNAs are shown in light gray and dark gray boxes, respectively. Open boxes indicate small transcripts with characteristics similar to the rRNA fragments; these may correspond to less-conserved regions of rRNA. The P. falciparum mitochondrial genome has been most thoroughly studied, with fewer rRNA fragments identified for T. parva and none as yet for T. gondii. Preliminary analyses show the presence of further rRNA sequences on both these mitochondrial genomes, in small scattered fragments as in *P. falciparum*.

Sequence comparisons showed changes at two positions important for electron transfer, matching data from atovaquone-resistant lines of *Plasmodium* (Srivastava *et al.*, 1997; McFadden *et al.*, 2000). Despite the easy development of resistance (Chiodini *et al.*, 1995), atovaquone has been successfully used for malaria treatment in combination with other antimalarials (Patel and Kain, 2005). Against *Toxoplasma*, in combination with other agents, atovaquone is recommended for prevention or treatment of toxoplasmosis when first line agents have failed or are not tolerated in HIV-infected individuals.

The predicted C-terminus of *T. gondii* COB is hydrophobic and likely buried in the mitochondrial membrane, whereas in other organisms it is usually hydrophilic. Extending past the stop codon, the predicted amino acid sequence would be a good match for *Plasmodium cob* genes (McFadden *et al.*, 2000). It is unclear whether translation of the *cob* mRNA somehow bypasses the stop codon or if *T. gondii* COB simply has an unexpected topology. Regardless of this consideration, the *cob* gene is expressed; *cob* mRNA and COB were detected in both tachyzoites and bradyzoites (McFadden *et al.*, 2000). Tags for both *cob* and *cox*1 are found in recently reported SAGE libraries. Based on relative abundance of SAGE tags, mitochondrial transcripts appear to be more abundant than those derived from the apicoplast genome (Radke *et al.*, 2005).

While transcription from the T. gondii mitochondrial genome has not yet been further investigated, some predictions may reasonably be made. It is common for mitochondrial genomes to be polycistronically transcribed, with the precursor RNA then cleaved to produce individual RNAs (Gillham, 1994). Mitochondrial transcripts have been reported for P. falciparum, P. voelii, P. gallinaceum, and T. parva (reviewed in Feagin, 2000), and data from P. falciparum support polycistronic transcription and post-transcriptional processing (Ji et al., 1996; Gillespie et al., 1999; Rehkopf et al., 2000). It is likely that mitochondrial transcription in T. gondii will have similar characteristics. The three P. falciparum mitochondrial mRNAs are coordinately regulated, and are most abundant in the late trophozoite and schizont stages (Feagin and Drew, 1995). Nuclearly encoded mitochondrial proteins also show a general up-regulation in trophozoite and schizont stages (Bozdech et al., 2003; Le Roch et al., 2003).

As yet, mitochondrial protein synthesis in *T. gondii* remains largely unexplored. Given the content and expression of mitochondrial genomes in other apicomplexans, it is likely that rRNAs will be encoded as small fragments. Ribosomal proteins and tRNAs will need to be imported. Indeed, several tRNAs have already been shown to be imported into the *T. gondii* mitochondrion (Esseiva *et al.*, 2004). Mitochondrial protein synthesis, like that of the plastid, is likely to be sensitive to inhibitors of eubacterial and organelle ribosomes. Analysis of such drug sensitivities are discussed in section 9.2.8.

9.3.4 Mitochondrial genome replication

Mitochondrial genome replication has been examined in *P. falciparum*, and appears to involve recombination as well as replication. Linear concatamers, likely produced by rolling circle replication from a few circular templates, appear to be capable of priming further rounds of replication on the templates and each other (Preiser *et al.*, 1996). Preliminary indications suggest that the *T. gondii* mitochondrial genome is not tandemly repeated. Thus even if it is replicated via the rolling circle method, it is unlikely that complex networks of mitochondrial DNA will be produced, as reported for *P. falciparum* (Preiser *et al.*, 1996).

9.3.5 Protein trafficking to the mitochondrion

As described above, proteins destined for secondary plastids traffic first to the secretory system and then enter the plastid. In contrast, entry into primary endosymbionts - both mitochondria and plastids - typically does not involve the secretory system. Rather, proteins are translated on free cytosolic ribosomes, or on ribosomes studding the organelle's outer membrane, and enter the organelle directly by virtue of translocation machinery. This is a largely understudied topic for apicomplexans, but a few points have been investigated, mostly in concert with plastid targeting studies (see section 9.2.7). Paradoxically, one of the first sequences shown to function as a mitochondrial targeting sequence in T. gondii was derived from the NEAT protein S9. At its N-terminal, T. gondii S9 gene encodes a signal sequence and a predicted plastid targeting sequence, which together are able to target GFP to the apicoplast. However, when only the transit sequence was present, GFP was targeted to the mitochondrion (DeRocher et al., 2000). Plastid transit peptides do resemble mitochondrial targeting sequences. Both types of topogenic signals show considerable flexibility in sequence and are rich in basic amino acids, but mitochondrial targeting sequences generally adopt an amphipathic structure, which is not essential for plastid transit peptides (reviewed in Haucke and Schatz, 1997). Delivery of proteins to the correct location is aided by diversion of *T. gondii* apicoplast proteins into the secretory system. Hence cryptic mitochondrial targeting sequences are not revealed until the signal sequence is cleaved, at which point mitochondrial targeting can no longer occur.

Brydges reported a similarly unexpected result when assessing the subcellular location of a T. gondii iron superoxide dismutase (FeSOD2) (Brydges and Carruthers, 2003). This gene has a predicted apicoplast targeting sequence consisting of an N-terminal signal sequence followed by a transit sequence. Unexpectedly, when the targeting sequence was fused to GFP and transfected into T. gondii, it was the mitochondrion and not the apicoplast which fluoresced. Changing a single amino acid in the signal sequence from arginine to alanine was sufficient to divert GFP to the apicoplast (Brydges and Carruthers, 2003). More recently, Soldati and colleagues further investigated targeting of T. gondii FeSOD2, this time looking at the full protein fused to a C-terminal epitope tag instead of just the targeting sequence fused to GFP. This construct localized to both the apicoplast and the mitochondrion. Addition of an ER-retention motif, HDEL, to the epitope-tagged FeSOD2 allowed trafficking to the mitochondrion but restricted targeting to the apicoplast by retention in the ER (D. Soldati, personal communication). These experiments suggest that the context of the N-terminal targeting sequence also affects localization, and point to the need for caution in ascribing cellular localizations on the basis of predicted trafficking sequences and experiments with tagged proteins. They also indicate that dual targeting of nuclearly encoded proteins is a factor in T. gondii organelle function.

Dual targeting allows a gene to do double duty, encoding products that function in more than one subcellular location. Examples of dual-targeted proteins are growing more abundant, especially in *Arabidopsis* (Duchene *et al.*, 2005). Most often, the proteins involved – tRNA synthetases, RNA polymerases, and various enzymes, to name a few – are needed for both mitochondrial and plastid functions (reviewed in Peeters and Small, 2001; Silva-Filho, 2003; Karniely and Pines, 2005). However, there are also examples of dual targeting to mitochondria and peroxisomes (Petrova et al., 2004), to mitochondria and the ER (Levitan et al., 2005), and to chloroplasts and the ER (Kiessling et al., 2004; Levitan et al., 2005), and triple targeting to mitochondria, nuclei, and the cytosol (Martin and Hopper, 1994). In many instances, alternative splicing or use of alternate initiation codons produces sufficient differences to direct the slightly different gene products to different parts of the cell. In other instances, post-translational modifications affect localization (Silva-Filho, 2003). As yet, FeSOD2 is the only T. gondii protein demonstrated to be targeted to multiple organelles. However, there are insufficient tRNA synthetases and ribosomal proteins encoded by the P. falciparum genome for each of the three translation compartments - cytosol, apicoplast, and mitochondrion - to have an exclusive set of genes of the necessary components (Gardner et al., 2002). Initial analyses of the T. gondii genome indicate low numbers for some of the corresponding genes (D. Soldati, personal communication), and it is likely that dual targeting will be a factor for many apicomplexans.

Comparatively little progress has been made concerning the identification of components of the T. gondii mitochondrial import system. The T. gondii genome encodes orthologs of at least four putative mitochondrial inner membrane translocases, Tim17, Tim22, Tim23, Tim44, and possibly Tim9. However, none of the corresponding outer membrane translocases have been identified in T. gondii or other apicomplexans. Only cpn60 (HSP60), a classical mitochondrial chaperone involved in import, has been characterized (Toursel et al., 2000). The HSP60 mRNA is alternatively spliced and both of the mRNAs are more abundant in bradyzoites than in tachyzoites, as is the HSP60 protein. Immunofluorescence analysis with anti-T. gondii HSP60 antibodies confirms mitochondrial localization in tachyzoites but curiously, early in the bradyzoite to tachyzoite conversion, the same antibodies detect two unknown bodies within or below the nucleus. This transitory localization is

lost by 18 hours, and its significance is unknown (Toursel *et al.*, 2000).

9.3.6 Energy metabolism

The mitochondrion is commonly described in introductory biology classes as the powerhouse of the cell. It is the site of the tricarboxylic acid cycle and the electron transport chain, which act in concert to efficiently convert the products of glucose catabolism to ATP. The other functions of the organelle often get little mention. However, while mitochondrial respiration is key for aerobically respiring cells, many parasite cells are found in environments with low oxygen tension and rely at least partly on alternate means for energy generation. This can result in modification of the energy-generating pathways by loss or replacement of some enzymes or steps. Evaluation of the enzyme content of the T. gondii mitochondrion currently waits on the annotation step of the T. gondii genome, and considerable additional effort will be needed to validate predictions and fully assess mitochondrial roles. Some comments can already be made, however. Importantly, the T. gondii mitochondrion can be stained with rhodamine 123, a fluorescent dye which accumulates in organelles that have a high transmembrane potential (Tanabe, 1985). Diaphorase staining is another classical way of marking active mitochondria. Active mitochondrial diaphorase uses NADH or NADPH as substrate to reduce nitroblue tetrazolium, producing a dense, black precipitate. In the presence of NADPH, T. gondii tachyzoites stain darkly while mitochondria in the host cell do not. The reverse is true for NADH (Seeber et al., 1998). These data point to generation of an electrochemical gradient, but the role of the T. gondii mitochondrion in energy generation is contested.

The relative importance of glycolysis and mitochondrial respiration in *T. gondii* energy generation is still under investigation. Tachyzoites have cristate mitochondria, typically associated with mitochondrial respiration, but they rely heavily on glycolysis for energy production. Bradyzoites do as well, but there are differences in enzyme activities between these stages. Activity of the glycolytic enzyme phosphofructokinase is two- to three-fold lower in bradyzoites than in tachyzoites (Denton *et al.*, 1996). Two other glycolytic enzymes, glucose-6-phosphate isomerase and enolase, are upregulated in bradyzoites (Dzierszinski *et al.*, 1999). On the other hand, isocitrate dehydrogenase activity, followed as a Krebs cycle indicator, was low and unchanged between the stages (Denton *et al.*, 1996). Together, these findings suggest that glycolysis is necessary for energy production in both tachyzoites and bradyzoites, although perhaps not to the same degree.

Much of the pyruvate produced by glycolysis is converted to lactate by lactate dehydrogenase. *T. gondii* has two lactate dehydrogenase genes, one expressed in both tachyzoites and bradyzoites while RNA from the other is bradyzoite-specific (Yang and Parmley, 1995, 1997). Bradyzoites have about three times the lactate dehydrogenase activity of tachyzoites (Denton *et al.*, 1996) but it is not known whether this increase is directly attributable to expression of the second isozyme. Denton *et al.* (1996) hypothesize that bradyzoites need lactate dehydrogenase for catabolism of amylopectin, a storage polysaccharide which is abundant in the encysted parasites.

Pyruvate is also usually funneled to the mitochondrion where pyruvate dehydrogenase (PDH) begins its conversion to acetyl CoA, the entry point to the Krebs cycle. However, both T. gondii and P. falciparum have only a single PDH, which resides in the plastid rather than the mitochondrion (Foth et al., 2005; Ralph, 2005). Other data indicate a role for aerobic respiration, especially in extracellular tachyzoites (see below); in the absence of a mitochondrial PDH, what is the source for mitochondrial acetyl CoA? The answer is still unclear, but pyruvate's conversion to acetyl CoA is accomplished by a three-enzyme cycle: PDH, dihydrolipoamide dehydrogenase (LipDH), and dihydrolipoamide S-acetyltransferase. There are two genes for each of the latter two enzymes in P. falciparum, with N-terminal sequences predicted to traffic one of each gene to the apicoplast or mitochondrion (McMillan et al., 2005). The predicted localization has been confirmed for the LipDHs using GFP fusions to the targeting sequences (McMillan *et al.*, 2005).

Multiple lines of evidence point to maintenance of a mitochondrial electron transport chain in T. gondii. The cell permeant dyes rhodamine 123 and MitoTracker (Invitrogen) serve as indicators of mitochondrial respiration, since they fluoresce only in the presence of high transmembrane potential. Extracellular tachyzoites and host mitochondria label brightly with rhodamine 123, while intracellular tachyzoites are not labeled (Tanabe, 1985; Melo et al., 2000). These results were interpreted as indicating that intracellular T. gondii had a non-functioning mitochondrion. However, MitoTracker labels intracellular as well as extracellular tachyzoites (Melo et al., 2000). Also, cytochemical staining for cytochrome c oxidase activity labels mitochondrial cristae in both stages (Seeber et al., 1998; Melo et al., 2000). Mitochondrial activity may decrease following transition from the extracellular to intracellular lifestyle, but these data strongly suggest that mitochondrial respiration continues after invasion of the host cell.

T. gondii mitochondrial biochemistry has been evaluated using extracellular tachyzoites permeabilized with digitonin. Oxygen consumption was measured in the presence of a variety of mitochondrial substrates and inhibitors. Rotenone inhibits complex I of the mitochondrial electron transport chain (NADH-ubiquinone oxidoreductase), thus decreasing electron flow and ultimately reducing oxygen consumption at complex IV. Exposure of T. gondii to rotenone produced little change in oxygen consumption (Vercesi et al., 1998), although the drug, like other mitochondrial inhibitors, stimulates transition to bradyzoites (Tomavo and Boothroyd, 1995). Multiple possibilities could explain this apparent contradiction, including the activity of rotenone at high concentration against other mitochondrial enzymes. In addition, the permeability of the T. gondii mitochondrion to rotenone is unknown. There are conflicting data regarding rotenone sensitivity for other apicomplexans (Ginsburg et al., 1986; Gozar et al., 1992; Uyemura et al., 2000, 2004; Krungkrai et al., 2002). Although the matter remains unsettled, PlasmoDB (Kissinger et al.,

2002) lists a *P. falciparum* NADH-oxidoreductase with a mitochondrial targeting signal and it shows strong similarity to a rotenone-insensitive ortholog in *Solanum tuberosum*. A *T. gondii* gene with strong similarity (E value of 10⁻⁸³) can be identified by BLAST query at ToxoDB.org.

The sensitivity of oxygen consumption to other inhibitors suggests that a typical ubiquinolcytochrome *c* oxidoreductase (complex III) and cytochrome *c* oxidase (complex IV) are present and contribute to generation of an electrochemical gradient (Vercesi *et al.*, 1998). Oligomycin, which specifically targets mitochondrial F_0F_1 ATP synthase, slows oxygen consumption in extracellular tachyzoites. It also slows growth of *T. gondii* in host cells that lack a mitochondrial genome and thus are unable to make a functional F_0F_1 ATP synthase. These data suggest that oligomycin is inhibiting a *T. gondii* mitochondrial ATP synthase (Bohne *et al.*, 1994).

Alternate oxidases have been described in plant, fungal, and protozoan mitochondria, in chloroplasts, and in some bacteria. These shuttle hydrogens to oxygen without passage through complexes III and IV. Cyanide targets complex IV, so detection of cyanide-insensitive respiration in P. falciparum suggested the activity of an alternate oxidase (Murphy et al., 1997). Additionally, salicylhydroxamic acid, which inhibits alternate oxidases, decreases growth of P. falciparum and T. gondii (Murphy and Lang-Unnasch, 1999). Despite these observations, no convincing candidate for an alternate oxidase gene has been identified in the P. falciparum or T. gondii genomes. In contrast, the C. parvum genome includes a gene with strong similarity to alternate oxidases and a mitochondrial targeting sequence (Roberts et al., 2004). Resolution of the question of alternate oxidase utilization in T. gondii awaits further investigation.

While numerous details remain unsettled, it is clear that both glycolysis and mitochondrial respiration are employed by *T. gondii*, possibly with variation between stages. The many resources coming to bear from the sequencing projects and related efforts should have a salutary effect on these questions.

9.3.7 Other metabolism

The importance of oxidative phosphorylation is so great in many cells that other functions of the mitochondrion have sometimes been overlooked (Figure 9.10). Among those functions are formation of Fe–S complexes, heme biosynthesis, redox reactions, and more. Genes for some of these additional mitochondrial functions have been described from *T. gondii*. At the time of preparing this chapter, annotation of the $10 \times T$. *gondii* shotgun genome sequence was in progress, so additional insights into mitochondrial function are expected soon. Data from the *P. falciparum* genome also suggest possible functions for the *T. gondii* mitochondrion, noted below.

Mitochondria, like chloroplasts, rely on Fe–S clusters for key functions. Apicomplexans appear to use two different biosynthetic pathways to produce Fe–S clusters. One resembles that of non-photosynthetic plants and is apicoplast-localized (see section 9.2.9). The other is similar to the pathway found in mitochondria, and some of its genes have predicted mitochondrial targeting sequences (Seeber, 2002).

Heme biosynthesis is normally a mitochondrial activity in cells lacking chloroplasts and a chloroplast activity in those that have them. In apicomplexans, as described in section 9.2.9, the process appears to be shared between the organelles. Based on localization studies with GFP fusion proteins, the heme biosynthetic pathway starts in the mitochondrion, moves to the apicoplast, and returns to the mitochondrion. This calls for passage of reactants between the organelles twice. Both EM and fluorescence microscopy show a zone of proximity between the two organelles (Hopkins *et al.*, 1999). It is possible that this close association assists movement of reactants.

Mitochondria are also known to participate in pyrimidine biosynthesis. In this pathway, the reduction of dihydroorotate to orotate is catalyzed by dihydroorotate dehydrogenase (DHOD), which passes electrons to ubiquinone in complex II of the mitochondrial ETC. As expected, given this relationship, *P. falciparum* DHOD localizes to the mitochondrion (Krungkrai, 1995). *T. gondii* DHOD activity may also be mitochondrially localized, since it is found with particulate matter following subcellular fractionation while other pyrimidine biosynthesis enzymes are in the soluble fraction (Asai *et al.*, 1983). While resident in erythrocytes, *P. falciparum* fills its energy needs by glycolysis, with prodigious production of lactate (reviewed in Sherman, 1979). Pyrimidine biosynthesis has been hypothesized to explain why a functional mitochondrial electron transport chain is still required in these cells (Gutteridge *et al.*, 1979). This may be one of the functions of the *T. gondii* mitochondrion as well.

9.3.8 Mitochondrial function and the tachyzoite to bradyzoite switch

The transition of active tachyzoites to encysted bradyzoites is a bit of a black box, but changes in certain environmental conditions stimulate the transition *in vitro*. Among these are changes in pH, alteration in nutrient accessibility, change in O_2 tension, and exposure to compounds that limit or block mitochondrial function. It is this latter point that is the focus here.

T. gondii tachyzoites transition quickly to *in vitro* bradyzoites following exposure to atovaquone and other mitochondrial inhibitors (Bohne *et al.*, 1994; Tomavo and Boothroyd, 1995). Bradyzoites appear to be less reliant on mitochondrial respiration than are intracellular tachyzoites (reviewed in Gross *et al.*, 1996). It is not clear whether the switch from active growth to an encysted stage reflects a drop in ATP availability, or interference with the mitochondrial redox machinery. In either case, identifying the molecular correlates that connect mitochondrial function to stage conversion provides an avenue to understanding this critical step.

9.4 PERSPECTIVES

The endosymbiotic organelles of apicomplexans have proven much more exciting than imagined years ago. The mitochondrion still searches for delineation of function, but its fragmented rRNAs provide a look at a minimal ribosome. From a multi-membraned organelle of unknown significance, we now have a relict plastid with pathways that are yielding targets for new drugs against the diseases caused by these parasites. Much research on both organelles has previously focused on P. falciparum, for good reasons: the organelle genomes were first identified in P. falciparum; its nuclear genome sequence was completed years earlier than for any other apicomplexan; and the magnitude of morbidity and mortality it causes is dramatically greater than for T. gondii. But now, with many tools in place and more being developed, it is likely (and indeed highly desirable) that there will be a rapid expansion of knowledge about the apicoplast and mitochondrion of T. gondii, with implications for treatment and control for diseases caused by numerous apicomplexans.

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Calcium Storage and Homeostasis in *Toxoplasma gondii*

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10.1 INTRODUCTION

A variety of cellular functions are regulated by fluctuations of the cytosolic concentration of Ca2+. The intracellular Ca2+ concentration [Ca2+]i is maintained at very low levels (of the order of 10⁻⁷ M) compared with that in the extracellular medium (about 10⁻³ M). This low figure of 10⁻⁷ M applies only to the free Ca²⁺ in the cytosol, and it is to this free Ca2+ level that Ca2+-dependent and Ca2+controlled enzymes respond. The total calcium inside the cell is much higher than 10⁻⁷ M, but the bulk of this calcium is either bound to proteins, polyphosphate, membranes, or other cellular constituents, or is sequestered inside intracellular organelles such as mitochondria, endoplasmic reticulum, Golgi apparatus, and nuclei (Irvine, 1986). Stimulation of Ca²⁺ influx across the plasma

membrane is one of the key events in transmembrane signaling. Ca^{2+} storage organelles capable of both high affinity uptake and rapid triggered release of Ca^{2+} are believed to be ubiquitous among eukaryotes (Carafoli, 1987). This chapter discusses the distinct peculiarities of the calcium homeostatic mechanisms in *Toxoplasma gondii*, as well as the methods used in their study.

10.2 FLUORESCENCE METHODS TO STUDY CALCIUM HOMEOSTASIS IN *T. GONDII*

Because of its importance, numerous methods for analyzing the mechanisms of cellular and/or subcellular Ca²⁺ activity have been established. Although each method has some advantages over the others, each also suffers drawbacks. Investigation of $[Ca^{2+}]_i$ was boosted dramatically with the development of fluorescent dyes of high sensitivity by Tsien and colleagues (Grynkiewicz *et al.*, 1985; Tsien, 1989). Many other techniques exist for measuring $[Ca^{2+}]_i$, including ⁴⁵Ca²⁺, microelectrodes, metallochromic indicators, and Ca²⁺-sensitive photoproteins. For example, ⁴⁵Ca²⁺ was used to measure Ca²⁺ release from microsomes of tachyzoites (Chini *et al.*, 2005). However, fluorescence methods have been more widely used in *T. gondii*. Here, the methods used so far for the study of Ca²⁺ in *Toxoplasma* are summarized.

10.2.1 Fluorescent Ca²⁺ indicators

The chemical fluorescent probes most widely used for Ca²⁺ measurements have been developed by Tsien and colleagues (Grynkiewicz et al., 1985). These fluorescent Ca2+ probes can be separated based on whether they are ratiometric versus nonratiometric indicators (single wavelength). Ratiometric indicators exhibit changes in their intensity with changing [Ca2+] and, in addition, the Ca2+-free and Ca2+-bound forms of the indicator have distinct spectra with the maxima of each at different wavelengths. This shift in the spectra is used for ratio calculations of the fluorescence intensities, and it is useful for minimizing the effect of artifacts that are unrelated to changes in [Ca²⁺]. Ratiometric indicators are preferable because the measured signal, being expressed as a ratio value, is insensitive to factors such as dye concentration, bleaching, variations in cell thickness, and uneven distribution throughout the cytoplasm. The most used ratiometric indicators are fura-2 and indo-1, both available from Molecular Probes (Invitrogen). Fura-2 shows a significant shift in its excitation spectra but not in its emission spectra, while a significant shift in the indo-1 emission spectra is observed. For single wavelength indicators like fluo-3 or fluo-4 (also available from Molecular Probes) the fluorescence intensity observed is the only measurement related to [Ca²⁺], and the intensity changes arising from factors unrelated to changes in $[Ca^{2+}]$ (uneven dye loading, dye leakage, photobleaching,

and changes in cell volume) can confound the interpretation of the intensity data.

The commonly used fluorescent indicators for Ca²⁺ are polycarboxylate anions that cannot cross the lipid bilayer, and as a consequence are not cell permeant. The AM (acetomethoxy) ester forms of these indicators, which are uncharged and hydrophobic, have been developed (Tsien, 1981). These forms can cross lipid membranes and gain entry into the interior of cells. This AM group is labile to enzymatic hydrolysis by the cell esterases, and it is removed to allow the carboxyl groups in the indicator to sense Ca2+. This Ca2+-sensitive polycarboxylate form, multiply charged, becomes trapped inside the cell and it accumulates up to hundreds of micromolar concentration (Takahashi et al., 1999). This could cause extra Ca²⁺ buffering and lead to an underestimation of Ca²⁺ transients. In addition, hydrolysis of the AM group leads to the formation of formaldehyde that could be toxic to the cells.

Fura-2-AM loaded cells have been used to study intracellular calcium stores in *T. gondii* (Moreno and Zhong, 1996). In addition, indo-1 was used to analyze the distribution of calcium in *T. gondii* infected cells (Pingret *et al.*, 1996) and the effects of dithiols on infected cells (Stommel *et al.*, 2001). Fluo-4 has been used to detect Ca²⁺ changes induced by motility or host cell attachment (Lovett and Sibley, 2003).

For fura-2 measurements, excitation is set at 340 and 380 nm and emission at 510 nm. The fura-2 fluorescence response to intracellular Ca²⁺ concentration should be calibrated from the ratio of 340/380-nm fluorescence values after subtraction of the background fluorescence of the cells at 340 and 380 nm as described (Grynkiewicz *et al.*, 1985). The $[Ca^{2+}]_i$ is calculated by titration with different concentrations of Ca²⁺–EGTA buffers (Moreno *et al.*, 1992; Vercesi *et al.*, 1993). Concentrations of the ionic species and complexes at equilibrium are calculated by employing an iterative computer program as described previously (Grynkiewicz *et al.*, 1985).

Fluo-3 is one of the most suitable Ca²⁺ indicators for confocal laser scanning microscopy and flow cytometry. The absorption and emission peaks of fluo-3 are 506 and 526 nm, respectively. The fluorescence emission increases with increasing $[Ca^{2+}]$, and fluo-3 has been reported to undergo a 40- to 200-fold increase in fluorescence upon binding Ca²⁺ (Kao, 1994). Fluo-4 is a derivative of fluo-3, which can be used at lower concentrations with better fluorescence signals.

10.2.2 Manipulation of [Ca²⁺]

Calcium buffers and ionophores are frequently used to lower or raise intracellular or extracellular calcium. The purpose of these protocols is to study Ca²⁺-dependent cellular processes (Kao, 1994).

EGTA is highly selective for binding Ca²⁺ over Mg²⁺, and because of this it is the most commonly used Ca²⁺ buffer. However, the Ca²⁺-binding activity by EGTA is very pH-dependent when used at physiological pH. This is because at these pH values EGTA exists primarily as protonated species (H₂EGTA²⁻) (highest pK_as 8.90 and 9.52). Upon binding Ca²⁺, 2 H⁺ will be released, suggesting that this reaction should have very steep pH dependence. In fact, a drop in pH from 7.2 to 7.1 changes the K_d(Ca) of EGTA by a factor of approximately 1.6. Acidification of the medium by high levels of EGTA was proposed to be responsible for the postulated requirement of extracellular Ca2+ for invasion of host cells by tachyzoites (Lovett and Sibley, 2003).

Tsien developed an analog of EGTA in which the methylene links between oxygen and nitrogen atoms were replaced with benzene rings to yield a compound called BAPTA (Tsien, 1980). This compound has a considerable lower pH sensitivity than EGTA at physiological pH values, since its highest pK_as are 5.47 and 6.36 (Tsien, 1980). Because of this, BAPTA is a less troublesome Ca²⁺ buffer to use – although it is significantly more costly than EGTA.

To study the correlation of a biological process with changes in intracellular calcium ($[Ca^{2+}]_i$), it is useful to be able to block the change in $[Ca^{2+}]_i$ with a calcium chelator. The easiest way to introduce extra Ca²⁺ buffering capacity into cells is by loading them with BAPTA/AM, the ester form of BAPTA (Vieira and Moreno, 2000). Cells can be loaded with AM esters of BAPTA and a calcium indicator simultaneously, since the same conditions can be used (Kao, 1994). This BAPTA buffering method has been widely used to understand the role of calcium in microneme secretion by T. gondii (Carruthers and Sibley, 1999), conoid extrusion (Mondragon and Frixione, 1996), gliding motility (Wetzel et al., 2004) and invasion (Vieira and Moreno, 2000). It is important to load the cells with BAPTA analogs unable to chelate Ca2+, as a control that the effect of BAPTA is because of Ca2+ chelation and not because of toxicity. Analogs such as 'half-BAPTA' (Vieira and Moreno, 2000) or D-BAPTA (Saoudi et al., 2004) can be used, although half-BAPTA is not currently available. It is important to know that BAPTA can display side effects. It has a potent microtubule depolymerizing effect, and decreases the ATP pool of the cells (Saoudi et al., 2004). It is also important to provide controls showing that the concentrations of BAPTA-AM used are able to chelate intracellular Ca²⁺ (Vieira and Moreno, 2000).

The ionophores Br-A23187 and ionomycin form lipid-soluble complexes with divalent metal cations and increase the permeability of biological membranes to Ca2+. There are significant differences in the properties of both ionophores that should be considered when using them in an experiment. The ability of Ca²⁺ transport by both ionophores is pH-dependent, and this pHdependence differs (Liu and Hermann, 1978). Transport of Ca²⁺ by Br-A23187 is best at pH 7.5, whereas Ca2+ transport by ionomycin does not reach a maximum until pH 9.5. In addition, ionomycin has better selectivity for Ca²⁺ over Mg²⁺, whereas Br-A23187 shows no preference for one cation over the other (Liu and Hermann, 1978). Both ionophores are inefficient in mediating Ca²⁺ transport at low Ca2+ concentrations. Br-A23187 (or ionomycin) should be used instead of A23187 for fluorescence microscopy, since A23187 is fluorescent.

Figure 10.1 shows a typical tracing with fura-2loaded *T. gondii* tachyzoites in suspension in a buffer containing 1 mM EGTA. Under these conditions, fluorescence changes reflect Ca²⁺ movements from intracellular calcium stores. The addition of



FIGURE 10.1 Effect of ionomycin and nigericin on $[Ca^{2+}]_i$ of tachyzoites. Tachyzoites were loaded with fura 2. Ionomycin (ION; 1 µM) or nigericin (NIG; 1 µM) were added where indicated. Trace a shows the initial addition of ION followed by NIG. Trace b shows the initial addition of NIG followed by ION. Reproduced with permission from Moreno and Zhong (1996), *Biochem. J.* **313**, 655–659.

ionomycin shows a large increase in intracellular calcium, indicating that calcium is released from an intracellular compartment with neutral pH into the cytosol (endoplasmic reticulum). When nigericin is added after ionomycin, a second increase in cytoso-lic Ca²⁺ occurs due to its release from an acidic compartment (acidocalcisomes). Similar results are observed if the order of additions is reversed (Figure 10.1). Nigericin is a potassium/proton exchanger, and because of this property it alkalinizes acidic compartments by allowing protons to be released in exchange for potassium. As a consequence, a postulated proton/calcium exchanger takes protons back and releases Ca²⁺ into the cytosol.

10.3 REGULATION OF [Ca²⁺]_i IN *T. GONDII*

The $[Ca^{2+}]_i$ in tachyzoites is about 70 ± 6 nM when measured in the absence of extracellular Ca^{2+} (with the Ca^{2+} chelator EGTA added to the medium) and 100 ± 9 nM in the presence of 1 mM extracellular Ca²⁺, as detected in fura-2-loaded cells (Moreno and Zhong, 1996). These concentrations are in the range observed in many studies with eukaryotic cells (Grynkiewicz *et al.*, 1985).

10.3.1 Ca²⁺ transport across the plasma membrane

Ca2+ enters eukaryotic cells across the plasma membrane through a number of channels, some of which are under the control of receptors (receptor-operated Ca²⁺ channels), the potential across the plasma membrane (voltage-gated Ca2+ channels), and the content of intracellular Ca2+ stores (store-operated Ca²⁺ channels), whereas others appear to be non-selective leak channels (Tsien and Malinow, 1990; Tsien and Tsien, 1990). There is no direct evidence for receptor-operated or store-operated Ca2+ channels. Although voltagedependent Ca²⁺ channels have been detected in free-living protozoa, no such channels have been reported in T. gondii. However, a sequence with the characteristic of a voltage-dependent calcium channel has been found in the T. gondii genome (20.m03897) (Chen et al., 2006) (http:// orthomcl.cbil.upenn.edu/cgi-bin/ OrthoMclWeb.cgi). The predicted protein has 2247 amino acids and 24 transmembrane domains.

The active export of calcium from eukaryotic cells is accomplished by the action of a Na⁺/Ca²⁺ exchanger and a Ca²⁺-ATPase (PMCA). There is no biochemical evidence for the presence of a Na⁺/Ca²⁺ exchanger in *T. gondii*, although a gene encoding for a protein of 501 amino acids has recently been annotated as a sodium/calcium exchanger (25.m01788) (Chen *et al.*, 2006). However, as occurs with yeast, it is possible that this gene encodes for a Ca²⁺/H⁺ exchanger, since there are no reports of the presence of Na⁺/Ca²⁺ exchangers in early eukaryotes (Pozos *et al.*, 1996).

A PMCA-type Ca²⁺-ATPase (*Tg*A1) has been characterized in these parasites, which is located in the plasma membrane and acidocalcisomes (Luo *et al.*, 2001) (Table 10.1; see also section 10.4.4). Mammalian PMCA Ca²⁺-ATPases are activated by the Ca²⁺ binding protein calmodulin REGULATION OF [Ca²⁺]_i IN T. GONDII

Protein	Name/accession number	Expressed/function confirmed	Reference
Plasma membrane- type Ca ²⁺ -ATPase (PMCA)	TgA1/AF151372	Yes/Yes*	Luo <i>et al.</i> , 2001
Sarcoplasmic- endoplasmic reticulum Ca ²⁺ - ATPase (SERCA)	<i>Tg</i> SERCA1/AAU93917	Yes/Yes*	Nagamune <i>et al.</i> , 2005
Calmodulin (CaM)	TgCaM/CAA69660	Yes/Yes**	Seeber <i>et al.</i> , 1999
Calnexin	583.m05347	No/No	http://orthomcl.cbil.upenn. edu/cgi-bin/OrthoMclWeb.cgi
Phosphatidylinositol- phospholipase C (PI-PLC)	<i>T</i> gPI-PLC/ AAQ75083/AAV70738	Yes/Yes**	Fang <i>et al.</i> , 2005
Sodium/hydrogen exhanger (Na+/H+ exchanger)	<i>T</i> gNHE1/858890	Yes/No	Arrizabalaga <i>et al.</i> , 2004
23 K calcium- binding major antigen precursor	<i>Tg</i> DAG1/A33839	No/No	Cesbron-Delaw <i>et al.</i> , 1989
Protein phosphatase 2B regulatory subunit (calcineurin)	unnamed/AAM97279	No/No	Available at NCBI
Calmodulin-domain protein kinase 1	TgCDPK1/AAG53993	Yes/Yes**	Kieschnick <i>et al.</i> , 2001
Calmodulin-domain protein kinase 2	TgCDPK2/AAG53994	Yes/Yes**	Kieschnick <i>et al.</i> , 2001
Calmodulin-domain- protein kinase	unnamed/CAD32376	No/No	Available at NCBI
Other calmodulin- domain protein kinases annotated	20.m00372; 37.m0003; 38.m00014; 42.m03394; 49.m05692; 541.m00134		http://orthomcl.cbil.upenn. edu/cgi-bin/OrthoMclWeb.cgi

TABLE 10.1 Proteins potentially involved in Ca2+ homeostasis in T. gondii

*Function confirmed by expression in the same parasite or in a heterologous system and correlation found between expression of the enzyme and Ca²⁺ transport.

**Function confirmed by expression and detection of activity.

In addition, several genes corresponding to calcium/calmodulin-dependent protein kinases have been annotated (http://orthomcl.cbil.upenn.edu/cgi-bin/OrthoMclWeb.cgi).

(CaM), and biochemical evidence for CaM stimulation has been reported for a Ca²⁺-ATPase activity from *T. gondii* (Bouchot *et al.*, 2001). However, *Tg*A1 appears to lack a typical CaM-binding domain, which might suggest the presence of a different domain able to bind CaM. A gene for a second possible PMCA has been found in the *T. gondii* genome (44.m02812) (Chen *et al.*, 2006). The deduced amino-acid sequence (1200 aa) contains a secretory signal sequence with a predicted cleavage site between amino acids 40 and 41. In addition, this enzyme shows 45 percent identity with *Tg*A1 (Luo *et al.*, 2001).

10.3.2 Ca²⁺-binding proteins

Once inside the cell, Ca²⁺ can either interact with so-called soluble Ca²⁺-binding proteins, or become sequestered into intracellular organelles. Some of these Ca2+-binding proteins, such as calmodulin (CaM), act as Ca2+ receptors. Other proteins appear to act as Ca2+-storing devices (e.g. calsequestrin, calreticulin families). T. gondii CaM is a small (16 kDa) acidic calcium-binding protein with four calcium-binding sites (EF hands) and a high level of identity (92.5 percent) with human CaM (Seeber et al., 1999) (Table 10.1). By immunofluorescence analysis using monoclonal antibodies reactive against CaM from different species, T. gondii CaM was found in the apical end of released tachyzoites (together with actin and myosin), and also beneath the membrane in intracellular parasites (Pezzella-D'Alessandro et al., 2001). Immunogold electron microscopy using monoclonal antibodies against mammalian CaM confirmed the localization of CaM in the anterior region of tachyzoites, together with actin (Song et al., 2004). The CaM inhibitors calmidazolium and trifluoperazine significantly reduced parasite invasion in vitro and caused changes in the tachyzoites' shape (Pezzella et al., 1997), although, in the case of calmidazolium, this effect could also be due to its ability to increase [Ca²⁺]_i and stimulate microneme secretion (Wetzel et al., 2004).

Two members of the calmodulin-like domain protein kinases, also known as calcium-dependent protein kinases (CDPKs), were detected in *T. gondii* (Kieschnick *et al.*, 2001) (Table 10.1). *Tg*CDPK1 activity is calcium-dependent, but does not require CaM or phospholipids and is inhibited by KT5926 at concentrations that block parasite attachment to host cells. *In vitro*, *Tg*CDPK1 phosphorylated three parasite proteins that migrated identical to three KT5926-sensitive phosphoproteins detected *in vivo*. On the basis of these results, it was proposed that *Tg*CDPK1 has a central role in regulating *T. gondii* motility and host-cell invasion (Kieschnick *et al.*, 2001).

A 23-kDa calcium-binding protein, named P24, was identified in tachyzoites and shown to be a major antigen and component of the dense granules (Cesbron-Delauw *et al.*, 1989) (Table 10.1). No studies have been reported on Ca²⁺-storing proteins, like calreticulin or calsequestrin, in *T. gondii*. However, a calnexin gene has been found in the toxoplasma genome (583.m05347) (Chen *et al.*, 2006) (Table 10.1). In addition, several sequences with similarity to CaMs have been found in the *T. gondii* genome. They are characterized by the presence of EF-hand domains, but their identity and function will require further investigation.

10.4 CALCIUM STORAGE

10.4.1 Endoplasmic reticulum

The largest store of Ca²⁺ in cells is usually found in the endoplasmic reticulum, with local concentration reaching millimolar levels. The endoplasmic reticulum also possesses two independent pathways for calcium influx and efflux. The influx is catalyzed by the very well-known sarco-endoplasmic reticulum Ca²⁺-ATPase (SERCA), which actively translocates two Ca2+ for the hydrolysis of one ATP molecule. Evidence for the presence of a SERCA-type Ca²⁺-ATPase in T. gondii was first provided by experiments using fura-2-loaded tachyzoites in which thapsigargin, a very specific inhibitor of this pump when used at low concentrations (Thastrup et al., 1990), was shown to increase [Ca²⁺], in tachyzoites (Moreno and Zhong, 1996). Molecular evidence for the presence of a SERCA-type Ca²⁺-ATPase in *T. gondii* has been reported recently (Nagamune and Sibley, 2005; Nagume *et al.*, 2005). The gene encoding this pump was able to complement yeast deficient in Ca²⁺ pumps, providing evidence of its function as a Ca²⁺ pump, and the encoded protein has an apparent molecular mass of 120 kDa. Inhibitors of this pump in other cells, such as thapsigargin (Thastrup *et al.*, 1990) or artemisinin (Eckstein-Ludwig *et al.*, 2003), were able to stimulate microneme secretion, a process that relies on elevated $[Ca²⁺]_i$ (see below, under 'Ca²⁺- signaling').

Ca²⁺ release from the endoplasmic reticulum of eukaryotic cells is mediated by ryanodine (RyR) and inositol 1,4,5-trisphosphate (InsP₃R) channels. RyR are activated by a rise in $[Ca^{2+}]_i$ (Ca²⁺induced Ca²⁺ release, CICR). In addition, there are RyR-like channels activated by cyclic ADP-ribose (cADPR), sphingosine, and a distinct Ca2+-release pathway activated by nicotinic acid adenine dinucleotide phosphate (NAADP). The T. gondii phosphoinositide-specific phospholipase C, the enzyme that generates the second messengers InsP₃ and diacylglycerol, was recently cloned, sequenced, and expressed in E. coli, and its enzymatic characteristics were investigated (Fang et al., 2006). InsP₃/ryanodine-sensitive stores had been postulated to be present in T. gondii, on the basis of pharmacological studies (Lovett et al., 2002). Treatment with ethanol increased InsP3 and [Ca²⁺]_i, and this pathway was sensitive to inhibitors of InsP₃R channels. T. gondii also responded to agonists of cADPR-gated channels, such as ryanodine and caffeine (Lovett et al., 2002). Evidence for the presence of cADPR cyclase and hydrolase activities, the two enzymes that control cADPR levels, has been found recently (Chini et al., 2005). T. gondii microsomes that were loaded with ⁴⁵Ca²⁺ released Ca²⁺ when treated with cADPR, and the RyR antagonists 8-bromo-cADPR and ruthenium red blocked this response. Although T. gondii microsomes also responded to InsP₃, the inhibition profiles of these calciumrelease channels were mutually exclusive (Chini et al., 2005). Although there is no molecular evidence for the presence of InsP₃R or RyR channels in T. gondii, this could be due to lack of homology

with the channels of animal cells, as occurs in plants that otherwise respond to the same second messengers (Nagata *et al.*, 2004).

10.4.2 Nucleus

The transport of Ca^{2+} across the nuclear membrane has been the subject of much controversy. Although even proteins permeate the nuclear membrane through the nuclear pores, some authors have shown that the movement of Ca^{2+} may be restricted and require a SERCA-type pump. The nuclear membrane of *T. gondii* is continuous with the endoplasmic reticulum (Hager *et al.*, 1999), and a similar composition in channels and pumps would be expected.

10.4.3 Mitochondria

Mitochondria possess a high capacity to sequester Ca²⁺, although under physiologic conditions the total mitochondrial Ca2+ levels and free Ca2+ reflect and parallel cytosolic Ca2+. The inner mitochondrial membrane possesses a uniport carrier for Ca²⁺, which allows the electrogenic entry of the cation driven by the electrochemical gradient generated by respiration or ATP hydrolysis. Calcium efflux, on the other hand, takes place by a different pathway, which appears to catalyze the electroneutral exchange of internal calcium by external sodium or protons. Biochemical evidence for mitochondrial Ca²⁺ uptake is available in malaria parasites (Uyemura et al., 2000), and preliminary evidence suggests the presence of a uniport mechanism in T. gondii (Vercesi and Moreno, unpublished observations). Unlike the mammalian mitochondria, where intracellular Ca²⁺ regulates the activity of several dehydrogenases, no such Ca2+-regulated dehydrogenases have been reported in T. gondii. Figure 10.2 shows a scheme of the Ca²⁺ homeostatic mechanisms of T. gondii.

10.4.4 Acidocalcisomes

The largest store of Ca^{2+} in *T. gondii* is found in the acidocalcisomes (Moreno and Zhong, 1996; Bouchot *et al.*, 1999; Luo *et al.*, 2001). These are



FIGURE 10.2 Schematic representation of the distribution of Ca2+ in T. gondii. Ca2+ entry is probably through Ca²⁺ channels (1). Once inside the cells, Ca2+ can be translocated back to the extracellular environment by a PMCA (plasma membranetype Ca2+-ATPase) (2). In addition, Ca2+ will interact with Ca2+-binding proteins or become sequestered by the endoplasmic reticulum (3), mitochondrion (4), acidocalcisome (5), or nucleus (6). The endoplasmic reticulum contains the SERCA (sarcoplasmic-endoplasmic reticulum Ca²⁺-ATPase) while the mitochondrion takes up Ca²⁺ through its uniport. Acidocalcisomes have a PMCA, a vacuolar H⁺-PPase, and a V-H⁺-ATPase. Ca²⁺ appears to diffuse freely into the nucleus. Further details are discussed in the text. ER, endoplasmic reticulum; M, mitochondrion; N, nucleus; $\Delta \Psi$, mitochondrial membrane potential. Illustration by Cheryl E. Reese. This figure is reproduced in color in the color plate section.

acidic calcium-storage organelles found in a diverse range of microorganisms from bacteria to man (Docampo *et al.*, 2005). They are characterized by their acidic nature, high density (both in weight and by electron microscopy), and high content of pyrophosphate, polyphosphate (poly P), calcium, magnesium, and other elements (Docampo *et al.*, 2005). Acidocalcisomes are similar to the volutin or metachromatic granules first described almost 100 years ago (Kunze, 1907) in coccidians, and detected in 1966 in *T. gondii* for their ability to stain red when treated with toluidine blue (metachromasia) (Mira Gutierrez and Del Ray Calero, 1966). More recently, they were also named 'black granules' (Bonhomme *et al.*, 1993).

The acidity of acidocalcisomes of T. gondii is easily demonstrated through the incubation of tachyzoites with the weak base acridine orange (AO) and subsequent observation by fluorescence microscopy. Cells incubated in the presence of AO show orange labeling of several acidocalcisomes, which are the most acidic compartments in the cell (Figure 10.3). Shaw and colleagues reported that when using DAMP (3-(2,4-dinitroanilino)-3'amino-N-methyldipropylamine), which differentially accumulates in acidic compartments, the only acidic compartments at the electron microscope level were mature and forming rhoptries (Shaw et al., 1998). No labeling of other organelles (micronemes, dense granules, endoplasmic reticulum, Golgi, or any other membrane-bounded organelles or anything resembling a lysosomal system) was observed. However, no orange staining of the rhoptries is observed by fluorescence microscopy (Figure 10.3). The reasons for this discrepancy are, first, that treatment of the cells for electron microscopy empties the acidocalcisome content (see below) and therefore it would be impossible to see any labeling by electron microscopy techniques that do not preserve its content; and second, even if the rhoptries are acidic, they are not as acidic as the acidocalcisomes. Figure 10.4 shows the lack of co-localization of acidocalcisome and rhoptry markers.

In thin sections, the acidocalcisomes of *T. gondii* appear as empty vesicles occasionally bearing an electron-dense material that sticks to the inner

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FIGURE 10.3 Detection of acidocalcisomes with acridine orange. Isolated tachyzoites were incubated with 25 μ M acridine orange in Dulbecco's phosphate buffer saline (PBS) for 10 minutes, washed twice in PBS and mounted in a light microscope slide. Images were obtained using a Zeiss Axioplan fluorescence microscope equipped with a 488-nm excitation filter, a Hamamatzu digital CCD camera (model C5810) and an image analysis system. A final composition of images of cells from different fields was obtained through the clone tool of the Adobe Photoshop program. Bars = 2 μ m.

This figure is reproduced in color in the color plate section.



FIGURE 10.4 Immunofluorescence microscopy of TgVP1.

- (A) The acidocalcisome localization of *Tg*VP1 in isolated tachyzoites is shown using polyclonal antibodies against the enzyme (red, 1 : 1000).
- (B) The lack of co-localization of the reaction against *Tg*VP1 and ROP1, a rhoptry protein, is shown, using polyclonal antibodies against *Tg*VP1 (red, 1 : 1000) and monoclonal antibodies against ROP1 (green, 1 : 1000). Bar = 10 μm.

This figure is reproduced in color in the color plate section.

face of the membrane (Figure 10.5B). Tachyzoites show acidocalcisomes with different degrees of preservation of the electron-dense material, from an almost totally empty vacuole (Figure 10.5A, left arrows) to others containing a considerable amount of electron-dense material (Figure 10.5A, right arrows). The membrane of the *T. gondii* acidocalcisome is about 8 nm thick (Figure 10.5B). In some circumstances the electron-dense material can be found in aggregates within the organelle, apparently surrounded by an internal membrane (Figure 10.5C, arrow), as occurs in some *Phytomonas* species (Miranda *et al.*, 2004), but no direct evidence for the presence of an internal membrane has been obtained. Occasionally acidocalcisomes of *Toxoplasma* can be seen fusing with each other, being found in a polymorphic organization (Figures 10.5D, 10.5E).

A large number of acidocalcisomes can be seen in electron spectroscopic images of whole cells directly dried on Formvar-coated grids (Figure 10.6A). The advantage of this type of preparation is the observation of the whole parasite (and whole organelles) without the addition of fixatives and



FIGURE 10.5 Transmission electron microscopy of tachyzoites of T. gondii.

- (A) Thin section of a *T. gondii* tachyzoite showing several acidocalcisomes in different regions of the cell, with different degrees of preservation of the electron-dense material (*arrows*). Bar = 500 nm.
- (B) and (C) High magnification of acidocalcisomes. Note the single membrane and the small amount of electron-dense material (B) and the amount of electron-dense material accumulated in the matrix of the organelle (C, arrow). Scale bars: (B) = 100 nm, (C) = 200 nm.
- (D) and (\tilde{E}) Acidocalcisomes fusing with each other (arrows). Bars: (D) = 100 nm, (E) = 200 nm.

Cells were washed twice in PBS, fixed in Karnovsky, post-fixed in OsO_4 and embedded in Polybed 812 epoxide resin. Sections were stained for 30 minutes in uranyl acetate and for 5 minutes in lead citrate, and observed in a JEOL 1200EX electron microscope operating at 80 kV.



FIGURE 10.6 Electron probe X-ray microanalysis of whole cells adhered to formvar-coated grids. (A) Electron spectroscopic image (ESI) of *T. gondii*. Bar = 1 μm.

(B) Corresponding X-ray spectrum obtained from the sample region arrowed in Figure 10.6A (arrow). Note the presence of high amounts of oxygen, sodium, magnesium, phosphorus, chlorine, potassium, calcium and zinc within these organelles. Copper signal comes from the electron microscope grid and titanium signal from the specimen holder. Specimens were analyzed in a LEO 912 Omega scanning transmission electron microscope. X ray point measurements were collected for 150 seconds using a Li-drifted Si-detector (front area 30 mm²) equipped with an ATW atmospheric window. The microscope was operated at 80 kV using a tungsten filament, in the scanning transmission (STEM) imaging mode, and spot size was 63 nm.

other chemicals used in the routine procedures for transmission electron microscopy. This reduces significantly the extraction of material from the acidocalcisomes, and therefore allows observation of the organelle in its 'native' state (Luo et al., 2001). In these preparations, acidocalcisomes can be seen as spherical electron-dense organelles randomly spread throughout the cell body (Figure 10.6A). Approximately 10 acidocalcisomes, with diameters varying between ~150 and ~400 nm, are observed per cell. X-ray microanalysis (Luo et al., 2001) (Figure 10.6B) reveals considerable amounts of oxygen, sodium, magnesium, phosphorus, chlorine, potassium, calcium, and zinc concentrated in these compartments, similarly to what has been reported previously in the acidocalcisomes of trypanosomatids (Scott et al., 1997; Rodrigues et al., 1999; Miranda et al., 2000, 2004; Lefurgey et al., 2001).

T. gondii acidocalcisomes have been shown to possess a plasma membrane-type Ca^{2+} -ATPase (PMCA), involved in Ca^{2+} influx, with similarity to

vacuolar Ca2+-ATPases of other unicellular eukaryotes (Bouchot et al., 2001; Luo et al., 2001), and two proton pumps - a vacuolar H+-ATPase (V-H+-ATPase) and a vacuolar H⁺-pyrophosphatase (V-H+-PPase) - involved in their acidification (Moreno et al., 1998; Rodrigues et al., 2000; Luo et al., 2001; Drozdowicz et al., 2003). No second messengers have been demonstrated to be involved in Ca2+ release from acidocalcisomes of T. gondii. However, a gene with similarity to the previously described two-pore channel 1 (TPC1), the Arabidopsis thaliana Ca2+-dependent Ca2+release channel (Furuichi et al., 2001), has been found in the genome of T. gondii (583.m05406) (Chen et al., 2006). The predicted protein sequence shows 12 transmembrane segments distributed in 2 domains typical of these kinds of channels, which are present in the plant vacuoles, and it is possible that this channel might be present in the acidocalcisomes.

Although the Ca²⁺ content of acidocalcisomes is very high (probably in the molar range), most of it is bound to poly P and can be released only upon alkalinization (Moreno and Zhong, 1996) or after poly-P hydrolysis (Rodrigues *et al.*, 2002).

A gene encoding the acidocalcisome Ca2+-ATPase (TgA1) (Table 10.1) was identified in T. gondii (Luo et al., 2001). This gene was able to complement yeasts deficient in the vacuolar Ca2+-ATPase gene PMC1, providing genetic evidence for its function (Luo et al., 2001). The protein product is closely related to the family of plasma membrane calcium ATPases (PMCA). A sequence analysis of conserved core sequences of all PMCA-type Ca2+-ATPases has identified a subcluster within these sequences that is formed by the acidocalcisome Ca²⁺-ATPases of T. gondii, Trypanosoma cruzi, T. brucei, and Dictyostelium discoideum, and the vacuolar Ca2+-ATPases of veast and Entamoeba histolytica (Luo et al., 2001). A common feature of these pumps is the lack of a calmodulin-binding domain, in contrast to other PMCA-type Ca²⁺-ATPases. Mutants deficient in TgA1 were shown to have decreased virulence in vitro and in vivo due to their deficient invasion of host cells (Luo et al., 2005). Biochemical analysis revealed that the tachyzoite poly P content was drastically reduced, and that the basal Ca²⁺ levels were increased and unstable. Microneme secretion under the conditions of stimulation by ionophores was altered. Complementation of null mutants with TgA1 restored most functions (Luo et al., 2005).

The V-H⁺-ATPase was first identified in *T. gondii* by its sensitivity to bafilomycin A₁, a specific inhibitor of this proton pump when used at low concentrations (Bowman *et al.*, 1988). In experiments using intact tachyzoites loaded with the fluorescent calcium indicator fura-2, bafilomycin A₁ was able to release calcium from an intracellular compartment of *T. gondii* (Moreno and Zhong, 1996). The V-H⁺-ATPase was also shown, by immunofluorescence microscopy, to localize in acidocalcisomes and in the plasma membrane, where it has a role in regulating intracellular pH homeostasis (Moreno *et al.*, 1998).

A V-H⁺-PPase activity was also found in *T. gondii* (Rodrigues *et al.*, 2000), and this enzyme was also shown to localize in acidocalcisomes of *T. gondii*

(Rodrigues et al., 2000; Luo et al., 2001; Drozdowicz et al., 2003) (Figure 10.4). The gene encoding the T. gondii enzyme (TgVP1) was cloned and sequenced, and a truncated version of the enzyme (without the N-terminal) could be functionally expressed in yeast (Drozdowicz et al., 2003). Interestingly, the V-H+-PPase-specific staining of T. gondii assumes a transverse radial distribution soon after the parasite has made contact with the host cell. A collar-like structure is generated that migrates along the length of the parasite in synchrony with and immediately anterior to the apicobasally propagating penetration furrow (Drozdowicz et al., 2003). Upon completion of infection, the V-H+-PPase-associated fluorescence disperses before reappearing again at the anterior apex of the intracellular tachyzoite (Drozdowicz et al., 2003). In recent work a chimera of the T. gondiiV-H+-PPase, with or without the N-terminal extension of T. cruzi V-H+-PPase at its N-terminus, has shown improved expression levels, enough to complement yeasts deficient in the soluble pyrophosphatase (Drake et al., 2004). The acidocalcisome enzyme belongs to the K+-stimulated group of V-H+-PPases (type I) (Rodrigues et al., 2000; Drozdowicz et al., 2003), and has been successfully used as a marker for acidocalcisome purification - not because is only localized in these organelles, but because it is heavily concentrated in them (Rodrigues et al., 2002).

A number of genes have been identified in the genome of *T. gondii* that could potentially encode for additional acidocalcisome transporters. For example, a Ca^{2+}/H^+ exchanger similar to those present in the vacuole of yeast and plants has been annotated, probably erroneously, as a Ca^{2+}/Na^+ antiporter (see section 10.3.1) (20.m03897); a putative phosphate transporter (49.m03192); two putative chloride channels (57.m01751 and 80.m02270); a neutral and basic amino-acid transporter (583.m05611); a Zn^{2+} transporter (52.m01632); and Na⁺/H⁺ exchangers (129.m00252 and 541.m01159) (Chen *et al.*, 2006). Alternatively, however, some or all of these transporters could be located in the plasma membrane.

All acidocalcisomes described so far have been found to have high levels of phosphorus in the

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Phosphorous compounds	Concentration (mM)	
Pyrophosphate	7.950 ± 0.160	
Short-chain poly P	24.000 ± 0.500	
Long-chain poly P	0.043 ± 0.005	

TABLE 10.2 Pyrophosphate and polyphosphate levels in tachyzoites

Data from Rodrigues *et al.*, 2002.

form of inorganic pyrophosphate (PP_i) and polyphosphate (poly P). Quantitative measurements of PP_i and different poly Ps in *T. gondii* are shown in Table 10.2. *T. gondii* acidocalcisomes are especially rich in short-chain poly Ps such as poly P₃ (Rodrigues *et al.*, 2000; Moreno *et al.*, 2001).

PP_i is a byproduct of many biosynthetic reactions (synthesis of nucleic acids, coenzymes, and proteins, activation of fatty acids, and isoprenoid synthesis), and its hydrolysis by inorganic pyrophosphatases makes these reactions thermodynamically favorable. None of these pathways has been found in T. gondii acidocalcisomes. One possibility is that PP_i is there as a byproduct of the hydrolysis of poly P, or as an intermediate for its synthesis. Only three reactions are known to use PP_i in T. gondii: one catalyzed by phosphofructokinase (Peng et al., 1995), another by the V-H+-PPase responsible for acidification of acidocalcisomes (Rodrigues et al., 2000; Drozdowicz et al., 2003), and the third by an inorganic pyrophosphatase (Luo and Moreno, unpublished results). Since PP_i is charged and polar, any movement through the acidocalcisome membrane probably involves a transporter. A transmembrane transporter that shuttles PPi between intracellular and extracellular compartments has been identified in several mammalian tissues (Ho et al., 2000) and a similar channel in the acidocalcisome membrane would explain PP_i accumulation after its synthesis through anabolic reactions occurring in the cytosol or other compartments, or its release into the cytosol to serve as substrate for the V-H+-PPase.

Poly P accumulates in very large amounts in acidocalcisomes (Table 10.2). The storage of phosphate as poly P reduces the osmotic effect of large

pools of this important compound. Short- and long-chain poly P levels also rapidly decreased upon exposure of tachyzoites to agents that mobilize Ca^{2+} , such as calcium ionophores (ionomycin), alkalinizing agents (NH₄Cl), or inhibitors of the V-H⁺-ATPase (bafilomycin A₁) (Rodrigues *et al.*, 2002). This would suggest a role for poly P in the adaptation of the parasites to environmental stress.

The low sulfur content detected by elemental analysis (Figure 10.6) suggested a low content of proteins within acidocalcisomes. However, in addition to the proton and calcium pumps, another enzymatic activity has been detected. Acidocalcisomes from *T. gondii* were shown to contain a polyphosphatase activity (Rodrigues *et al.*, 2002).

10.4.5 Methods to study acidocalcisomes

In addition to the light- and electron-microscopy techniques used to visualize acidocalcisomes, and described above, spectrofluorometric techniques using fura-2-loaded parasites have been used to detect changes in acidocalcisome Ca2+ (Moreno and Zhong, 1996; Rodrigues et al., 2002). Acidocalcisomal Ca²⁺ is released when the cells are submitted to alkalinizing agents (NH₄Cl), inhibitors of the V-H+-ATPase (bafilomycin A1) and Na⁺/H⁺ (monensin), and K⁺/H⁺ exchangers, or when incubated with ionophores (ionomycin) after their alkalinization (Moreno and Zhong, 1996). Phosphorous compounds were investigated by ³¹P NMR spectroscopy (Rodrigues et al., 2000; Moreno et al., 2001), or by biochemical techniques (Rodrigues et al., 2002). Isolation of acidocalcisomes has been done using iodixanol gradient centrifugation using enzymatic markers (Rodrigues et al., 2002). This procedure is depicted in Figure 10.7. In contrast to procedures used before to isolate acidocalcisomes from other cells, this method used lower concentrations of iodixanol in the gradient steps but with the sample added in the middle of the gradient in a 20 percent iodixanol layer rather than applied to the top of the gradient without added iodixanol. This strategy allowed a better separation of acidocalcisomes



FIGURE 10.7 Schematic representation of the fractionation procedure used to purify acidocalcisomes of *T. gondii* tachyzoites. The 15 000 × g pellet is applied in the middle layer of a 10–30 percent iodixanol stepgradient. Insets on the right are X-ray microanalysis of an isolated acidocalcisome showing the presence of carbon, oxygen, magnesium, phosphorus, and calcium, and a direct transmission electron micrograph (TEM) of the fraction showing the electron-dense acidocalcisomes. Bar = 1 µm. Reproduced with permission from Rodrigues *et al.* (2002), *J. Biol. Chem.* **277**, 48 650–48 656.

from the ghosts present in the intermediate fractions, and a better recovery of the pellet fraction at the base of the gradient (Rodrigues *et al.*, 2002).

10.5 Ca²⁺ FUNCTION IN *T. GONDII*

10.5.1 Motility

Gliding motility is dependent on an actomyosin motor, and is essential for active invasion of host cells by *T. gondii* (Dobrowolski and Sibley, 1996). In time-lapse microscopy experiments of parasites loaded with fluo-4, it was found that during parasite

motility the cytosolic calcium levels underwent periodic increases (as occurs in muscle cells during contractions), and that intracellular stores and not extracellular calcium were involved in these changes (Lovett and Sibley, 2003).

10.5.2 Conoid extrusion

Conoid extrusion occurs during host invasion and egress from the host cells, and is stimulated by agents that increase $[Ca^{2+}]_i$, like ionomycin, calcium ionophore A23187, and thapsigargin (Mondragon and Frixione, 1996; Monteiro *et al.*, 2001). Ionophore-induced conoid extrusion is prevented by pre-incubation of tachyzoites with

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the intracellular Ca²⁺ chelator BAPTA-AM, but not by agents that reduce extracellular Ca²⁺, like EGTA (Mondragon and Frixione, 1996).

10.5.3 Microneme secretion, attachment, and invasion of the host cell

The invasion process of T. gondii is marked by the sequential secretion of parasite organelles. Micronemes, rhoptries, and dense-granule contents are released by invading parasites, and participate in attachment, vacuole formation, and intracellular survival (Carruthers and Sibley, 1997). Micronemes are the first secretory organelles to be discharged. Host-cell contact triggers a burst of microneme release. Microneme proteins participate in attachment to host-cell surfaces (Fourmaux et al., 1996; Carruthers et al., 2000; Garcia-Reguet et al., 2000; Brecht et al., 2001), and the transmembrane form of the adhesin MIC2 may link parasite and host-cell membrane during invasion (Lovett et al., 2002). Increases in intracellular Ca2+ mediate microneme secretion even in the absence of host cells, as shown when cells are treated with calcium ionophores like ionomycin or A23187, the ATPase inhibitor thapsigargin (Carruthers and Sibley, 1999), ethanol (Carruthers et al., 1999a; Matthiesen et al., 2003), or the anti-calmodulin agent calmidazolium (Wetzel et al., 2004).

Changes in the intracellular Ca²⁺ concentration of T. gondii during their interaction with host cells have been directly demonstrated by digital fluorescence microscopy of parasites loaded with fura-2 (Vieira and Moreno, 2000), and by time-lapse microscopy of parasites loaded with fluo-4 (Lovett and Sibley, 2003). In experiments using fura-2 (Vieira and Moreno, 2000), an increase in calcium was detected in the tachyzoites upon attachment. Similarly, a peak of fluorescence was detected in tachyzoites immediately upon attachment (Figure in Lovett and Sibley, 2003), and after attachment the Ca²⁺ fluxes stopped. When Ca²⁺ transients are prevented by loading the cells with BAPTA-AM at concentrations able to chelate intracellular Ca²⁺, but not with the chemical analog half-BAPTA-AM that does not chelate Ca²⁺,

a decrease in host invasion by tachyzoites is observed (Vieira and Moreno, 2000). Inhibition of microneme release by chelation of intracellular calcium with BAPTA-AM also inhibits parasite invasion of host cells (Carruthers *et al.*, 1999b). In summary, these results indicate that a Ca²⁺ increase that occurs upon attachment of tachyzoites to the host-cell surface is possibly associated with conoid extrusion, microneme secretion, and invasion.

The source of the Ca²⁺ increase needed for invasion is an intracellular store. Although previous reports using EGTA to chelate extracellular Ca²⁺ indicated that this was important for invasion (Pezzella *et al.*, 1997), it was found that this effect was due to acidification of the culture medium by EGTA and not to chelation of Ca²⁺ (Lovett and Sibley, 2003).

Previous studies showed that ryanodine and caffeine enhances Ca2+ release and microneme secretion, and that ethanol (which is known to cause Ca2+ release) stimulates an increase in inositol 1,4,5-trisphosphate (InsP₃) (Lovett *et al.*, 2002). In addition, xestospongin C, an InsP₃ receptor antagonist, inhibited microneme secretion and blocked parasite attachment and invasion of host cells (Lovett et al., 2002). These studies indicate that InsP₃ is acting as a second messenger and T. gondii possesses an intracellular Ca²⁺-release channel with properties of the InsP₃/ryanodine receptors (RyR) superfamily. More recent work has established that another second messenger involved in intracellular Ca2+ release, cyclic ADP ribose (cADPR), is also present in T. gondii. Inhibition of this pathway by incubation of the parasites with the non-hydrolyzable analog 8-Br-cADPR or the RyR inhibitor dantrolene decreased microneme secretion and gliding motility (Chini et al., 2005). Taken together, these results indicate that both InsP3 and RyR channels are important for efficient motility and cellular invasion, suggesting that they may work cooperatively, as in other systems (Chini et al., 2005).

10.5.4 Egress from the host cell

Changes in intracellular Ca²⁺ have also been involved in *T. gondii* egress from the host cells on the basis of the use of Ca²⁺ ionophores (Endo *et al.*, 1982;

Black et al., 2000; reviewed in Arrizabalaga and Boothroyd, 2004). Addition of the Ca²⁺ ionophore A23187 to infected macrophages stimulated the movement and egress of tachyzoites, resulting in host-cell lysis (Endo et al., 1982). Most of the parasite population exited from infected human foreskin fibroblasts after only a 2-minute exposure to 1 µM A23187, and this event was temperaturedependent (Black et al., 2000). The importance of Ca²⁺ for T. gondii exit from the host cell was confirmed by experiments showing that microinjection of intracellular Ca2+ also stimulated the exit of the parasites (Schwab et al., 1994). A T. gondii mutant that is altered in its response to the Ca²⁺ ionophore A23187 was found to be defective in an Na⁺/H⁺ exchanger located on the parasite's plasma membrane. These mutant cells have increased levels of intracellular Ca²⁺, which explains their decreased sensitivity to A23187 (Arrizabalaga et al., 2004).

A role for a T. gondii PI-PLC in parasite egress from the dying host has also been postulated on the basis of studies with the PI-PLC inhibitor U73122 (Moudy et al., 2001). It was shown that permeabilized Toxoplasma-infected cells preincubated with U73122 but not with the inactive analog U73343 prevented parasite egress in the presence of extracellular buffer, and it was proposed that parasite egress depended on the intracellular Ca2+ increase stimulated by the decrease in the external K⁺ concentration (Moudy et al., 2001). Since the inhibitor U73122 apparently affects PI-PLC activity in mammalian cells through its effects on heterotrimeric G proteins (Thompson et al., 1991), and these have not been described in T. gondii, direct measurements of the products of TgPI-PLC activity (InsP₃, diacylgycerol) will be necessary to confirm this proposal.

Exposure of tachyzoites to 5-mM dithiotretitol (DTT) was also shown to activate egress of previously non-motile intravacuolar parasites within 60 seconds. This was accompanied by an increase in the intra-parasitophorous vacuole (PV) fluorescence ratio of indo 1-loaded infected human fibroblasts. The parasite activation and Ca^{2+} increase were prevented by chelation of extracellular Ca^{2+} by EGTA

and BAPTA-AM, although ionomycin was still able to increase Ca²⁺ in the PV, and the motility and egress of the parasite (Stommel *et al.*, 2001). Since DTT was known to activate the nucleoside triphosphate hydrolase (NTPase) of the parasite, a link with this effect was suggested, although no direct evidence of this link was provided (Stommel *et al.*, 2001).

10.6 CONCLUSIONS

Ca²⁺ regulation in *T. gondii* differs in several aspects from the processes that occur in other eukaryotic cells, providing great opportunities for targeting them for new therapies. Acidocalcisomes are distinct calcium-storage organelles present in T. gondii, in which calcium is mostly bound to poly P, although no information is available on second messengers involved in Ca2+ release from these organelles. Further studies are necessary to understand the biogenesis and function of acidocalcisomes in T. gondii. We do not know how acidocalcisomes multiply and distribute in daughter cells upon cell division, or the reason for the morphological changes that occur in T. gondii acidocalcisomes during invasion of host cells. PP_i, poly P, and cations are accumulated in large amounts in acidocalcisomes, but their transport mechanism or their functions in T. gondii are largely unknown. Ca2+-ATPases are present, but apparently different from their mammalian counterparts. SERCA-type Ca²⁺-ATPases are sensitive to artemisinin, which does not inhibit the mammalian pump, while the PMCA-type Ca²⁺-ATPase, which also localizes in acidocalcisomes, does not possess a typical calmodulin-binding domain. Intracellular Ca²⁺ increase preceding conoid extrusion, microneme secretion, invasion of host cells, and egress from host cells are the only well-established functions for Ca2+ in T. gondii, but with the information provided by microbial genome sequencing and further work in the field it might soon be possible to predict many other functions and utilize such information to direct effective therapeutic agents to specific pathways within T. gondii.

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Toxoplasma Secretory Proteins and their Roles in Cell Invasion and Intracellular Survival

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11.1 INTRODUCTION

Being an obligate intracellular parasite, *T. gondii* has a limited capacity to survive outside of a cell during infection. Invading a new host cell is therefore crucial for survival and expansion of infection. Invasion mechanisms seem to be highly conserved in the Apicomplexa, meaning that results obtained with one organism can often be extrapolated to other members of the phylum. Accordingly, in places in this chapter we will describe *T. gondii* invasion using supplemental findings obtained from other Apicomplexa. Almost all of our knowledge on *T. gondii*–host cell interactions is derived from *in vitro* studies using

tachyzoites, the stage that is the most amenable to experimentation.

T. gondii shows two major peculiarities when compared to other members of the phylum. First, it has almost no host specificity, being able to invade all cell types – from mammalian to fish and even insect cells. Only plant protoplasts have proven refractory to invasion (Werk and Fischer, 1982). Second, contrasting with most other Apicomplexa that multiply by schizogony, *T. gondii* tachyzoites proliferate by endodyogeny, bypassing the dedifferentiation stage that occurs in schizonts. Tachyzoites are thus invasive at any stage of their cell cycle, except during the short period of cytokinesis.
Host-cell invasion by *T. gondii* is an active, parasite-driven process (Morisaki *et al.*, 1995). It leads to the formation of a new subcellular compartment in which the parasites settle and multiply. The organelles of the apical complex – micronemes, rhoptries, and dense granules – successively play their part in the process by exocytosing their contents (Dubremetz *et al.*, 1993; Carruthers and Sibley, 1997).

This chapter will highlight the key elements involved in invasion and emphasize the sequential exocytosis of secretory organelles, including some peripheral aspects such as protein trafficking to organelles and biogenesis of the parasitophorous vacuole.

11.2 INVASION: A RAPID AND ACTIVE PROCESS DEPENDING ON GLIDING MOTILITY

Host-cell invasion by T. gondii is fundamentally different from phagocytosis or endocytosis induced by intracellular pathogens such as viruses, bacteria, or Trypanosoma cruzi (Finlay and Cossart, 1997; Antoine et al., 1998, Sibley and Andrews, 2000). Invasion is essentially completed in less than 20 seconds, and occurs with apparent passivity of the host cell (i.e. without inducing host-cell membrane ruffling, actin microfilament reorganization, or tyrosine phosphorylation of the host cell) (Morisaki et al., 1995). Entry mechanisms are largely driven by the parasite itself and therefore termed 'active invasion', instead of the term 'induced invasion' used for the entry of bacteria. The parasite penetrates into a tight-fitting vacuole formed by invaginating the host plasma membrane. This active invasion has been observed in many different cell types, including professional phagocytes, and the parasite then resides within a specialized compartment, called the parasitophorous vacuole (PV), which does not acidify (Sibley et al., 1985) or fuse with lysosomes (Jones and Hirsch, 1972). The PV also does not intersect with the host endocytic or exocytic systems (Mordue et al., 1999a).

Contrasting with this active invasion, opsonized parasites are internalized by macrophages over a period of 2-4 minutes in a spacious vacuole, involving a series of profound changes of the host cell similar to those triggered when a bacterium is captured (Morisaki et al., 1995). The vacuole surrounding the parasite is then a traditional phagosome, which quickly acquires the markers of endocytosis, fuses with endosomes/lysosomes, and rapidly acidifies (Sibley et al., 1985; Morisaki et al., 1995, Mordue and Sibley, 1997). The parasite is then destroyed by lysosomal enzymes. Opsonized internalization does not involve reorientation of the parasite to bring the apical end into contact with the host cell, as observed for active invasion, and does not involve secretion of apical proteins stored in secretory organelles (Morisaki et al., 1995). The fate of the parasite is therefore largely dependent on its ability actively to invade.

Like other apicomplexan zoites, the invasive stages of T. gondii are motile. Motility and invasion are closely linked, and the molecular mechanisms driving these two phenomena are rather similar. Non-motile parasites are unable to penetrate a cell. Toxoplasma cells do not have specialized organelles for motility, such as cilia or flagella, yet they are able to move along a substrate or a hostcell surface by gliding, with antero-posterior polarity. This gliding motility, which is specific for Apicomplexa and highly conserved within the phylum, is defined by the absence of shape change, in contrast to amebae and other vertebrate cells that use crawling motility accompanied by the emission of pseudopods and lamellipods in the direction of the motion. Apicomplexan locomotion is exceptionally fast, reaching speeds up to $10 \mu m/s$ in vitro – i.e. 10–50 times faster than the rates of motility of the majority of the other cells (keratinocytes, amebae, etc.).

Videomicroscopy of extracellular *T. gondii* tachyzoites reveals that this gliding motility consists of a succession of several stereotyped behaviors:

- circular gliding, which commences while the crescent-shaped parasite lies on its right side, from where it moves in a counter-clockwise manner
- upright twirling, which occurs when the parasite is attached to the substrate by its posterior end, producing clockwise spinning, and

 helical rotation, which is a horizontal twirling movement resulting in forward displacement (Hakansson *et al.*, 1999).

Helical gliding is the only long-distance productive movement observed *in vitro*, but it should be noted that *Plasmodium* sporozoites exhibit only circular gliding *in vitro* yet they are able to migrate considerable linear distances during liver invasion (Frevert *et al.*, 2005). The directional and twisting nature of helical motility strongly suggests a connection between the driving system of the parasite and the helical organization of the subpellicular microtubules. This connection could be established indirectly by the intramembrane particles (IMP) present on the protoplasmic faces of the inner membrane complex (see Chapter 2).

During helical gliding, Toxoplasma comes into direct contact with the host cell by its apical end. A counter-clockwise torsion of the body of the parasite is sometimes visible during entry (Nichols, 1985). An apical attachment step is difficult to visualize, as invasion is a rapid process and both gliding locomotion and invasion appear continuous except for an approximately five-fold reduction in speed during entry. However, there is some evidence of attachment with apical reorientation, since treatment of invading parasites with cytochalasin D (cytD), an actin-disrupting drug which blocks motility and invasion, does not affect attachment (Miller et al., 1979; Dobrowolski and Sibley, 1996), suggesting that attachment and active invasion are uncoupled.

The conoid, a thimble-shaped cytoskeletal structure at the extreme apex of the parasite, extends and retracts repeatedly as the parasite moves along the host-cell surface. A compound (inhibitor 6) identified in a recent small molecule screen selectively blocks conoid extrusion and parasite invasion (Carey *et al.*, 2004a). However, conoid extrusion is not involved in motility, since this same inhibitor has no effect on either parasite motility or microneme secretion. Although its precise role in invasion is unknown, the movements of the conoid may contribute to bringing the apex of the parasite in close proximity to the host-cell surface. Pretreatment with cytochalasin D inhibits conoid extrusion (Mondragon and Frixione, 1996) but, as previously mentioned, does not affect attachment, indicating that a direct role of conoid in intimate attachment can be excluded. The projection of this structure during motility or invasion is evident in *Toxoplasma, Eimeria,* and *Sarcocystis* – apicomplexan species that initiate infection through the gut. The absence of this organelle in *Plasmodium* sp. suggests it may be an accessory apparatus used to penetrate particularly robust barriers such as the intestinal epithelium (Mondragon and Frixione, 1996).

The area of close contact between the cell and the extended conoid invaginates as a small depression of the host-cell membrane to create the moving junction (MJ) through which apicomplexan zoites propel themselves into the nascent PV (Aikawa et al., 1978). The MJ is a region of tight apposition but not fusion between parasite and host-cell membranes, the latter being markedly thickened at this level (Aikawa et al., 1978). Freezefracture of invading tachyzoites (Porchet and Torpier, 1977; see also Chapter 2 of this volume) shows that the MJ contains rhomboidally arrayed particles and is identical in appearance to that formed by invading Plasmodium knowlesi merozoites (Aikawa et al., 1981). The MJ moves as a circumferential ring around the parasite as invasion proceeds (see Chapter 2). It coincides generally but not systematically with a prominent constriction of the parasite at the site of penetration. This constriction may result from physical constraints imposed by the host cytoskeleton. Analogous to an iris diaphragm, the MJ expands as it slides over the midsection of the parasite, then closes at the posterior end when entry is completed by fusion of host membrane behind the parasite (Aikawa et al., 1978; Lebrun et al., 2005). The parasite is now located within the PV, the membrane of which originates mainly from host plasmalemma (Suss-Toby et al., 1996). A striking difference in the density of IMP between the P face of the host cell and P face of the PV is observed (Aikawa et al., 1981; Dubremetz et al., 1993), illustrating the diffusion barrier that excludes transmembrane proteins at the MJ (Mordue et al., 1999b).

The parasite is then isolated from the cytoplasm of the host cell in a PV, which increases in size thereafter to allow its development. Sequestered within the PV, the parasite salvages essential nutrients from the host cell and multiplies rapidly. Schwab and colleagues demonstrated that the PVM surrounding T. gondii permits free bi-directional diffusion of small charged and uncharged molecules (< 1 300 Da) between the host-cell cytoplasm and the vacuolar space (Schwab et al., 1994). This finding is consistent with a pore of protein origin crossing the PVM. Since integral membrane proteins from the host are excluded from the parasitophorous membrane vacuole (PVM) (Mordue et al., 1999a), the pore is probably parasite derived. Parasite proteins released from two different secretory organelles (the rhoptries and dense granules) are associated with the PVM (Saffer et al., 1992; Lecordier et al., 1999) and could contribute to pore formation.

In contrast to the tight-fitting PV formed by tachyzoites, sporozoite entry *in vitro* is characterized by a large primary vacuole containing a single parasite and lacking molecular pores (Speer *et al.*, 1997; Tilley *et al.*, 1997). The parasite does not replicate in this vacuole; instead, within 24 hours, it forms and exits into a secondary vacuole, accompanied by the secretion of dense-granule proteins (Tilley *et al.*, 1997). The significance of this two-step invasion process is unknown, but since the parasite replicates only within the secondary vacuole (Speer *et al.*, 1995), specific secretory modification of the compartment is probably crucial for parasite intracellular survival and multiplication.

11.3 INVASION: TIGHTLY COUPLED SECRETION MACHINERY

As described above, the successive steps of invasion are well characterized morphologically. The molecular mechanisms are far less well understood, in particular those leading to building of the MJ and formation of the PV. The involvement of the apical organelle contents in the invasion process has been clearly demonstrated. Microneme proteins, followed by rhoptry proteins, are sequentially secreted to play distinct functions coordinated in time and space (Carruthers and Sibley, 1997). Dense-granule secretion has also been described (Leriche and Dubremetz, 1990; Dubremetz *et al.*, 1993; Carruthers and Sibley, 1997), but it occurs mainly at the end of the invasion process, after parasitophorous vacuole formation, and densegranule proteins are therefore most likely involved in PV remodeling.

11.3.1 Invasion and motility: central role of micronemes

The first insight into understanding gliding motility and invasion by apicomplexan zoites came from observing relocalization of surface-bound molecules. Vanderberg described the circumsporozoite reaction whereby antibodies were capped on the trailing end of gliding *Plasmodium berghei* sporozoites (Vanderberg, 1974). Then, Dubremetz and Ferreira showed that cationized ferritin bound the *Eimeria* sporozoite surface and was rapidly capped posteriorly (Dubremetz and Ferreira, 1978). The velocity and susceptibility to cytochalasin D and to low temperature of this capping phenomenon and of gliding motility were identical (Dubremetz and Ferreira, 1978; Russell and Sinden, 1981).

Further studies eventually led to a model in which parasite gliding results from antero-posterior translocation of surface proteins interacting with cell-surface or substrate receptors, while being coupled to a submembranous actomyosin motor (Menard, 2001; Soldati et al., 2001; Opitz and Soldati, 2002). Several studies have shown that these proteins were not resident at the parasite surface, but came from microneme exocytosis. Microneme exocytosis is an inducible process that has been first observed to occur at the apical attachment site between parasite and host cell before invasion (Carruthers and Sibley, 1997, 1999; Carruthers et al., 1999a). Inhibition of microneme exocytosis is correlated to inhibition of invasion (Carruthers et al., 1999b). After apical exocytosis, MIC proteins are translocated distally and released posteriorly as the parasite glides on a substrate or enters a cell (Carruthers and Sibley, 1997; Kappe *et al.*, 1999; Garcia-Reguet *et al.*, 2000; Opitz *et al.*, 2002). This antero-posterior relocalization matches exactly the progression of the invasion. In addition, it is inhibited by cytochalasin D, suggesting a link between these proteins and the inner actin cytoskeleton.

The molecular characterization of microneme proteins (MICs) has shown that adhesive motifs usually found in higher eukaryote proteins are present in these proteins (Tomley and Soldati, 2001). A functional part played by these adhesins in hostcell attachment, motility, and invasion, and a synergistic role of MICs in the infectious process (Huynh et al., 2003; Cerede et al., 2005; Mital et al., 2005) have been demonstrated, as well as a critical role of the C-terminal cytosolic domain (CD) of transmembrane MICs in motility and invasion (Kappe et al., 1999; Jewett and Sibley, 2004). This domain, which in many cases is highly conserved in Apicomplexa, is indeed the link between the extracellular domains of MICs interacting with host-cell receptors and the submembranous actomyosin motor (Kappe et al., 1999; Jewett and Sibley, 2003).

In the molecular model of invasion that has emerged, secreted MICs serve as ligands recognizing both the host-cell surface and the submembranous cytoskeleton associated motor, called the glideosome (Figure 11.1). The parasite myosin contractile system is anchored to the inner membrane complex (IMC) and allows anterior to posterior translocation of MICs, the outermost membrane of the IMC providing a conveyor belt-like system. When a transmembrane MIC protein is tethered to a fixed extracellular receptor, the parasite is propelled forward. The posterior release of the interaction needed for efficient motion or invasion involves specific proteolytic activities. The molecular actors of gliding and invasion are described below.

11.3.2 Parasitophorous vacuole formation and host-cell reprogramming: role of rhoptries

There is no formal proof of the rhoptry role in PV formation. However, rhoptry exocytosis is visualized

at the beginning of invasion, and rhoptry proteins (ROPs) are found associated with the PVM at early stages of biogenesis. The rhoptry contents sometimes appear as multilamellar vesicles both before and after exocytosis in T. gondii and in Plasmodium (Nichols et al., 1983; Bannister et al., 1986; Stewart et al., 1986). When invasion is interrupted with cytD, the intracellular accumulation of this membranelike material (called evacuoles) is particulary conspicuous even though the PV is not formed (Hakansson et al., 2001). Invagination of the host-cell membrane leads to PV formation, this latter being modified by fusing with these rhoptryderived secretory vesicles (Hakansson et al., 2001). Consistent with this, rhoptry discharge is closely tied to the abrupt spike of conductance that is detected upon initial binding of the parasite to the host cell (Suss-Toby et al., 1996). How these vesicles assume a multilamellar structure is not known, but their morphology suggests they contain lipids organized in sheets or bilayers. Their lipid contents are strictly parasite derived (Hakansson et al., 2001). Subcellular fractionation of rhoptries has also shown an abundance of lipids in these organelles (Foussard et al., 1991). Therefore, rhoptry exocytosis serves to export proteins and lipids modifying the membrane of the developing PV.

A growing number of studies have indicated that ROPs may also manipulate the host cell to the parasite's benefit. Indeed, a *Toxoplasma* kinase protein targeted to the PVM which may originate from rhoptries is responsible for the signaling cascade that leads to resistance to apoptotic inducers (Molestina and Sinai, 2005). In addition, at least two ROPs are relocated in the host nucleus early after invasion (Coller *et al.*, 2005; Gilbert *et al.*, 2005). These emerging roles are discussed below.

11.3.3 Moving junction formation: cooperative role between micronemes and rhoptries

The molecular structure of the MJ has long been an enigma. MIC protein complexes associated with host-cell receptors were believed to build up the junction, although no convincing data could



- FIGURE 11.1 *Toxoplasma* ultrastructure and molecular components of the moving junction during invasion.
 (A) Transmission electron micrograph of a *Toxoplasma* tachyzoite actively invading a Caco2 cell. Relevant structures of the parasite and host cell are labeled, including the parasitophorous vacuole (PV), which is created by invaginating the host plasma membrane (HPM) to form the parasitophorous vacuolar membrane (PVM). Parasite internal structures including the nucleus (N), mitochondrion (M), dense granule (DG), and apicoplast (Ap) are labeled. Also shown is the moving junction (bracket and rectangle), the site of intimate contact between the parasite's plasma membrane and the HPM where receptor engagement occurs. Photomicrograph courtesy of David A. Elliott.
- (B) Model of the glideosome system at the moving junction. As discussed in greater detail in the text, TM MIC complexes are thought to bind host receptors and transmit the glideosome's mechanical force by connecting through aldolase to F-actin, which is translocated posteriorly by the ATP-driven myoA complex anchored into the inner membrane complex (IMC). Intramembrane particles (IMP) consisting of TM proteins may connect the IMC with sub-pellicular microtubules and intermediate filament-like (IF) structures on the cytosolic face of the IMC. Also shown is the AMA1-RON2/4/5 complex, which is proposed to form the MJ exclusion zone that prevents most host surface proteins from entering the PV. The precise arrangement of RONs in association with AMA1 is not known, neither is that of receptors for the complex. RON2 is predicted to be an integral membrane protein and therefore may be anchored into the parasite plasma membrane (as depicted here) or the host plasma membrane. AMA1 is associated with RONs in the MJ but also resides on either side of the MJ without RONs.

support this hypothesis. The only protein clearly associated with a MJ was the merozoite cap protein-1 (MCP-1), described 15 years ago in *Plasmodium falciparum* (Klotz *et al.*, 1989; Hudson-Taylor *et al.*, 1995). This protein has been located under the merozoite plasmalemma, suggesting that it is not directly involved in the parasite–red cell interaction.

Recent studies of T. gondii have led to the characterization of new components of the MJ, an important step toward solving the molecular architecture of this structure central to apicomplexan invasion (Alexander et al., 2005; Lebrun et al., 2005). Surprisingly, among these MJ proteins are hypothetical proteins restricted to Apicomplexa and derived from the anterior part of the rhoptries, known as rhoptry neck proteins (RONs) (Figure 11.1). Several RONs build up a macromolecular complex that is secreted at the apical tip of the parasite and coincides with the MJ during invasion (Alexander et al., 2005; Lebrun et al., 2005). Alexander and colleagues reported that the essential microneme protein apical membrane antigen 1 (AMA1) helps to organize the complex at the surface of the parasite during invasion (Alexander et al., 2005). These results strongly suggest that the MJ derives from collaboration between MICs and RONs.

11.3.4 Maturation of the parasitophorous vacuole: a prominent role of dense granules

Toxoplasma and *Plasmodium* have evolved two distinct types of parasitophorous vacuole (PV), linked to differential strategies of intracellular parasitism. While initial formation of both types of vacuole is driven by the same active invasion process, maturation of the nascent PV is different in terms of both architecture and metabolism, and leads to two distinct modes of parasite replication – endodyogeny *versus* schizogony.

The fate of the *Toxoplasma*-containing vacuole strictly correlates with the intravacuolar release of dense-granule proteins (GRAs) and the formation of multilamellar structures at a posterior invaginated pocket of the parasite, formed 10–20 minutes post-invasion (Sibley *et al.*, 1995). These structures extend into the vacuolar lumen as a membranous

nanotubular network (MNN) that connects with the parasitophorous vacuole membrane (PVM) (Sibley et al., 1986, 1995). The GRA proteins are segregated to either the MNN or PVM (Mercier et al., 2005). The role of the MNN remains unclear, but, based on its spatial importance within the vacuole, current hypotheses favor a role in supporting metabolic exchange between the parasite and the host cell, and/or an architectural role in the spatial organization of the vacuole. Both GRA proteins and the network persist during replication of the parasite within the PV. Later, when parasite stage conversion takes place, the vacuolar structures redistribute and contribute to the formation of an intracellular cyst (Torpier et al., 1993; Ferguson, 2004). Hence, an essential function of some GRAs might be at the encystation stage for the construction and maintenance of the cyst wall.

By contrast, the Plasmodium-containing vacuolar space remains tightly apposed to the parasite plasma membrane. The parasite expands its membranes outside from the limits of the initial vacuole, thus developing a prominent tubovesicular network (TVN). The Plasmodium PV behaves as a sorting compartment for the secretory machinery, leading to the export of parasite proteins of major importance for host-parasite interactions. Although the presence of electron-dense organelles distinct from both micronemes and rhoptries has been reported (Blackman and Bannister, 2001), there is no evidence that these are functionally similar to the Toxoplasma dense granules. In particular, so far no GRA orthologs have been found in the Plasmodium databases. The dense granules might thus reflect an evolutionary divergence required for specialized intracellular development and the host-parasite relationship.

11.4 MICRONEMES

11.4.1 Trafficking of micronemes through the secretory pathway

The apical portion of invasive *Toxoplasma* zoites is populated by 50–100 micronemes (from the Greek for 'small threads'). Micronemes are often seen in an arc-like pattern owing to their association with the cytoplasmic face of the inner membrane complex in the apical region. This association is likely mediated by binding to microtubules, as was recently shown in *P. falciparum* (Bannister *et al.*, 2003).

The first evidence of microneme-specific forward targeting elements was reported by Di Cristina and colleagues (Di Cristina et al., 2000). By creating a chimera consisting of the major surface antigen SAG1 ectodomain, CD46 transmembrane anchor (TM), and the MIC2 cytosolic domain (CD), they showed that the MIC2 CD was sufficient for microneme targeting. The key targeting elements were narrowed down to two motifs: YHYY, located immediately adjacent to the cytosolic side of the TM anchor; and EIEYE, located 11 aa further downstream. Altering these motifs caused mistargeting to the PV, presumably via the dense granules. Although it was proposed that these motifs function as tyrosine-based sorting signals that interact with medium chains (u-chain) within adaptor protein (AP) complexes for sorting into clathrin-coated vesicles, the YHYY motif is probably too close to the TM anchor to be accessible for µ-chain binding. Also, the EIEYE(AD...) element does not conform to the YXXF consensus µ-chain binding sequence, in contrast to the tyrosinebased signals seen in rhoptry proteins (see below).

Nonetheless, further support for tyrosine-based motifs in targeting to the micronemes came from showing that the CD of the lysosomal protein Lamp was sufficient for microneme targeting, and that the tyrosine in the sequence GYQTI was crucial since a mutant expressing GAQTI was retained in a Golgi-associated compartment (Hoppe et al., 2000). The GYQTI conforms to the YXXF consensus sequence, suggesting that *Toxoplasma* expresses microneme directive APs that can recognize this canonical element. It is unclear why mutation of the MIC2 CD sorting sequences (YHYY and EIEYE) resulted in secretion into the PV (i.e. a targeting defect) whereas altering the Lamp tyrosine-based motif led to retention (i.e. a trafficking defect), but this presumably reflects the fine specificity of APs functioning at different steps in the pathway. Mutational analysis of tyrosine-based motifs in the context of full-length MIC proteins expressed in a corresponding knockout strain may further clarify their role in targeting to the micronemes.

To investigate targeting elements in the soluble microneme protein MIC3, Striepen and colleagues examined a series of deletion mutants of MIC3-GFP and showed that it uses multiple targeting sequences (Striepen et al., 2001). Although deletion of the ~50 aa C-terminal domain had no effect on microneme targeting, expression of this domain alone in conjunction with GFP showed staining consistent with at least partial localization to the microneme. These results indicate that additional signals are present in MIC3. Since these results were obtained in wild-type parasites expressing endogenous MIC3, which is homodimeric, the ability of endogenous MIC3 to interact with some of the truncated forms of MIC3 complicates the interpretations, especially when the 65 aa C-terminal dimerization domain is present.

New light has been shed on the sorting signals of MIC3, by complementation of a mic3KO (El Hajj and Lebrun, unpublished). Deletion of the N-terminal propeptide caused mistargeting to the PV, showing that the MIC3 pro-sequence contains microneme targeting information. In addition to the propeptide, each one of the three EGF domains is sufficient for MIC3 delivery to micronemes. Also, both the dimerization of MIC3 and the chitin-binding-like sequence which are crucial for host-cell binding are dispensable for proper targeting. These findings suggest that the two elements, propeptide and EGFlike domain, probably function in different steps in the microneme pathway. Since the propeptide suppresses the receptor binding activity of MIC3's chitin-binding-like domain (CBL) (Cerede et al., 2002), this element may facilitate trafficking by preventing inappropriate association of MIC3 with other parasite proteins early in the pathway. Alternatively, the MIC3 propeptide may function by binding to a cargo receptor for microneme targeting, but at a step that is downstream of the requirements for other targeting elements in MIC3 or its partner, MIC8. These studies indicate the multifactorial nature of microneme targeting.

Reiss and colleagues revealed the interdependence of microneme proteins for trafficking and targeting by showing that the TM microneme protein MIC6 serves as an escorter for MIC1 and MIC4 (Reiss et al., 2001). Targeted deletion of MIC6 resulted in secretion of MIC1 and MIC4 into the PV. Together with their finding that the MIC6 CD was sufficient to confer microneme targeting, these results strongly suggested that MIC6 uses elements in its CD for targeting of MIC1 and MIC4 to the micronemes, thereby verifying the earlier findings of Di Cristina et al. (2000). Targeting interdependence was further revealed by the discoveries that MIC8 is an escorter for MIC3 (Meissner et al., 2002a), and likewise MIC2 for MIC2-associated protein (M2AP) (Huynh et al., 2004). These studies support the concept that soluble MICs are escorted to the micronemes by binding to TM MICs containing CD forward targeting information.

Although the CDs of MIC2 and MIC6 are sufficient for microneme targeting when isolated from the rest of the protein, several studies show that soluble MICs are also necessary for correct trafficking. For example, the absence of MIC1 causes arrest of MIC4 and MIC6 in the ER/Golgi, and failure to reach the micronemes (Reiss et al., 2001). Similarly, MIC2 accumulates in the ER/Golgi of m2apKO parasites (Huynh et al., 2003). It was initially proposed that retention was due to recognition of the inappropriately assembled complex by the secretory pathway's quality control system. However, more recent findings argue against this, and illuminate a trafficking role for the M2AP propeptide in a manner akin to MIC3 discussed above. A propeptide deletion mutant (M2A Δ Ppro) expressed in m2apKO parasites accumulated in the post-Golgi compartment associated with the endosomal system, despite correctly oligomerizing with MIC2. Collectively, these findings indicate that the propeptides of certain MICs contain forward targeting information that supplements the tyrosine-based sorting signals expressed in the CDs of transmembrane MICs.

Table 11.1 summarizes the properties of *Toxoplasma* secretory microneme proteins.

11.4.2 Microneme proteins

T. gondii expresses a large, diverse array of MICs, including transmembrane and soluble proteins.

Several approaches have been used to identify MICs. Before the genome sequencing era, MICs were discovered using monoclonal antibodies (MIC1, MIC2, MIC3), followed by immunoscreening of cDNA libraries (Achbarou et al., 1991a; Fourmaux et al., 1996; Garcia-Reguet et al., 2000) or immunoaffinity purification (Donahue et al., 2000). Genome sequencing of T. gondii has better allowed the characterization of additional MICs, either by similarities to MICs of other Apicomplexa (AMA1, MIC2, SUB1) (Wan et al., 1997; Hehl et al., 2000; Miller et al., 2001) or by searching transmembrane domains and CDs with homologies to that of TRAP/MIC2 (MIC6, MIC7, MIC8, MIC9, MIC12) (Meissner et al., 2002a). Novel MICs were identified by cell fractionation and calcium-modulating compounds that enhance or block microneme secretion, and N-terminal sequencing (MIC4, MIC5, MIC10, MIC11) (Brydges et al., 2000; Brecht et al., 2001; Hoff et al., 2001; Harper et al., 2004a). Co-precipitation allowed the identification of M2AP (Rabenau et al., 2001). More recently, highly sensitive and complementary technologies such as 2-DE/MS and LC/ESI-MS-MS have added new candidates to the already long list of MICs previously identified (Zhou et al., 2005). This study is only indicative, since the assignment of each candidate to its secretory compartment has not been done, but reveals that proteins of unknown function constitute the largest category (54 percent). Among the hypothetical proteins, three displayed significant homology to hypothetical proteins of P. falciparum and several others were without homology proteins or domains in the public databases.

11.4.2.1 MICs sharing homologies with structural domains of eukaryotic proteins involved in protein–protein or protein–carbohydrate interactions

The molecular characterization of MICs has revealed a striking conservation of structural domains known in higher eukaryotic cells (see Table 11.1). These domains are found on both soluble and transmembrane MICs, in single or multiple copies and in a variety of combinations,

	1 5	1	51	I			
Location/ protein	Calculated MW (kDa) ¹	Domains (no.) ²	Interacting partners	Mutant phenotypes	Function	Post- secretory trafficking	References
Microneme							
MIC1	49	Degenerate TSR (2), galectin-like domain	MIC4, MIC6	Non-essential protein; KO mutant is less invasive and less virulent in mice	Adhesion, folding, assembly of the MIC1/4/6 complex	Secreted and posterior capping	Fourmaux <i>et al.</i> , 1996; Cerede <i>et al.</i> , 2005; Saouros <i>et al.</i> , 2005
MIC2	83	A/I-domain, TSR (5), degenerate TSR (1), TM (1)	M2AP	Essential protein; C-terminal truncation mutant is non-viable; substitution with <i>Eimeria</i> MIC1 is viable but less invasive	Escorter, adhesion	Secreted and posterior capping	Carruthers <i>et al.</i> , 1999b; Huynh <i>et al.</i> , 2004; Jewett and Sibley, 2004; Wan <i>et al.</i> , 1997
MIC3	38 (90 dimer)	CBL, EGF (5)	MIC8	Non-essential protein; KO mutant has reduced virulence in mice	Adhesion	Secreted and posterior capping	Garcia-Reguet <i>et al.</i> , 2000; Cerede <i>et al.</i> , 2002, 2005
MIC4	63	Pan/Apple (6)	MIC1, MIC6	Non-essential protein		Secreted and posterior capping	Brecht <i>et al.</i> , 2001; Reiss <i>et al.</i> , 2001; Brydges and Carruthers, unpublished

 TABLE 11.1 Properties of Toxoplasma secretory proteins – microneme proteins

MIC5	20	Parvulin-like PPIase motif		Non-essential protein; KO mutant Shows elevated proteolysis of other secretory proteins		Secreted and posterior capping	Brydges <i>et al.</i> , 2000; 2006	
MIC6	37	EGF (3), acidic	MIC1, MIC4	Non-essential protein	Escorter		Reiss <i>et al.,</i> 2001	
MIC7	36	EGF (5), TM (1)					Meissner <i>et al.</i> , 2002a	
MIC8	75	CBL, EGF (10), TM (1)	MIC3				Meissner <i>et al.</i> , 2002a	
MIC9	32	EGF (3), TM (1)					Meissner <i>et al.</i> , 2002a	
MIC10	23					Secreted	Hoff <i>et al</i> ., 2001	
MIC11	22	Strong charge asymmetry				Secreted	Harper <i>et al.,</i> 2004a	MICRC
MIC12 (Ts3273)	234	EGF (31), TSR (4), TM(1))			Secreted	Opitz <i>et al.,</i> 2002	NEMES
AMA1	60	Pan/Apple (2), TM (1)	RON2, RON4, RON5	Essential protein; conditional knockdown mutant can attach but cannot penetrate; fails to organize the MJ	MJ organization	Secreted; detected on both the external and the internal regions of invading parasites; visible at the MJ in conditional knockdown mutant	Donahue <i>et al.</i> , 2000; Hehl <i>et al.</i> , 2000; Alexander <i>et al.</i> , 2005; Howell <i>et al.</i> , 2005; Mital <i>et al.</i> , 2005	

Continued

Location/ protein	Calculated MW (kDa) ¹	Domains (no.) ²	Interacting partners	Mutant phenotypes	Function	Post- secretory trafficking	References
M2AP	35	Beta, coil	MIC2	Non-essential protein; KO mutant shows defects in MIC2 trafficking; deficient attachment and invasion		Secreted and Posterior capping	Rabenau <i>et al.</i> , 2001; Huynh <i>et al.</i> , 2003
SUB1	85	Subtilase, GPI		Non-essential protein	Proteolysis	Secreted and posterior capping	Miller <i>et al.</i> , 2001; Binder and Kim, 2004; Brydges <i>et al.</i> , 2006
ROM1	28	Rhomboid, TM (7)			Proteolysis		Brossier <i>et al.</i> , 2005; Dowse <i>et al.</i> , 2005

 TABLE 11.1 Properties of Toxoplasma secretory proteins – microneme proteins—cont'd

¹Based on the complete open reading frame including signal sequence or GPI anchor signal, if present.

²Abbreviations: CBL, chitin-binding-like domain; EGF, epidermal growth factor; TSR, thrombospondin type-1 repeat; TM, transmembrane.

so that every MIC protein is structurally unique. The repertoire of MICs in T. gondii constitutes a patchwork of proteins, some of them sharing orthologs in the other Apicomplexa. Several of these conserved structural domains are known in higher eukaryotes to be responsible for protein-protein or carbohydrate-protein interactions. The cell surfacebinding properties of MICs have been demonstrated (MIC1, MIC2, MIC3) (Fourmaux et al., 1996; Carruthers et al., 1999b; Garcia-Reguet et al., 2000), but the presence of such domains has not been systematically associated with an attachment function (for example, MIC4 and MIC6, which do not directly bind host receptors) (Saouros et al., 2005). This section reviews the various structural domains found in MICs; additional discussion of some of these domains is provided elsewhere in this book (see also Chapter 17).

I- or A-domain MIC2 is the only characterized Toxoplasma protein known to possess this domain (Wan et al., 1997). MIC2 is a member of the TRAP family, highly conserved among the phylum (Robson et al., 1988; Spano et al., 1998; Clarke et al., 1990). It possesses a single integrin-like A-domain. This domain is present in the α -chain of some integrins, which are type 1 integral membrane proteins that promote cell-cell and cell-extracellular matrix (ECM) contacts (Pytela, 1988; Larson et al., 1989). A similar domain is also found in various plasma proteins (e.g. von Willebrand factor), in soluble matrix proteins, and in proteins involved in cell-cell and cell-ECM matrix interactions during homeostasis, inflammation or cell migration (Whittaker and Hynes, 2002). These domains bind to various ligands, including collagens, laminin, fibronectin, ICAM-1, and the complement product iC3b. A unique feature of A-domains is that they possess a metal ion dependent adhesion site (MIDAS) motif composed of five noncontiguous amino acids (Lee et al., 1995). In vitro assays of binding to several different putative receptors revealed that the MIC2 A-domain binds specifically to heparin (Harper et al., 2004b), a ubiquitous sulfated proteoglycan found in the extracellular matrix (ECM). This binding is not dependent on the MIDAS site, a feature also

observed for binding of $\alpha 2\beta 1$ integrin to laminin (Dickeson et al., 1998) and of the P. falciparum TRAP domain with heparin (McCormick et al., 1999). In contrast, a considerable loss of infectivity and host-cell invasiveness has been observed for P. berghei sporozoite expressing TRAP mutated in the MIDAS site (Matuschewski et al., 2002), showing the importance of the domain in these functions, and suggesting that its function is at least partly MIDAS-dependent. Homology modelingbased structural analysis of the TRAP A-domain identified a cluster of basic residues on the surface that probably confers heparin-binding ability (Akhouri et al., 2004). It shows also a spatial separation of the putative heparin-binding site from the MIDAS region, indicating that these distinct molecular surfaces may be involved in the recognition of multiple receptors (Akhouri et al., 2004). Recently, it was shown that the TRAP A-domain interacts with fetuin-A on hepatocyte membranes, and that this interaction enhances the parasite's ability to invade hepatocytes (Jethwaney et al., 2005). In T. gondii, MIC2 binds to ICAM-1 and this interaction facilitates migration across polarized epithelial cells (Barragan et al., 2005).

Thrombospondin type 1 repeat domain The thrombospondin type 1 repeat (TSR) domain is found in six tandem copies in MIC2 and two copies in MIC1 (Fourmaux *et al.*, 1996; Wan *et al.*, 1997), with one degenerated motif in each case. TSR domain is present in many proteins from distantly related organisms. These proteins are usually involved in cell–cell and cell–matrix interactions (Lawler, 1986) in clotting or innate immunity (Haefliger *et al.*, 1989). This domain allows thrombospondin and properdin to bind sulfated sugars, and especially glycosaminoglycans (GAGs) (Holt *et al.*, 1990; Chen *et al.*, 2000). It shows also a high affinity for heparin (Guo *et al.*, 1992).

There are no reports of a function for the TSR domain of MIC2. However, in *Plasmodium*, the TRAP TSR domain binds heparan sulfate proteoglycans on the hepatocyte surface *in vivo* and *in vitro* (Muller *et al.*, 1993). The importance of the TRAP TSR-heparan sulfate interaction in the host-cell invasion by *Plasmodium* sporozoite has been

demonstrated by Matuschewski and co-workers (Matuschewski *et al.*, 2002). Recent structural data on the TSP-1 of thrombospondin (Tan *et al.*, 2002) suggests that the MIC2 TSR likely forms an extended 'stalk' on which the A-domain sits, optimally positioned for interaction with host receptors. Although the TSR domain appears to participate in the interaction with M2AP (Harper and Carruthers, unpublished), additional studies will be required to determine whether it also binds to a host-cell receptor.

MIC1 is a monomeric protein which binds to the surface of fibroblasts *in vitro* (Fourmaux *et al.*, 1996). Cell adhesion properties of MIC1 require the presence of both intact TSR domains (Saouros *et al.*, 2005). Furthermore, the tandem TSR domains are also responsible for the interaction between MIC1 and MIC4 (Saouros *et al.*, 2005).

Epidermal growth factor-like domain Epidermal growth factor-like (EGF) domains are found in a large variety of proteins, mainly of animal origin (growth factors, lipoprotein receptors, selectins, clotting factors, extracellular matrix proteins, etc.) and are frequently seen in tandem repeats with various degrees of conservation. The functional significance of these domains is not yet understood. A common feature of these repeated domains is that they are found in the extracellular portion of transmembrane proteins or in secreted proteins engaged in protein interactions (Appella et al., 1988; Davis, 1990). EGF domains contain six cysteine residues that form disulfide bridges. The sub-domain lengths between cysteines vary extensively. Some EGF-like domains have the capacity to bind calcium, and are therefore termed calcium-binding EGF (cbEGF) domains. cbEGF domains form a more rigid, protease-resistant structure upon binding calcium, as recently shown for the Eimeria protein EtMIC4 (Periz et al., 2005) - a transmembrane protein containing 31 EGF-like and 12 TSR domains (Tomley et al., 2001). Similar to the TSP domain of MIC2, this property may help to form an elongated stalk-like structure to maximally project cbEGFcontaining proteins from the cell surface.

EGF-like domains are found in apicomplexan MICs and on resident surface proteins of

Plasmodium (Tomley and Soldati, 2001). Four of the EGF-containing proteins in *T. gondii* are transmembrane (MIC6, MIC7, MIC8, and MIC9) (Meissner *et al.*, 2002a), and the last one is soluble (MIC3) (Garcia-Reguet *et al.*, 2000). MIC3, MIC6, and MIC8 are expressed by tachyzoites and bradyzoites (Garcia-Reguet *et al.*, 2000; Meissner *et al.*, 2002a), whereas MIC7 and MIC9 are predominantly expressed by bradyzoites (Meissner *et al.*, 2002a). A *Toxoplasma* ortholog of EtMIC4 exists in the ToxoDB database. This protein likely corresponds to *T. gondii* MIC12, since the deduced amino-acid sequence is virtually identical to the partial sequence of MIC12 published by Opitz and colleagues (Opitz *et al.*, 2002).

The involvement of MIC EGF-like domains in the interaction of these proteins with the host cell has not been shown. The MIC3 EGF-like domains are not involved in MIC3 binding to cells. They might, however, be involved in the appropriate exposure of the MIC3 binding site located in the chitin-binding-like domain (Cerede *et al.*, 2002). MIC6 contains three EGF-like domains, but does not bind to cells (Saouros *et al.*, 2005). These EGFlike domains likely allow the formation of heteromeric MIC protein complexes. For instance, MIC6 associates with MIC1 via its third EGF-like domain (Reiss *et al.*, 2001).

PAN/Apple module The Apple module contains a conserved core of three disulfide bridges. In some members of the family, an additional disulfide bridge links the N- and C-termini of the domain. This later type is commonly seen in tandem repeats (Tordai et al., 1999), and mediates protein-protein or protein-carbohydrate interactions. It is found in the N-terminal domain of members of the plasminogen/hepatocyte growth factor family, in the plasma prekallikrein/coagulation factor XI family (Tordai et al., 1999), in various nematode proteins, and, recently, in apicomplexan MIC proteins (Brown et al., 2001). The Apple domains of plasma prekallikrein are known to mediate its binding to high molecular weight kininogen (Herwald et al., 1996), and the Apple domains of factor XI bind to factor XIIa, platelets, kininogen, factor IX, and heparin (Ho et al., 1998). This domain was described for the first time in MIC4 through searching sequence homologies (Brecht *et al.*, 2001). It is also found in the micronemes of two other Apicomplexa: *Eimeria tenella* MIC5, which contains 11 apple domains (Brown *et al.*, 2000), and the *Sarcocystis muris* lectin (SML), which contains 2 of these domains (Klein *et al.*, 1998). Brecht and co-workers have suggested that MIC4 could be an adhesin (Brecht *et al.*, 2001), but this was based on the cell-binding activity of the MIC1/MIC4 complex. Subsequent studies have shown that MIC4 does not directly bind host cells, and that MIC1 is responsible for receptor recognition (Lourenco *et al.*, 2001; Saouros *et al.*, 2005).

The Apple domain is a subfamily of the PAN module that has recently been detected in the AMA1 (Pizarro et al., 2005), a microneme protein found in all Plasmodium species and in at least six other Apicomplexa, including T. gondii, Neospora caninum, Sarcocystis neurona, Eimeria tenella, Babesia bovis, and Theileria. The crystal structure of the complete ectoplasmic region of P. vivax AMA1 shows its organization into three domains, as previously predicted from the pattern of cysteine-bridge formation. The core domains I and II are based on the PAN folding motif. The structural homology between domains I and II suggests that they are probably the product of gene duplication but have since diverged significantly. Domain III does not fit into any currently known fold (Nair et al., 2002). The generally low sequence similarity between PAN domains, the absence of the usual cysteine signature in AMA1, and the insertion of additional segments into the PAN scaffold of domains I and II explain why this structural homology was not initially recognized from the primary sequence. The same pattern of cysteine residues and of residues playing a critical role in the three-dimensional structure of PvAMA1 is also present in T. gondii AMA1 (Chesne-Seck et al., 2005). Erythrocyte binding has been obtained on COS-7 cells expressing a construct comprising domains I and II of P. yoelii AMA1 (Fraser et al., 2001). However, other investigations have failed to demonstrate binding of Plasmodium AMA1 to host cells (Howell et al., 2001). In T. gondii, direct evidence of a receptor-binding function has not been obtained.

Proteomic analysis of secretory products in *T. gondii* reveals four additional proteins containing PAN/Apple domains (Zhou *et al.*, 2005). Although the mass spectrometry data were unable to distinguish between these very closely related proteins, only one member of this family (corresponding to TgTwinScan_2359) is abundantly expressed in tachyzoites based on EST data. Over-expression of this protein fused to YFP revealed only partial co-localization with micronemes, but this unusual pattern may be due in part to the use of a heterologous promoter (Zhou *et al.*, 2005).

The chitin-binding-like domain MIC3 and MIC8 contain in their N-terminal region a domain homologous to one found in chitin-binding proteins ('hevein domain' or 'chitin-binding' motif), followed by several EGF-like domains (Garcia-Reguet et al., 2000; Meissner et al., 2002a). This chitin-bindinglike (CBL) domain is also found in an E. tenella protein (Et_v1_Twnscn_Contig4083.tmp1) (www.sanger.ac.uk/Projects/E_tenella/). The chitinbinding domain is a well conserved 30-40 aa stretch found in plants and fungi (Wright et al., 1991). It binds specifically to N-acetyl glucosamine oligosaccharides, and is involved in the cross-linking of chitin subunits. One of the best-known plant lectins is the wheat germ agglutinin (WGA), a homodimeric protein. Each subunit contains four repeats of the CBL domain (Wright and Kellogg, 1996), which features eight conserved cysteine residues implicated in four disulfide bridges that are responsible for structural conformation. A conserved serine residue forms a hydrogen bond with the nonreduced end of chitin polymers, and stabilizes the interaction. This serine residue is followed by an aromatic aa pocket responsible for sugar binding (Wright et al., 1991). In MIC3, the CBL domain has been shown to be responsible for cell surface interaction (Cerede et al., 2002). This domain could allow binding to a large array of cell types (Garcia-Reguet et al., 2000). Similar to WGA, the binding ability of MIC3 involves aromatic amino acids (Cerede et al., 2005). However, all carbohydrate binding assays with MIC3 failed (Lebrun, unpublished), suggesting a significant alteration in binding motif specificity compared to chitin-binding domain.

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The CBL domain is also found in three additional ORFs of the *T. gondii* genome (TgTwinScan_6002, TgTwinScan_6003, and TgTwinScan_1708). Of these, TgTwinScan_1708 looks as though it has a TM anchor.

Galectin-like domain A galectin-like domain is present in the C-terminal part of MIC1 (Saouros et al., 2005). Galectins are a unique family of soluble carbohydrate binding proteins (Barondes et al., 1994). The carbohydrate recognition involves critical amino acids in the central region of the sixstranded beta-sheet, and comprises an array of hydrophilic residues and a key aromatic side-chain (Seetharaman et al., 1998; Umemoto et al., 2003). These positions are not conserved in the equivalent locations within MIC1 which shares a hydrophobic environment in this region, reminiscent of protein-protein interface (Saouros et al., 2005). All the carbohydrate experiments showed no detectable carbohydrate binding except for 100-mM lactose, which substantiates an altered binding motif (Saouros et al., 2005). A role in protein-protein interaction has been confirmed. Indeed, the galectin domain of MIC1 is involved in the interaction with MIC6, assisting the correct folding of MIC6 and stabilizing the C-terminal fragment encompassing the third EGF and the acidic region (Saouros et al., 2005). This allows the MIC1/4/6 complex to exit the ER (Reiss et al., 2001).

11.4.2.2 Other MICs

Although many MICs contain structural motifs suggestive of a role in protein–protein interactions and parasite attachment, a growing subset of microneme proteins (MICs) does not possess obvious adhesive features, suggesting that these may play alternative functions (MIC10, MIC11). MIC10, an 18-kDa protein lacking a putative transmembrane domain, is secreted during invasion, but does not remain associated with the parasite membrane after microneme discharge like other MICs, and does not bind to host cells (Hoff *et al.*, 2001). MIC11 is also a small, soluble protein of unknown function that displays a pronounced charge asymmetry, with an acidic N-terminal region (pI 4.3) and a basic C-terminal region (pI 8.2) (Harper *et al.*, 2004a), reminiscent of the rhoptry protein ROP1 (Ossorio *et al.*, 1992). It is hypothesized that ROP1 and MIC11 proteins may act as molecular organizers within rhoptries and micronemes, respectively, using their charge asymmetry to form an organized scaffold based on homotypic ionic interactions (Hoff *et al.*, 2001).

MIC5 encodes a previously identified immunodominant antigen called H4 (Johnson and Illana, 1991; Brydges *et al.*, 2000). Although MIC5 possesses a consensus sequence characteristic of members of the parvulin family of peptidyl-prolyl cis-trans isomerases (PPIases), no PPIase activity was detected with recombinant MIC5 (Brydges, Kamphausen, Fischer and Carruthers, unpublished). Targeted deletion of *TgMIC5* results in hyperproteolysis of MICs on the parasite surface suggesting that *TgMIC5* regulates protease activity or the susceptibility of other MICs to proteolysis (Brydges *et al.*, 2006).

At least two proteases are found in micronemes. SUB1 is a subtilisin-type serine protease. It is recognized by an anti-*Plasmodium* SUB1 antibody, suggesting that the two subtilases share areas of conserved antigenic structure (Miller *et al.*, 2001). Another serine protease belonging to the rhomboid family has been identified in micronemes, named ROM1. SUB1 and ROM1 are likely to play a role in MIC processing before or after secretion of the organelles; this is discussed in section 11.4.4.2.

Proteins possessing a membrane attack complex (MAC)/perforin domain and a metalloprotease are also probably localized in micronemes, and may play a role in infection by easing the migration/ dissemination of the parasites during infection (Zhou *et al.*, 2005).

11.4.2.3 MICs assemble in complexes

Another characteristic feature of *Toxoplasma* MICs is that they assemble into protein complexes that work in concert. The first demonstration of the occurrence of such a complex was obtained by *MIC6* gene deletion (Reiss *et al.*, 2001). Indeed, the latter led to MIC1 and MIC4 missorting to the

PV instead of microneme. In addition, artificial expression of MIC6 at the parasite plasma membrane induced the same localization for MIC1 and MIC4. Co-immunoprecipitation assay confirmed the association of the three proteins into a stable complex (Reiss et al., 2001). Then, the MIC2/M2AP complex was also found (Rabenau et al., 2001). In this case, a 450-kDa hexameric complex was found to form in the ER to be targeted in micronemes and to persist on the surface during invasion (Rabenau et al., 2001; Jewett and Sibley, 2004). Last, the existence of a MIC3/MIC8 complex has been strongly suggested by the expression of MIC3 on the surface of the parasite expressing artificially a GPI-anchored version of MIC8 (Meissner et al., 2002a).

The density of MICs in complexes may serve two functions. They are likely to be important for proper trafficking in the secretory pathway. A role in quality control has been shown for MIC1 (Reiss et al., 2001; Saouros et al., 2005). Indeed, soluble MIC1 and MIC4 require interaction with the membranebound protein MIC6 to ensure proper targeting to the micronemes. As mentioned previously, the galectin domain of MIC1 binds MIC6 and ensures proper folding of MIC6 to pass the ER quality control. Then, MIC6 ensures targeting of the complex to micronemes. A similar role has been postulated for the soluble protein M2AP (Huynh et al., 2003, 2004). It stabilizes the complex formed with MIC2, and influences the correct targeting to micronemes. In the absence of M2AP, MIC2 appears to trimerize correctly but does not target efficiently to the micronemes, and as a consequence undergoes degradation.

A second function of MIC complexes may be in cooperative binding to enhance cell recognition and facilitate cell entry. Indeed, oligomerization is often critical for enhancing affinity – also, to cluster receptors and other components within complexes to induce signaling. Multimerization of MIC2 has been shown to increase the number of interactions with host-cell receptors, thereby forming a multivalent adhesive protein (Harper *et al.*, 2004b). MIC3 is a homodimer, and this oligomerization is required for host-cell binding (Cerede *et al.*, 2002). The MIC3 homodimer contains two CBL domains and the transmembrane protein MIC8 possesses one, the complex therefore expressing three CBL domains. CBL domain oligomerization has been shown to increase its avidity (Cerede *et al.*, 2002). CBL domain oligomerization in the MIC3/MIC8 complex would therefore be used to increase the affinity with the host-cell surface. Last, MIC1 has affinity for host-cell surfaces when dimerized (Fourmaux *et al.*, 1996). Thus, all microneme proteins described so far as showing affinity for host cells work as oligomers.

11.4.2.4 Cytosolic domain of transmembrane MICs

The cytosolic domain of several transmembrane MICs is highly conserved in Apicomplexa. Replacing the cytosolic domain of TRAP with that of TgMIC2 in P. berghei sporozoites resulted in normal motility, invasive capability, and infectivity in vivo (Kappe et al., 1999). This underscores the functional conservation of the invasive mechanism in Apicomplexa. This domain contains three essential motifs: two of them include first, a stretch of acidic residues and second, tyrosine residues involved in microneme targeting; the other has C-terminal amino acids and especially a tryptophan involved in the interactions of MICs with the glideosome (Kappe et al., 1999). In MIC2 and in TRAP, this tryptophan is required for binding aldolase (Jewett and Sibley, 2003). Aldolase is a glycolytic enzyme that binds actin filaments in vitro and in vivo (Wang et al., 1997; Schindler et al., 2001). In Apicomplexa, it links the transmembrane MICs interacting extracellularly with their receptors and the submembranous actomyosin motor of the parasite (see also below) (Jewett and Sibley, 2003).

11.4.3 Microneme secretion

Microneme secretion is a regulated process (also known as stimulus-coupled secretion). The external stimulus that triggers MICs secretion is unknown, but fluorescence imaging studies have revealed that parasites in association with host cells show elevated levels of cytoplasmic calcium (Vieira and Moreno, 2000). Chelation of extracellular calcium

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with EGTA or BAPTA or addition of excess calcium has little effect on microneme secretion, parasite motility, or cell invasion (Lovett and Sibley, 2003). Chelation of host-cell intracellular calcium prior to invasion with the cell-permeable chelator BAPTA-AM also has no effect on attachment and invasion (Lovett and Sibley, 2003). In addition, host-cell calcium levels remain relatively constant during invasion (Lovett and Sibley, 2003). These observations indicate that parasite invasion occurs independently of host calcium. By contrast, secretion of micronemes is inhibited by chelation of parasite intracellular calcium (by pre-loading of parasites with BAPTA-AM), indicating that parasite calcium is critical to the process of invasion (Carruthers et al., 1999b; Lovett and Sibley, 2003).

Apicomplexans have multiple calcium stores, including the endoplasmic reticulum (ER), mitochondria, and acidocalcisomes (Moreno and Docampo, 2003), but relatively little is known about the intracellular origin of the releasable intracellular calcium or how the release of calcium is regulated (see also Chapter 10). Like most cells, T. gondii utilizes the ER as a source of mobilizable calcium. Indeed, inhibition with thapsigargin of the ER ATP-dependent calcium transporter, which normally resequesters calcium that has leaked from intracellular stores (Thastrup et al., 1989), inhibits microneme secretion (Carruthers and Sibley, 1999). The presence of an active SERCA protein in ER has been recently obtained (Nagamune and Sibley, 2005).

T. gondii also possesses channels of the IP3/ryanodine superfamily (Lovett *et al.*, 2002), mediating an increase in intracellular calcium, which in turn promotes microneme secretion. Ryanodine is a chemical that allows the liberation of calcium from the ER lumen via the ER-ryanodine receptor (Sorrentino *et al.*, 2000). It has been shown to stimulate microneme secretion in *T. gondii* (Lovett *et al.*, 2002). Ethanol is a potent trigger of microneme secretion in the absence of host cells (Carruthers *et al.*, 1999a), and a rapid increase in the second messenger IP3 is observed after addition of ethanol (Lovett *et al.*, 2002). IP3 release precedes secretion of MICs. Using xestospongin as a competitive inhibitor of IP3 to block calcium

release from IP3 receptors, Lovett and colleagues showed that secretion by ethanol is reduced (Lovett et al., 2002). These results suggest that IP3 catalyzes intracellular calcium release in the parasite through an intracellular channel related to the IP3/ryanodine receptor during T. gondii invasion of the host cells. IP3 receptors and the ryanodine receptors are ER calcium-release channels, and are structurally similar (Sorrentino et al., 2000). In mammalian cells, IP3 induces calcium release from ER channels and is produced after hydrolysis of phosphatidylinositol 4,5-bisphosphate by inositolphospholipid-specific phospholipase C (PLC) (Berridge, 1993). A hypothetical signaling cascade has been proposed in which a putative parasite PLC converts PIP2 to IP3, which in turn activates calcium release from IP3/ryanodine channels. Attempts at identifying IP3 or ryanodine receptor channels in the T. gondii genome have been unsuccessful (Lovett et al., 2002), suggesting that T. gondii possess intracellular calcium-release channels that are unconventional in sequence.

In addition, some studies have demonstrated that T. gondii can mobilize calcium from at least one additional intracellular store with acidic characteristics, sensitive to alkalinizing agents such as ammonium chloride or nigericin, which probably corresponds to the acidocalcisomes (Moreno and Zhong, 1996). First, ethanol and acetaldehyde have been shown to increase parasite intracellular calcium levels and stimulate microneme secretion in the absence of host cells, but ethanol stimulation of thapsigargin-pretreated parasites generates an additional increase in intracellular calcium, implying that they posses a thapsigargin-insensitive calcium pool (Carruthers et al., 1999a). A large proportion of intracellular calcium is also stored within acidocalcisomes. A calcium ATPase is associated with the T. gondii acidocalcisome and plasma membrane (Luo et al., 2001). Deletion of the corresponding gene alters the regulation of intracellular calcium concentrations in tachyzoites and microneme secretion (Luo et al., 2005).

A homolog of parafusin, a protein involved in calcium-regulated exocytosis in *Paramecium*, named PRP1 (parafusin related protein), is associated with a subset of micronemes in *T. gondii*, particularly those situated near the apical end (Matthiesen *et al.*, 2001, 2003). Its involvement in calcium-regulated microneme exocytosis has been suggested based on its dynamic redistribution to the cytoplasm during microneme discharge.

The kinase inhibitors staurosporine (KT5926) and compound 1 block microneme secretion and attachment/invasion when added to parasites before infecting HFF cells (Dobrowolski et al., 1997a; Carruthers et al., 1999b; Kieschnick et al., 2001; Wiersma et al., 2004), supporting a role for a parasite protein kinases in these processes. The effect of staurosporine is not reversed by treatment with the calcium ionophore A23187 (Carruthers et al., 1999b), indicating that the staurosporine block is downstream of the calcium step in the microneme secretion signal transduction pathway. A calcium-dependent calmodulin domain protein kinase sensitive to KT5926 (CDPK1) that phosphorylates three unknown parasite proteins has been identified in tachyzoites (Kieschnick et al., 2001). These CDPK1 substrates may be involved in microneme secretion. Also, the activity of a cyclic GMP-dependent protein kinase (PKG) sensitive to compound 1 is required for microneme secretion, gliding motility, and invasion (Donald et al., 2002; Wiersma et al., 2004). Inhibition of PKG activity by compound 1 was not reversed by calcium agonist, suggesting that it functions downstream of the calcium-dependent events in the signaling pathway. Precisely how PKG regulates microneme secretion and motility awaits identification of its substrates.

11.4.4 Post-secretory traffic of MICs

11.4.4.1 Parasite surface exposition and posterior capping of MICs

As the parasite penetrates the host cell, most MICs are excluded from entering the vacuole and are progressively capped behind the MJ, remaining confined to the portion of the parasite that still protrudes from the host cell (Carruthers and Sibley, 1997; Carruthers *et al.*, 1999b; Garcia-Reguet *et al.*, 2000). As a consequence of MIC protein capping, binding to a fixed substrate would lead

to forward locomotion, and binding to cell-surface receptors would lead to penetration of the cell. The backward capping of *T. gondii* MICs is an actin-dependent process, implying that either actin polymerization or actin filaments are required (Jensen and Edgar, 1976; Ryning and Remington, 1978; Miller *et al.*, 1979; Russell and Sinden, 1981).

The current view is that the actomyosin motor, located beneath the plasma membrane of the parasite, interacts indirectly with the cytoplasmic tails of the transmembrane MICs and translocates them toward the posterior end of the parasite (Sibley, 2003; Keeley and Soldati, 2004; Soldati and Meissner, 2004). Thus, as an immobilized myosin walks along the actin filament, the MIC-cell receptor complexes are capped backwards, and the parasite propels itself on the substrate or into the host cell. Substantial evidence of this capping model has been obtained by reverse genetic approaches in *Plasmodium*. Deletion of the TRAP C-terminus abolishes gliding motility and cell invasion (Kappe *et al.*, 1999).

Directional invasion and helical gliding motility are sustained by connection of the actomyosin motor with the IMC. This additional membrane layer is supported by microtubules that provide the pitch for the spiral gliding action and serve as the 'tramline' for the capping reaction. The myosin is indeed anchored in the inner membrane complex of the pellicle, and generates the mechanochemical force for translocating the actin filaments (Gaskins *et al.*, 2004). The different players of the invasion apparatus are called glideosomes. The following sections describe the components of the glideosome, their origins, and how they interact together in building this elaborate machine.

Actin By using cytochalasin-resistant mutants of either the host cell or *Toxoplasma*, Dobrowolski and colleagues have shown that actin dynamics in the tachyzoite, but not in the host cell, are critical for parasite invasion (Dobrowolski and Sibley, 1996). *T. gondii* possesses a single conventional actin gene, *ACT1* (Dobrowolski *et al.*, 1997b), and this protein has been detected biochemically in its monomeric, globular form (G-actin) – i.e. it is

recoverable in the soluble fraction following cell fractionation experiments. In situ, actin filaments have not been detected under physiological conditions in any Apicomplexa using conventional methods. The inability to stain apicomplexan microfilaments with phalloidin *in situ* is not due to a failure of F-actin to bind phalloidin (Schuler et al., 2005), but more likely to an unusually short filament length (Sahoo et al., 2005; Schmitz et al., 2005). A pool of parasite actin must exist under an assembled form, as cytochalasins, which are known to interfere mainly with actin elongation, and which are efficient in blocking parasite movement. The limited intrinsic polymerizability of Plasmodium actin has been recently overcome by the combined action of two non-physiological factors, gelsolin and phalloidin (Schuler et al., 2005). This suggests that parasite-specific factors may perform similar tasks in a regulated fashion.

Thus far, cytoplasmic actin filaments have only been visualized after treatment of parasites with the actin filament-stabilizing drug jasplakinolide (JAS) (Poupel and Tardieux, 1999; Shaw and Tilney, 1999). Under these conditions, actin filament polymerization is induced at the apical end of motile cells and leads to a prominent apical protrusion of actin filaments (Shaw and Tilney, 1999). These filaments were clearly visible, but their polarity could not be resolved. Together, these findings suggest that filaments might be very short and possibly undergo high turnover due to the abundant presence of actindepolymerizing factors (Allen et al., 1997), and actin polymerizes only in response to signaling generated by substrate interaction or host-cell contact at the time of invasion. Finally, by using cytoskeleton stabilizing protocols on membrane-extracted parasites and novel imaging with high-resolution low-voltage field emission scanning electron microscopy (LVFESEM), Schatten and colleagues were able to visualize for the first time a network of actin-sized filaments just below the cell membrane (Schatten et al., 2003). This location is ideal for facilitating translocation of cell-surface MICs.

In contrast to agents that depolymerize actin (such as cytD) and block all forms of motility, JAS treatment increases the speed of *T. gondii* gliding motility, indicating that filament turnover must be tightly controlled to regulate the timing of motility (Wetzel *et al.*, 2003). In addition, the polarity of actin filaments must be ordered to drive unidirectional gliding, since JAS treatment induces bidirectional movement not normally displayed by tachyzoites (Wetzel *et al.*, 2003).

Aldolase The cytoplasmic tails (CD) of MIC2 and TRAP indirectly associate with actin via aldolase (Jewett and Sibley, 2003). As mentioned earlier, aldolase is a glycolytic enzyme that binds to actin filaments in vitro and in vivo (Arnold and Pette, 1968; Bronstein and Knull, 1981; Wang et al., 1996, 1997). Aldolase is tetrameric, and each monomer can bind to F-actin (Wang et al., 1997). The interaction between the MIC2 or TRAP CD with aldolase is direct, and involves a conserved tryptophan within the CD (Jewett and Sibley, 2003). The presence of additional aldolase-contacting residues has been described in Plasmodium TRAP (Buscaglia et al., 2003). It is likely that two non-contiguous acidic stretches in the TRAP tail interact with positively charged regions scattered throughout the surface of the aldolase. A specific association of F-actin, aldolase, and TRAP in a ternary complex has been demonstrated in Plasmodium (Buscaglia et al., 2003).

T. gondii aldolase displays an apically skewed localization in non-motile parasites, and undergoes a partial posterior redistribution during motility similar to that observed with MIC2 (Jewett and Sibley, 2003). No evidence for secretion of aldolase was seen, confirming that it functions within the parasite. These results may reflect the association of aldolase with the CD of MIC2 that extends from the micronemes into the cytosol. This was confirmed by ultrastructural studies in salivary gland Plasmodium sporozoites showing that aldolase localizes to the periphery of the secretory micronemes containing TRAP (Buscaglia et al., 2003). Thus, the interaction between aldolase and the MIC2 or TRAP tail likely takes place during or preceding the biogenesis of the micronemes. Upon contact with the targets cells, the parasite releases the contents of micronemes in the anterior pole of the parasite; MIC2-containing micronemes may fuse with the parasite membrane (Carruthers

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and Sibley, 1999), bringing the adhesive domains of MIC2 to the parasite surface and the aldolase– actin complex beneath the parasite plasma membrane. Aldolase thus provides a link between adhesins engaged with host-cell receptors and the actin cytoskeleton on which the myosin can walk, pushing the MIC2/aldolase complex toward the posterior end of the parasite. The mediators of actin polymerization in apicomplexan parasites are unknown. No homologs to any of the proteins that constitute an F-actin nucleating Arp2/3 complex have been identified.

Myosin A role for myosin in invasion and motility was initially suggested by studies using butanedione monoxime (BDM), an inhibitor of myosin ATPase (Dobrowolski et al., 1997a). Subsequently, a functional role of myosin was confirmed by conditional gene disruption of T. gondii MyoA (Meissner et al., 2002b). Depletion of MyoA completely abrogates the gliding motility of these parasites, and impairs both host-cell invasion and egress, whereas no effect on intracellular replication and secretion of micronemal proteins is observed. Members of the myosin superfamily are mechanoenzymes that convert chemical energy stored in ATP into force directed along actin filaments (Spudich, 1994). Myosin molecules are modular motors made up of three domains: the N-terminal domain is the actin-binding motor, the middle neck domain associates myosin light chain, and the divergent tail domain is thought to target a given myosin to its site of action and thereby reflects the specific task of the motor. The current repertoire of myosins in T. gondii is comprised of four genes giving rise to five distinct proteins (MyoA-E), all of class XIV, which is restricted to the Apicomplexa (Hettmann et al., 2000; Delbac et al., 2001; Heintzelman and Schwartzman, 2001). With a molecular mass ranging between 90 and 125 kDa, these myosins are the smallest molecular motors identified so far. By using a reverse genetic approach and generating a tail-specific antibody, Hettman and colleagues have shown that MyoA is distributed beneath the plasma membrane of tachyzoites (Hettmann et al., 2000). While MyoB and MyoE are not expressed in tachyzoites, MyoC appears to play a role in parasite division (Delbac *et al.*, 2001). MyoD appears enriched in the peripheral region of the parasite, but this is not as sharply defined as for MyoA (Delbac *et al.*, 2001).

The unique localization of MyoA under the plasma membrane places it in an ideal position to transmit mechanical energy for forward motion and cell invasion (Hettmann et al., 2000). Close homologs of MyoA are present in the genomes of Plasmodium spp. (Heintzelman and Schwartzman, 1997; Pinder et al., 1998; Hettmann et al., 2000; Matuschewski et al., 2001), Babesia, Cryptosporidium and Theileria. Actin and myosin share a very similar pattern beneath the plasma membrane. Consistent with this observation, T. gondii MyoA binds to F-actin in an ATP-dependent manner in vitro (Dobrowolski et al., 1997a; Hettmann et al., 2000; Herm-Gotz et al., 2002). Despite some unusual features, MyoA fulfils the biochemical requirements for a fast motor with a step size of 5.3 nm and a velocity of 5.2 µm/s towards the plus end of actin filaments, and theoretically would drive gliding motility at a speed of $3-5 \,\mu\text{m/s}$, which is in the observed range of speed of T. gondii tachyzoites (Herm-Gotz et al., 2002). The directionality of MyoA implies that the actin filaments are oriented with their plus ends toward the posterior pole.

A pair of basic residues in the tail of T. gondii MyoA is essential to target it to the periphery, and this localization is independent of the actin cytoskeleton (Hettmann et al., 2000) but is likely due to specific and saturable interaction with a component of the inner membrane complex. MyoA is associated with a light chain, MLC1, and two other proteins, GAP45 and GAP50, the latter being an integral protein that anchors the complex in the membrane of the IMC (Gaskins et al., 2004). Immunofluorescence analysis of transgenic parasites has shown that the complex is assembled in multiple stages during cell division in T. gondii (Gaskins et al., 2004). GAP50 is found in the inner membrane complex of both mature parasites and immature daughters; in contrast, MyoA, MLC1, and GAP45 are only found associated with the inner membrane complex of mature parasites, and are entirely absent from immature daughters.

All apicomplexan parasites for which sequence data are available possess highly similar orthologs of GAP45 and GAP50, suggesting that their function is conserved throughout the entire phylum. During their synthesis, MyoA, MLC1, and GAP45 are synthesized on cytoplasmic ribosomes and associated with each other into a complex, the proto-glideosome. GAP50, on the other hand, is co-translationally inserted into the parasite ER and is transported to the inner membrane complex where, in mature parasites, it associates with the MyoA/GAP45/MLC1 proto-glideosome to form the functional, membrane-associated glideosome.

In order to power an efficient posterior capping of MICs and propulsion of the parasite inside the cell, the glideosome must be immobilized within the plane of the membrane. This could be accomplished by direct or indirect interaction of the glideosome with stable elements of the *Toxoplasma* cytoskeleton. Freeze-fracture analysis of the inner membrane complex has revealed candidates that could fulfil this function in the form of alignments of intramembranous particles present in both membranes and distributed in a manner suggesting they are associated with both a submembrane skeleton and the microtubules (Morrissette *et al.*, 1997; Porchet and Torpier, 1977).

11.4.4.2 Proteolytic cleavages during invasion

MIC proteins are extensively processed on the parasite surface. These post-exocytosis processing events likely regulate adhesion, and permit the disruption of the adhesive MIC complexes and the dissociation of the parasite–host interaction at the end of the invasion process. Post-exocytosis proteolysis is achieved by at least three distinct protease activities, the proteases being known as microneme protein proteases 1, 2, and 3 (MPP1, MPP2, and MPP3) (Carruthers *et al.*, 2000; Zhou *et al.*, 2004).

MPP2 trims off a short N-terminal extension upstream of the A-domain from MIC2, and is responsible for multiple cleavages of M2AP within its C-terminal domain (Zhou *et al.*, 2004). Based on partial inhibition by chymostatin and PMSF, and nearly complete inhibition by ALLN and ALLM, MPP2 has been predicted to be a chymotrypsin-like serine protease or calpain-like cysteine protease. The global substrate repertoire of MPP2 was analyzed by comparison of ESA proteins from ALLN-treated parasites with ESA from untreated parasites (Zhou et al., 2004). This predicts that at least two additional MICs are also processed by MPP2. MPP2 is likely involved in the cleavage of the N- and C-terminus of MIC4, as previously suggested (Brecht et al., 2001), and in the processing of SUB1. MPP2 is likely an apical surface resident protease, since its cleavage products are only seen after microneme secretion and processing is markedly elevated by treatment with cytD, which blocks the capping of MICs toward the posterior end of the parasite and artificially traps surface MICs in the same vicinity, thereby facilitating processing before release into the supernatant (Carruthers et al., 2000; Zhou et al., 2004). MPP2 cleavage of MIC2 has been shown to facilitate interaction with host receptors or clustering of adhesive MICs on the parasite surface (Barragan et al., 2005). Indeed, MIC2 binds to ICAM-1, and this binding requires maturation by MPP2, presumably exposing the A-domain for interaction.

The most C-terminal cleavage of M2AP is not blocked by ALLN, suggesting the presence of a distinct proteolytic activity, MPP3 (Zhou *et al.*, 2004). Although MPP2 and MPP3 processing events have been proposed to regulate adhesive activity (Carruthers and Blackman, 2005), this hypothesis not been tested. Interestingly, parasites lacking SUB1 are devoid of MPP2 and MPP3 activity, based on analysis of MICs secreted from SUB1 KO parasites (Binder, Carruthers and Kim, unpublished). Whether SUB1 is directly responsible for MPP2 and/or MPP3 activities or indirectly regulates these proteases is under active investigation.

The post-exocytosis C-terminal cleavage of MIC2, MIC6, MIC8, MIC12, and AMA1 has been demonstrated (Carruthers *et al.*, 2000; Donahue *et al.*, 2000; Reiss *et al.*, 2001; Meissner *et al.*, 2002a; Opitz *et al.*, 2002). This event in MIC2 constitutes an essential step during host-cell invasion, since mutation-based impairment of MIC2 C-terminal proteolysis impairs host-cell entry (Brossier *et al.*, 2003). Genetic and biochemical evidence shows that C-terminal cleavage occurs within the transmembrane (TM) domain by regulated intramembrane proteolysis (RIP) (Opitz *et al.*, 2002; Zhou *et al.*, 2004; Howell *et al.*, 2005). The protease responsible for RIP is named MPP1, but it has not been unequivocally identified. MPP1 activity is blocked by the serine protease inhibitor 3,4-dichloroisocoumarin (DIC) (Howell *et al.*, 2005), which has also been found to inhibit host-cell invasion by *T. gondii* (Conseil *et al.*, 1999).

The TM cleavage site is conserved between transmembrane MICs of *Toxoplasma* and other Apicomplexa (Dowse *et al.*, 2005). These cleavage sites resemble the recognition sequence for rhomboid-like proteases, whose activity is also sensitive to DIC (Urban and Freeman, 2003). Rhomboids are multipass membrane serine proteases that cleave within the TM of their substrate. First evidence that a parasite rhomboid may cleave transmembrane MICs came from a study by Urban and Freeman (2003), showing that Rhomboid-1 from *Drosophila* and RHBDL2 from humans cleave chimeric proteins containing the TM domain of MIC2, MIC6 or MIC12 (Urban and Freeman, 2003).

Rhomboid-like genes are present in the genome of all apicomplexan parasites currently sequenced. T. gondii contains six rhomboid genes (ROM1-6) (Dowse and Soldati, 2005). ROM2 and ROM3 are mainly expressed in sporozoites, ruling out their role in MPP1 activities in all stages of the parasite (Brossier et al., 2005). MPP1 activity is predicted to be constitutive on the parasite surface (Opitz et al., 2002). ROM1 localizes to micronemes, and ROM4 and ROM5 are expressed at the plasma membrane (Brossier et al., 2005; Dowse et al., 2005). ROM5 is the best candidate for MPP1 activity. Indeed, ROM5 localizes at the posterior pole of the parasite, where the cleavage of MICs is thought to occur, and can cleave the transmembrane domains of MICs in vitro (Brossier et al., 2005).

11.4.5 Why does *T. gondii* exhibit this patchwork of MICs?

The vast MIC repertoire of *T. gondii* may be correlated with the broad host-cell specificity *in vitro* and the spreading of infection in all organs in toxoplasmosis, contrasting with the high cell and organ specificity found in *Plasmodium*. Apicomplexa show variable cell specificity, particularly at different stages of infection: specificity may be related to the MIC repertoire, quite different from one genus to the other, and dependent upon the stage in the life cycle.

MIC gene deletion in T. gondii has shown that at least some of the MICs are not essential for invasion in vitro (M2AP, MIC1, MIC3, MIC4, MIC5, MIC6) (Reiss et al., 2001; Huynh et al., 2003; Cerede et al., 2005), whereas complete abrogation of MIC2 and AMA1 could not be obtained (Huynh et al., 2004; Mital et al., 2005), thus demonstrating their essential character. MIC3 gene disruption does not modify fibroblast invasion, but induces a death delay in mice (Cerede et al., 2005). Consistent with this, MIC3 could be essential for invading specific cell types other than those that have been tested thus far *in vitro*. This hypothesis is supported by the demonstration that the binding ability of MIC3 to host cells is crucial for parasite virulence in vivo (Cerede et al., 2005). This shows also that the importance of MICs is better evaluated in vivo than in vitro. Indeed, motility, adhesion, and invasion, which are functions postulated for MIC proteins, are multifactorial phenomena, the expression of which is likely to differ in vivo from that which occurs in the static environment found in a culture dish.

Deletion of the *MIC1* gene results in defective targeting of both MIC4 and MIC6 in micronemes (Reiss *et al.*, 2001), and *mic1KO* parasites are 50 percent impaired in invasion (Cerede *et al.*, 2005). This defect in invasion can thus be assigned to the absence of the MIC1/4/6 complex, without further precision. As previously mentioned, only MIC1 binds host cell *in vitro* (Saouros *et al.*, 2005). Therefore, MIC6 might anchor the two other proteins and interact with the underlying motor, whereas MIC1 would establish specific interactions with host-cell receptors necessary for host-cell invasion.

The disruption of M2AP gives also a partial phenotype. M2AP knockout parasites were > 80 percent impaired in host-cell entry (Huynh *et al.*, 2003), and showed delayed death in mice (Harper and colleagues, submitted). In these parasites, MIC2 partially accumulates in the parasite endoplasmic reticulum and Golgi apparatus, and

is poorly secreted. This invasion defect is likely due to defective expression of the MIC2/M2AP complex.

The importance of MIC protein diversity has been further stressed by simultaneous disruption of MICs (Cerede *et al.*, 2005). Indeed, a spectacular decrease in virulence *in vivo* was observed by simultaneous disruption of the *MIC1* and *MIC3* genes (which corresponds in fact to an MIC1/3/4/6 functional KO). This result demonstrates that MICs have a synergistic effect on infection *in vivo*.

Essential MICs Repeated attempts to disrupt the gene encoding MIC2 were unsuccessful (L.D. Sibley, personal communication; Huynh et al., 2004), suggesting that, as for TRAP in Plasmodium sporozoites, MIC2 is an essential protein. Moreover, although parasites bearing a targeted deletion of the MIC2 C-domain could be initially detected by flow cytometry in a transfected population, they were rapidly lost upon further growth (Jewett and Sibley, 2004). Conditional knockdown of MIC2 (mic2KO) has been recently achieved (Huynh and Carruthers, 2006). These parasites express MIC2 at approximately 5 percent of normal levels, and were >80 percent impaired in invasion because of a defect in attachment. Interestingly, they also display almost exclusively one type of motion circular gliding.

mic2KO parasites are massively attenuated in mice, requiring a >600-fold higher inoculation for lethal infection. No defect in intracellular replication or egress was seen. Whether MIC2 is involved in the MJ formation has not been established, although it has been shown to occupy the interface between the parasite and host membranes during invasion (Carruthers et al., 1999b). In Plasmodium, the two adhesive motifs of TRAP have been suggested to be involved in MJ formation. Indeed, independent or simultaneous mutation in Plasmodium TRAP A-domain and TSP does not alter sporozoite motility, but specifically decreases (or abolishes in the case of simultaneous mutations) host-cell invasion in vitro and in vivo (Matuschewski et al., 2002). Therefore, gliding motility and host-cell invasion likely involve distinct extracellular associations, perhaps with invasion requiring stronger binding to host receptors than gliding motility needs.

AMA1 is an integral membrane protein first described in Plasmodium 15 years ago (Marshall et al., 1989). Like TRAP, it is a widely conserved apicomplexan MIC protein. The precise function of AMA1 is not known, but considerable evidence points toward a role in invasion. The demonstration of such a role came from the inhibitory effects of anti-Plasmodium AMA1 antibodies (Thomas et al., 1984; Gaffar et al., 2004; Silvie et al., 2004) or anti-Toxoplasma AMA1 antibodies (Hehl et al., 2000) on invasion. AMA1 was proposed to be involved in the formation of a tight binding interface between merozoite and erythrocyte surfaces (Mitchell et al., 2004). Indeed, the initial random surface attachment of merozoites to red blood cells is not affected by the presence of inhibitory antibodies, but the normal apical reorientation of merozoites does not occur and the close junctional contact (6 nm) is absent.

Consistent with this, Mital and colleagues generated a conditional knockdown of T. gondii AMA1 and showed that AMA1 is not involved in gliding motility, or in the initial step of attachment, or in microneme release, but fails to attach intimately to host cells (Mital et al., 2005). A role for T. gondii AMA1 in building the MJ has been confirmed by Alexander and colleagues, who showed that secreted AMA1 is associated at the parasite surface with a complex of secreted rhoptries neck proteins that relocalize at the MJ (Alexander et al., 2005). During invasion, AMA1 displays a surface localization with a high steadystate level (Howell et al., 2005). AMA-1 is present at the MJ, but the majority of AMA1 is clearly on both sides of this adhesion zone (Alexander et al., 2005). However, in knockdown parasites expressing a low level of AMA1, this protein precisely co-localizes with RON4 at the MJ.

AMA1-deficient parasites attach but do not invade (Mital *et al.*, 2005); they secrete RON4 apically, but the ring staining of RON4 is replaced by a bleb-like labeling (Alexander *et al.*, 2005). These studies suggest a model in which AMA1, with the cooperation of RONs, is involved in the formation of the MJ, being a major player in invasion. Intriguingly, however, AMA1 is not required for egress or for organizing the RON ring during this exit event (Alexander *et al.*, 2005), underscoring the emerging differences between invasion and egress that were previously unappreciated (Hoff and Carruthers, 2002). Together, findings from the conditional expression of MIC2 and AMA1 suggest that these proteins perform essential roles in distinct steps in *Toxoplasma* adhesion, with MIC2 functioning in attachment and AMA1 being required for formation of the tight binding interface at the MJ.

11.5 RHOPTRIES

11.5.1 Biogenesis of rhoptries and traffic of ROPs in the secretory pathway

Rhoptries are present at a multiplicity of 6–12 per cell in *T. gondii*. Rhoptries are club-shaped organelles with a bulbous base and an extended duct that reaches to the anterior pole of parasite. They are formed first as immature rhoptries (or pre-rhoptries) (see Chapter 2).

Rhoptries and pre-rhoptries are thought to be the only acidified organelles in the parasite (Shaw et al., 1998) and are packaged with specialized hydrolases (Que et al., 2002), characteristics of a secretory lysosome. Rhoptries are hypothesized to be derived from both the secretory and endocytic pathways (Ngo et al., 2004; Yang et al., 2004). Members of the ROP2 gene family (ROP2, ROP4, ROP7, etc. see below) are type 1 transmembrane proteins, which contain in their cytoplasmic tails both tyrosinebased and dileucine sorting signals (Hoppe et al., 2000; Ngo et al., 2003). Mutation of either of these signals diminishes the delivery of ROP2 or ROP4 to mature rhoptries, and proteins accumulate in a multivesicular body (MVB). This compartment is distinct from the dense granules, micronemes, and Golgi/TGN, and is co-localized with a multivesicular endosomal compartment marked by the endosomal marker VPS4 (vacuolar protein sorting 4).

These results suggested that rhoptry targeting occurs along the endocytic pathway and is mediated by adaptins. Consistent with this, the YXX ϕ motif facilitates protein sorting by binding to the *T. gondii* μ -chain component (Tgµ1) of the AP-1 clathrin adaptator (Hoppe et al., 2000). In mammalian cells, AP-1 is an essential regulator of membrane trafficking by mediating budding of clathrin-coated vesicles from the TGN, immature secretory granules, and endosomes. Tgu1 localizes to Golgi/TGN, to juxtaposed coated Golgi-associated vesicles, and both the rhoptry membrane and membranous lumen. Dominant-negative expression of Tgu1 disrupts rhoptry sorting and rhoptry biogenesis (Ngo et al., 2003). Large tubular and multivesicular structures were mostly observed, and ROP2 accumulated in MBV resembling the vesicles to which ROP2 with cytoplasmic targeting signal mutations localized. There was no significant alteration of dense granules and micronemes. These results indicate that ROPs are delivered from the TGN to an endosomal compartment and subsequently to the mature rhoptry. A direct transport of ROPs from the TGN to rhoptries is not favored because tyrosine-based sorting mutations in ROP2 do not retain the protein in the TGN. It was then proposed that rhoptries may be analogous to secretory lysosomes (Ngo et al., 2004).

As for MICs, tyrosine-based sorting is not applicable to soluble rhoptry proteins. Mapping of the domains involved in targeting of soluble ROP1 involved two domains, the propeptide of ROP1 and a central peptide (Bradley and Boothroyd, 2001; Striepen et al., 2001). An escorter process similar to that used for soluble MICs proteins has not been described for rhoptries in T. gondii, but exists in *Plasmodium*, in which soluble rhoptry proteins RAP2 and RAP3 form an oligomeric complex with the transmembrane RAP1 protein (Baldi et al., 2000). In the course of identification of signals responsible for sorting into specific compartments with a green fluorescent protein (GFP)-based motif-trap, Bradley and colleagues have shown that a ROP4-GFP fusion lacking the two previously identified targeting signals (a dileucine and YXX**(**) motif) is correctly delivered to rhoptries (Bradley et al., 2004). Similarly, ROP17 and ROP18, two others members of the ROP2 family, localize to rhoptries although they do not possess these motifs (El Hajj et al., unpublished). These results clearly demonstrate that multiple mechanisms for targeting to rhoptries exist.

Most characterized *T. gondii* rhoptry proteins are proteolytically cleaved during transit in the secretory pathway. The importance of the cleavage site in trafficking is unknown; it is just reported that targetting of ROP1 to rhoptries does not depend on its proteolytic processing (Bradley *et al.*, 2002). The processing of ROP1 is blocked by brefeldin A (Soldati *et al.*, 1998) and is not inhibited by the dominantnegative Tgµ1 construct (Ngo *et al.*, 2003), indicating that the processing event occurs post-Golgi and before the intervention of the AP-1 complex. The maturases involved are unknown, but potential candidates are discussed later in this chapter.

11.5.2 ROPs and RONs

Identification of the major components of the rhoptries in T. gondii has been obtained by subcellular fractionation and generation of monoclonal antibodies (Leriche and Subremetz, 1991; see also Table 11.2). More recently, purified rhoptry proteomics has allowed the characterization of 38 previously unidentified proteins (Bradley et al., 2005), of which some have been confirmed to be localized in the organelles. All of the ROPs contain a signal peptide and many have at least one predicted transmembrane domain or a GPI anchor, suggesting an association with membranes. In addition, rhoptry proteomic analysis has led to the characterization of proteins specifically localized in the rhoptry neck - i.e. RONs. Many ROPs and RONs contain repeated motifs that may be involved in protein-protein interactions.

Virtually all the information regarding the contents or functions of the rhoptries has come from the tachyzoite stage. Only one bradyzoite-specific ROP has been described (Schwarz *et al.*, 2005). This specific bradyzoite rhoptry protein 1 (BRP1) was identified by a bioinformatic analysis of previously identified genes that are highly expressed during bradyzoite development and prediction of genes encoding secretory proteins (Cleary *et al.*, 2002; Schwarz *et al.*, 2005). BRP1 is also expressed in the merozoite stages in the gut of infected cats. The only homolog known is in the closely related parasite *N. caninum. In vitro* and *in vivo* analysis of *BRP1* knockout parasites show that

BRP1 does not play an essential role in development of the bradyzoite stage, development of brain cysts, or oral infection of new hosts.

11.5.2.1 ROP2 family

The ROP2 family was first described as three rhoptry proteins recognized by a single Mab (Sadak et al., 1988). Additional members of the family have been successively identified using various approaches. El Hajj and colleagues used proteomics, in silico searches, and gene cloning to obtain a comprehensive survey on the family that they described as comprising at least 12 members sharing several common features such as a C-terminal putative transmembrane domain, a similar size (50-kDa range), and a basic aa-rich N-terminal area (El Hajj et al., 2006a). The most intriguing feature of the family, however, is the presence in the C-terminus of the protein kinase-like domain. Most of the members lack the glycine loops and the conserved aspartic acid in the catalytic loop critical for phosphotransferase activity. The exceptions are ROP17 and ROP18, this latter having been shown to be capable of phosphorylation in vitro (El Hajj, 2006b). The family comprises the closely related ROP2, ROP4, ROP7, and ROP8, and the more distantly related ROP5, ROP11, ROP17, and ROP18, along with four other members dubbed ROP2-like proteins awaiting further characterization.

11.5.2.2 Proteases

Rhoptries contain several kinds of proteases. A subtilisin-like serine protease or subtilase (SUB2) has been identified in *T. gondii* rhoptries by homology to *P. falciparum* SUB2 (Miller *et al.*, 2003). Another rhoptry serine protease has been identified in the proteome study (Bradley *et al.*, 2005). SUB2 is predicted to be a type 1 transmembrane protein with a conserved catalytic domain. It is autocatalytically processed at the N-terminus. The mapping of the endogenous cleavage site of SUB2, harboring a similar cleavage site to ROP cleavage sites, supports the proposal that TgSUB2 may also cleave ROP1 and proteins of the ROP2 family that are processed *en route* to rhoptries (Bradley and Boothroyd, 1999;

Location/ protein	Calculated MW (kDa) ¹	Domains (no.) ²	Interacting partners	Mutant phenotypes	Function	Post-secretory trafficking	References
Rhoptry							
Rhoptry body							
ROP1	46	Strong charge asymmetry		Non-essential protein; KO shows normal growth, invasion and virulence, but abnormal rhoptry morphology		Associated with the PVM	Ossorio <i>et al.</i> , 1992; Saffer <i>et al.</i> , 1992; Soldati <i>et al.</i> , 1995
ROP2	64	Degenerate kinase (1), TM (1)		Essential protein; abnormal rhoptry morphology in ROP2 antisense treatment	PVM-host mito- chondria association	Associated with the PVM	Sadak <i>et al.</i> , 1988; Beckers <i>et al.</i> , 1994; Sinai and Joiner, 2001; Nakaar <i>et al.</i> , 2003
ROP4 (ROP2 family)	64	Degenerate kinase (1), TM (1)				Associated with the PVM and phosphorylated	Carey <i>et al.</i> , 2004b
ROP5 (ROP2 family)	61	Degenerate kinase (1), TM (1)				Associated with the PVM	Leriche and Dubremetz, 1991; Bradley <i>et al.</i> , 2005; El Hajj <i>et al.</i> , 2006a
ROP7 (ROP2 family)	63	Degenerate kinase (1), TM (1)				Associated with the PVM	Bradley <i>et al.</i> , 2005; Hajj <i>et al.</i> , 2005

TABLE 11.2 Properties of Toxoplasma secretory proteins – rhoptry proteins

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Location/ protein	Calculated MW (kDa) ¹	Domains (no.) ²	Interacting partners	Mutant phenotypes	Function	Post-secretory trafficking	References
ROP8 (ROP2 family)	64	Degenerate kinase (1), TM (1	 _)				Beckers <i>et al.</i> , 1994
ROP9 (P36)	39						Reichmann <i>et al.</i> , 2002
ROP10	61						Bradley <i>et al.,</i> 2005
ROP11 (ROP2 family)	57	Degenerate kinase (1), TM (1	1)				Bradley <i>et al.,</i> 2005
ROP12	25						Bradley <i>et al.</i> , 2005
ROP13	45						Bradley <i>et al.</i> , 2005
ROP14	122	TM (2), DUF1222 (1)					Bradley <i>et al.</i> , 2005
ROP15	34						Bradley <i>et al.</i> , 2005
ROP16 (ROP2 family)	76	Putative kinase (1) TM (1)				Associated with the host cell nucleus	Bradley <i>et al.</i> , 2005; Coller <i>et al.</i> , 2005
ROP17 (ROP2 family)	2 70	Putative kinase (1), TM (1)				Bradley <i>et al.,</i> 2005; El Hajj and Dubremetz,
ROP18 (ROP2 family)	62	Putative kinase (1), TM (1)				unpublished Bradley <i>et al.</i> , 2005; El Hajj <i>et al.</i> , 2006b

TABLE 11.2 Properties of Toxoplasma secretory proteins – rhoptry proteins—cont'd

SUB2	142	Subtilase (1), TM (1)	Essential protein; abnormal rhoptry formation in SUB2 antisense treatment	Rhoptry protein maturase		Miller <i>et al.</i> , 2003; Binder and Kim, 2004	
Toxopain-1 (cathepsin protease B)	62	Cathepsin (Clan CA)		Rhoptry protein maturase; rhoptry biogenesis		Que <i>et al.,</i> 2002; Shaw <i>et al.,</i> 2002	
Insulinase	177	Metalloprotease (M16 family)		Proteolysis	Associated with the PVM	Bradley <i>et al.</i> , 2005; Lebrun <i>et al.</i> , unpublished	
Toxofilin	27			Actin binding		Poupel <i>et al.,</i> 2000; Bradley <i>et al.,</i> 2005	RHOPT
Rab11 (associated with the cytoplasmic side of the rhoptry membrane)	25	Small GTPase		Vesicular trafficking?		Bradley <i>et al.,</i> 2005	RIES
NHE2	91	Sodium hygrogen exchanger	Non- essential protein; KO is defective in calcium- dependent egress	Ion homeostasis?		Karasov <i>et al.,</i> 2005	
BRP1 (bradyzoite- specific rhoptry protein 1)	18		-			Schwarz <i>et al.</i> , 2005	29

Continued

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Location/ protein	Calculated MW (kDa) ¹	Domains (no.) ²	Interacting partners	Mutant phenotypes	Function	Post-secretory trafficking	References
Rhoptry neck							
RON1	127	TM (1), CobT (1), CCP (1)					Bradley <i>et al</i> ., 2005
RON2	155	TM (2)	RON4, RON5, AMA1				Bradley <i>et al.</i> , 2005; Lebrun <i>et al.</i> , 2005
RON3	223	TM (1)					Bradley <i>et al.,</i> 2005
RON4	107		RON2, RON5, AMA1			MJ localization and posterior translocation	Bradley <i>et al.</i> , 2005; Lebrun <i>et al.</i> , 2005
RON5	179		RON2, RON4, AMA1				Bradley <i>et al.</i> , 2005; Lebrun <i>et al.</i> , 2005

 TABLE 11.2 Properties of Toxoplasma secretory proteins – rhoptry proteins—cont'd

¹Based on the complete open reading frame including signal sequence or GPI anchor signal, if present.

²Abbreviations: CCP, domain abundant in complement control proteins; CobT, cobalamin biosynthesis protein; DUF1222, domain of unknown function; TM, transmembrane.

El Hajj *et al.*, unpublished). Interestingly, SUB2 co-immunoprecipitates with ROP1 (Miller *et al.*, 2003) and also with ROP2 and ROP4 (Binder and Kim, 2004). These results suggest that SUB2 is a rhoptry protein maturase responsible for cleaving ROP1 and ROP2 family members. Consistent with this hypothesis, ROP5, a member of the ROP2 family, does not possess the SWLE/QE motif and is not cleaved (El Hajj *et al.*, 2006c). A knockout of SUB2 could not be obtained (Miller *et al.*, 2003), but SUB2 antisense treatment of parasites induced abnormal rhoptry formation (Binder and Kim, 2004), accumulation of vesicular structures, and impaired replication, leading to the hypothesis that SUB2 is involved in organelle biogenesis.

Based on homology with eukaryotic cysteine proteinases, a cathepsin B-like protein named toxopain-1 or cathepsin protease B (CPB) has been identified in T. gondii rhoptries (Que et al., 2002). CPB is produced as pro-protein that is autocatalytically cleaved to generate an active enzyme. A role of CPB in rhoptry protein processing and rhoptry biogenesis has been suggested. Indeed, inhibition of cysteine protease activity leads to an inactive CPB, a delay in ROP2 family protein processing, abnormal rhoptry biogenesis and reduced invasion (Que et al., 2002). Since uncharacterized cathepsin homologs are also present in the Toxoplasma database, further studies will be necessary to confirm the link between CPB and ROP protein processing which seems crucial for rhoptry biogenesis (Shaw et al., 2002).

An insulinase-like protein has been identified in the rhoptry fraction (Bradley *et al.*, 2005). This protein belongs to the M16 family of metalloprotease that generally depend on divalent cations for their activity (Rawlings *et al.*, 1991). The function of this insulinase is unknown.

11.5.2.3 Other ROPs with homologs or domains present in eukaryotic cells

Rab11 is found in purified rhoptries (Bradley *et al.*, 2005). In higher eukaryotes, Rab11 belongs to the family of small GTPases involved in the regulation of vesicular traffic. It is usually localized at early endosomes and perinuclear recycling endosomes, as well as at the trans-Golgi network, and it is

considered to control slow endosomal recycling, as well as traffic to the Golgi apparatus (Ullrich *et al.*, 1996; Chen *et al.*, 1998). *Toxoplasma* Rab11 is likely associated with the cytoplasmic side of the rhoptry membrane through geranyl–geranyl modification of the two cysteines of the CCXX site, a usual feature of Rab11 (Bradley *et al.*, 2005). It is therefore proposed that Rab11 may act as a regulator of trafficking to the rhoptries (Bradley *et al.*, 2005).

In searches of the Toxoplasma genome, three homologs of sodium-hydrogen exchangers have been found. These proteins catalyze the Na⁺/H⁺ exchange, and are involved in regulation of internal pH and cell volume. One of them, NHE2, is associated with rhoptries (Karasov et al., 2005). The pH of rhoptries changes from acidic (estimated to be approximately pH 3.5-5.5) for nascent rhoptries to more neutral (approximately pH 5.0-7.0) in mature organelles (Shaw et al., 2002). It is therefore hypothesized that NHE2 may be involved in pH regulation during the course of rhoptry biogenesis and rhoptry protein processing. However, the disruption of NHE2 does not affect targeting of several rhoptry proteins, including ROP1 and ROP2/3/4, nor does it alter egress or rate of growth (Karasov et al., 2005). In addition, in vivo studies of virulence yielded no difference compared to a wild-type strain.

The presence of a phosphatase 2C in rhoptries is very attractive (Bradley *et al.*, 2005). PP2C-type protein phosphatases are monomeric enzymes present in both prokaryotes and eukaryotes. Members of this family of phosphoprotein phosphatases are involved in the regulation of several signaling pathways, including regulation of the cell cycle, adaptation, and cell recovery after DNA double-strand breaks or environmental stress response (Schweighofer *et al.*, 2004).

ROP16 is a 76-kDa protein which contains a putative kinase domain in its C-terminal (Bradley *et al.*, 2005). As for ROP17 and ROP18, it displays all the signatures of an active kinase (http://www.expasy. ch/prosite/).

11.5.2.4 ROPs of unknown function restricted to Toxoplasma

There is a group of proteins that have no homologs outside of *Toxoplasma*. This includes the ROP2

family proteins, ROP1, ROP10, ROP12, ROP13, ROP15, ROP17, and toxofilin (Ossorio *et al.*, 1992; Bradley *et al.*, 2005). It can therefore be suggested that these proteins have been subjected to evolutionary pressure and may have derived from some specialized property of these organisms, such as their ability to form tissues cysts or from their complex heteroxenous life cycle, especially with their very unusual proliferation as tachyzoites (by endodyogeny) that is unique to *Toxoplasma* and its nearest relatives.

ROP1 was the first ROP described in Toxoplasma (Ossorio et al., 1992). It is a soluble protein whose function is as yet unknown. Although it has no transmembrane anchor, it is associated with the PVM after invasion (Saffer et al., 1992) and then later is completely degraded, suggesting that by then it has fulfilled its role. ROP1KO parasites are not impaired in growth, invasion, or virulence, but do show altered rhoptry ultrastructure (Kim et al., 1993; Soldati et al., 1995). Toxofilin is a 27-kDa protein that has been shown to bind mammalian G-actin (Poupel et al., 2000). It is an actin sequestering protein that caps actin filaments. Toxofilin has been suggested to be present in the cytosol of the apical end of the parasite and involved in control of parasite actin polymerization during invasion and motility (Poupel et al., 2000). More recently, toxofilin has been localized in rhoptries, suggesting it may be secreted and play a role in host-cell actin polymerization (Bradley et al., 2005). In vitro, the control of actin dynamics by toxofilin has been shown to depend on a casein kinase II and a phosphatase 2C (Delorme et al., 2003).

11.5.2.5 ROPs of unknown function restricted to Apicomplexa

Until the recent work by Bradley and colleague, there were no homologies described for rhoptry proteins within Apicomplexa, except for *T. gondii* ROP9 (P36) (Reichmann *et al.*, 2002), which has a homolog in *Plasmodium*. This is fairly surprising, since these organelles are likely to perform the same function. Indeed, in both *Plasmodium* and *Toxoplasma*, membranous structures containing protein and lipids are secreted from the rhoptries during invasion and are incorporated into the expanding PVM (Bannister *et al.*, 1986; Hakansson *et al.*, 2001). The proteomic study by Bradley and colleagues identified some ROPs (ROP9 and ROP14) that have homologs in other Apicomplexa (Bradley *et al.*, 2005). ROP14 also shows homologies with a hypothetical membrane protein of mammalian cells. Other hypothetical proteins found in the *T. gondii* rhoptry fraction have apicomplexan homologs and may have conserved roles; however, their localization in the organelle has not been confirmed.

A specific group of proteins showing homologies restricted to Apicomplexa is located in the rhoptry neck (Bradley *et al.*, 2005). As mentioned above, these proteins are named RONs, for rhoptry neck proteins (RON1–4). RON2 and RON4 associate as a complex, with a third partner (Tg TwinScan_4705), which has not been clearly located but has been tentatively named RON5. RON4 is a 109-kDa soluble protein; RON2 and RON5 possess two and three putative transmembrane domains respectively, in addition to the signal peptide. *Plasmodium* spp., *Eimeria, Neospora*, and *Theileria* possess homologs for each of these RONs.

11.5.2.6 Lipids

T. gondii rhoptries also contain lipids, including large amounts of cholesterol and phospholipids (Foussard et al., 1991). Phosphatidylcholine is the major rhoptry phospholipids, and significant amounts of phosphatidic acid and lysophospholipids were also found, but not phosphatidylserine, phosphatidylinositol, or sphingomyelin. The lipid to protein ratio is estimated to be 0.26, and the cholesterol to lipid molar ratio is 1.5 to 1 (Foussard et al., 1991). This ratio indicates that lipids stored within rhoptries are present in a non-lamellar state. It has been suggested that secretion of phosphatidic acid and lysophospholipids may facilitate initial vacuole membrane formation (Foussard et al., 1991). A recent report establishes that cholesterol is present in the PVM at the time of Toxoplasma invasion, and that parasites depleted of rhoptry cholesterol (16-23 percent) are still able to invade cells (Coppens and Joiner, 2003). These studies suggest that PVM cholesterol is largely derived from the host and that the contribution of parasites is minor, but does not exclude an essential role for cholesterol in PVM formation.

11.5.3 Secretion of rhoptries and post-secretion trafficking of ROPs and RONs

Rhoptry discharge occurs early during the invasion process. Electron microscopic observations show empty rhoptries as early as the initial apical contact creating the MJ. The signals and mechanisms of rhoptry contents secretion are still entirely unknown. The importance of this secretion into the host-cell cytosol and/or membrane during invasion has been recently documented (Mital et al., 2005). Indeed, ROP protein discharge is reduced in the absence of AMA1, and these parasites fail to invade. These results indicate that the intimate attachment of the parasite to the host cell is a prerequisite to efficient rhoptry bulb secretion. Whether RON and ROP protein discharge are simultaneous or sequential is not known, although some evidence suggest simultaneity (Lebrun et al., 2005). Co-immunostaining of MICs and RONs show that MICs are exocytosed before RONs (Lebrun et al., 2005). How ROPs are introduced in the cell is unknown. Details of rhoptry structure during redcell invasion by Plasmodium show a dome-like structure of the red-cell membrane that enters the tip of the rhoptry duct which is difficult to interpret (Bannister and Mitchell, 1989). Once secreted, rhoptry proteins associate with different compartments and most likely perform a wide variety of roles:

- · RONs remain associated with the MJ
- some ROPs associate with the PVM, and
- other ROPs are found in the hostcell nucleus (Coller *et al.*, 2005; Gilbert *et al.*, 2006).

11.5.3.1 Moving junction-associated proteins

The RON4 protein is secreted during invasion and perfectly follows the invasion progress, being strictly associated with the MJ, with no spreading on the posterior area of the parasite as observed for MICs (Alexander *et al.*, 2005; Lebrun *et al.*, 2005). The RON4 sequence shows no transmembrane domain, suggesting that it must be secreted in the space between the parasite and host membrane, and could therefore bridge molecules from both sides. This is confirmed by accessibility of RON4 to antibodies in the absence of permeabilization (Alexander *et al.*, 2005; Lebrun *et al.*, 2005). The presence of RON2 and RON5 at the junction has not been demonstrated. Their accessibility to antibodies may be restricted by transmembrane insertion. If RON4 plays a part in bridging the parasite and host-cell surfaces, the counterparts of such a junctional protein on the host-cell side and parasite side must also be identified.

The microneme protein AMA1, which is secreted during invasion and relocalizes on the parasite surface, becomes associated with the RON complex. AMA1 is therefore likely to anchor the RON complex on the parasite surface (Alexander et al., 2005). RON2 could also be inserted into the parasite surface and play a similar role. On the host-cell side, either RON4 or another member of the complex could bind a cell surface molecule on which the force propelling the parasite inside the vacuole would be exerted. This transmembrane molecule could be a host-cell protein or, alternatively, a parasite protein (perhaps a member of the complex) inserted in the host-cell membrane (Lebrun et al., 2005). In the latter case, the parasite would generate all the junction machinery, just as some bacteria, like enteropathic E. coli (EPEC), insert their own receptor in the host cell to build up their pedestal (Kenny et al., 1997).

An attractive hypothesis is that AMA1, which is a type 1 transmembrane protein sharing homologies with MIC2 in its CD, could interact with the glideosome to ensure the translocation of the MJ during invasion. However, AMA1 does not possess in its C-terminus the critical tryptophan seen in TRAP, TRAP-C1, MIC2, MIC6, MIC8, and MIC12 that is necessary for interaction with submembranous cytoskeleton machinery (Jewett and Sibley, 2003). Its C-domain is 20 amino acids longer than most TRAP family C-domains, and it is not required for either form of gliding motility (Mital *et al.*, 2005). Therefore an indirect interaction seems

more likely, perhaps through interactions between the AMA1/RON complex and other TM MICs occupying the MJ region.

At the MJ, host-cell proteins are segregated depending on their anchoring in the membrane, leading to the exclusion of transmembrane proteins but not GPI-anchored proteins from the forming parasitophorous vacuole (Mordue *et al.*, 1999b). Whether the RONs participate in the selective segregation is as yet unknown. Likewise, whether the junctional complex facilitates PV closure and fusion remains to be investigated.

11.5.3.2 PVM-associated ROPs

Rhoptry-derived vesicles are secreted in host-cell cytoplasm. These vesicles contain lipid components, derived presumably from the rhoptry lipids; and some rhoptry proteins, such as ROP1, and proteins of the ROP2 family (at least ROP2, ROP4, ROP5, and ROP7) (Saffer *et al.*, 1992; Beckers *et al.*, 1994; Carey *et al.*, 2004b; El Hajj *et al.*, 2005, 2006c). They are likely to contain other rhoptry components as well. These vesicles fuse with the nascent PVM (Hakansson *et al.*, 2001), and ROPs proteins become exposed to the host-cell side (Beckers *et al.*, 1994; Carey *et al.*, 2004b).

The role of the PVM-associated ROPs is not clear. In any intracellular parasite surrounded by a vacuolar membrane, the membrane serves as a critical functional interface between the parasite and the host-cell cytoplasm. For T. gondii, nutrient import occurs across the PVM; host mitochondria and endoplasmic reticulum are recruited and physically tethered to the PVM (Sinai et al., 1997). The host-cell microtubules (Melo et al., 2001; Romano et al., 2005) and the intermediate filament network are reorganized around the PVM (Halonen and Weidner, 1994), and the intracellular parasite is capable of inhibiting host-cell apoptosis from within the PVM. Transmembrane rhoptry proteins of the PVM are well situated to play an important role(s) in PVM function. The punctate or ribbon-like distribution of ROP2 family proteins in the PVM suggested that these proteins are organized in higher order structures in the PVM and may form a pore-like structure involved in nutrient acquisition (Schwab *et al.*, 1994).

ROP2 has been shown to be one of the mediators of the PVM-organelle association, which may ensure lipid transfer in a manner analogous to traffic of lipids on sites of membrane continuity between mitochondria and ER (Sinai et al., 1997; Sinai and Joiner, 2001). The N-terminal domain of ROP2, which contains a characteristic mitochondrial targeting signal, is exposed to the host-cell cytosol and allows the recruitment of mitochondria and endoplasmic reticulum to the PVM (Sinai et al., 1997; Sinai and Joiner, 2001). Insertion in the mitochondria involved the 30 N-terminal aa, and an unidentified ER-targeting signal is also present in ROP2 (Sinai and Joiner, 2001). The mechanism of mitochondrial membrane insertion of the peptide is not clearly known, but seems to occur by an unconventional import pathway.

ROP2 is described as a PVM-integral transmembrane protein, with the C-terminal tail exposed in the vacuolar space (Beckers et al., 1994). The same topology has been observed for ROP4 (El Hajj and Dubremetz, unpublished), but ROP5 is found inserted in the PVM, exposing its C-terminal tail to the host cytosol, in a reverse topology compared to ROP2 and ROP4 (El Hajj et al., 2006c). How and why these related proteins adopt inverted topologies remains unexplained. Moreover, the putative transmembrane anchor of these proteins is located within the kinase-like domain in a position that is normally buried within the hydrophobic core of the protein (pfam00069.11) (El Hajj et al., 2006c). Whether this domain unfolds to expose this putative anchoring element remains to be determined.

ROP4 has recently been shown to be phosphorylated on several serine/threonine residues inside the infected cell after secretion (Carey *et al.*, 2004b). The results suggest that ROP4 is a target for host or parasite kinases that are activated in response to invasion. Identification of this kinase(s) will be an important area for future investigation.

ROP2 has been shown to be essential. Indeed, targeted depletion of ROP2 using a ribozymemodified antisense RNA strategy results in disruption of rhoptry biogenesis, impairment of cytokinesis, reduction in the association of host-cell mitochondria with the PVM, reduced sterol uptake from the host cell, reduced capacity of parasites to invade and replicate in human fibroblasts, and attenuation of virulence in mice (Nakaar *et al.*, 2003). The importance of ROP2 and the fact that the parasite is apparently synthesizing simultaneously several homologs suggest that they serve crucial functions, yet the apparent indispensability of ROP2 suggests they may not complement one another and raises questions on their individual roles. ROP5 may also be essential, as several attempts at *ROP5* gene deletion have failed (El Hajj

et al., 2006c). ROP4 probably fulfils a redundant role, since a functional knockout obtained by a recent GFP protein trap screen suggests that it is dispensable (Bradley *et al.*, 2004).

11.5.3.3 ROPs targeting to the host nucleus

Cells infected with T. gondii undergo up- or downregulation of a subset of genes and, depending of the virulence, different T. gondii strains show dramatic differences in the ability to modulate host-cell genes. These genes encode chemokines (GRO1, GRO2, LIF, and MCP1), cytokines (IL-1β, IL-6), cell matrix and adhesion proteins (ICAM1 and matrix metalloproteinase 3), apoptotic (superoxide dismutase 2) and transcriptional regulatory factors (REL-B, NF-κB p105, I-κBα) (Blader et al., 2001). Many of these gene modulations are dependent on direct interaction with tachyzoites, and are not triggered by soluble secreted products. These results reveal that parasite molecules may interfere with the host-cell machinery, but the T. gondii proteins involved in such host gene expression control are unknown.

Two recent reports indicate that rhoptry proteins may participate in this host modulation. Indeed, the rhoptry TgPP2C is targeted to the host nucleus early following infection, suggesting that it may interact directly with host-cell molecules and control of the host-cell cycle (Gilbert *et al.*, 2005). Similarly, the rhoptry protein ROP16 localizes to the host nucleus (Coller *et al.*, 2005). Consistent with its nuclear location, ROP16 contains a nuclear import signal. In addition, it possesses a kinase domain likely to phosphorylate

host-cell molecules and then interfere with host-cell functions. Interestingly, the *ROP16* gene belongs to a locus containing several genes involved in manipulation of host-cell gene expression (Coller *et al.*, 2005).

11.6 DENSE GRANULES

11.6.1 The dense-granule proteins: GRAs and others

Characterization of dense-granule molecules started with the production of monoclonal antibodies against in vitro excreted-secreted antigens (ESA) and subcellular fractionation of tachyzoites (Cesbron-Delauw et al., 1989; Charif et al., 1990; Leriche and Dubremetz, 1991). The list of densegranule proteins that have been identified and characterized has grown steadily over the past 15 years since Cesbron-Delauw and colleagues first described P23 (Cesbron-Delauw et al., 1989), which was later renamed GRA1 according to the nomenclature proposed by Sibley et al. (1991) (see Table 11.3). Dense-granule proteins constitute a group of relatively small proteins, all presenting an N-terminal hydrophobic sequence which fits the characteristics of a signal peptide (for review, see Mercier et al., 2005). Except for a few where a specific enzymatic or regulatory function has been defined (see below), the majority of dense-granule proteins do not present any significant similarity either to each other or to proteins with known function. These were named GRA proteins solely on the basis of their subcellular localization within the dense granules of the tachyzoite stage (Sibley et al., 1991). Up to now, nine proteins given the GRA designation have been characterized (Mercier et al., 2005).

GRA1 is an abundant protein – approximately 2 percent of the total ESTs derived from an RH strain cDNA library (Ajioka *et al.*, 1998) – that is soluble and characterized by two predicted EF-Hand domains (aa 149–180 and 197–223, respectively) whose calcium-binding property was confirmed experimentally (Cesbron-Delauw *et al.*, 1989). With the exception of GRA1, all GRA proteins described so far

Location/ protein	Calculated MW (kDa) ¹	Domains (no.) ²	Interacting partners	Mutant phenotypes	Function	Post-secretory trafficking	References
Dense grant	ule						
GRA1	20	EF-Hand (2)			Calcium binding	Lumen of the PV or loosely associated with MNN	Cesbron- Delauw <i>et al.</i> , 1989; Charif <i>et al.</i> , 1990; Dubremetz <i>et al.</i> , 1993
GRA2	20	Amphipathic α-helix (2)	GRA4, GRA6	Non-essential protein; KO mutant shows complete disruption of the tubular architecture of MNN; less virulent in mice	MNN architecture	Associated with MNN	Charif <i>et al.</i> , 1990; Dubremetz <i>et al.</i> , 1993; Mercier <i>et al.</i> , 1998a, 1998b, 2002; Labruyere <i>et al.</i> , 1999
GRA3	24	TM (1)				Associated with the MNN and PVM	Achbarou <i>et al.</i> , 1991b; Dubremetz <i>et al.</i> , 1993; Henriquez <i>et al.</i> , 1991
GRA4	36	TM (1), ATP/GTP binding domain (1)	GRA2, GRA6			Associated with the MNN	Mevelec <i>et al.,</i> 1992; Labruyere <i>et al.,</i> 1999
GRA5	13	TM (1)		KO mutants display no obvious phenotype		Associated with the PVM	Lecordier <i>et al.</i> , 1993, 1999; Mercier <i>et al.</i> , 2001
GRA6	24	TM (1)	GRA2, GRA4	KO mutant results in vesiculation of the MNN;	MNN architecture	Associated with the MNN	Lecordier <i>et al.</i> , 1995; Labruyere <i>et al.</i> , 1999,

 TABLE 11.3 Properties of Toxoplasma secretory proteins – dense-granule proteins

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			less virulent in mice			Mercier <i>et al.</i> , unpublished
GRA7	26	TM (1), RGD (1)			Associated with the MNN and PVM	Bonhomme <i>et al.</i> , 1998; Fischer <i>et al.</i> , 1998; Jacobs <i>et al.</i> , 1998
GRA8	29	TM (1)			Associated with the PVM	Carey <i>et al.</i> , 2000
GRA9	35	Amphipathic α-helix (1)			Associated with the MNN	Adjogble <i>et al.</i> , 2004
NTPase I	69		Targeted reduction by antisense RNA inhibits parasite proliferation	Nucleotide triphos- phatase, egress	Lumen of the PV or loosely associated with MNN	Bermudes <i>et al.</i> , 1994; Sibley <i>et al.</i> , 1994; Asai <i>et al.</i> , 1995
NTPase II	70			Nucleotide triphos- phatase, egress	Lumen of the PV or loosely associated with MNN	Asai <i>et al.</i> , 1995
PI-1	33	Kazal (4)		Serine protease inhibitor	Lumen of the PV	Pszenny <i>et al.</i> , 2000, 2002; Morris <i>et al.</i> , 2002
PI-2	27	Kazal (4)		Serine protease inhibitor	Lumen of the PV	Morris and Carruthers, 2003
Cyp18	20	Cyclophilin		Protein folding		High <i>et al.</i> , 1994; Carey <i>et al.</i> , 2000

¹Based on the complete open reading frame including signal sequence or GPI anchor signal, if present.

²Abbreviations: TM, transmembrane.

DENSE GRANULES

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are predicted to encode membrane-associated domains on secondary structure analysis. These are either a single transmembrane domain (GRA3, GRA4, GRA5, GRA6, GRA7, and GRA8) or amphipathic α -helices (GRA2 and GRA9). Indeed, all these GRA proteins were detected associated with membranous systems of the PV (i.e. the MNN or PVM).

Another common feature of GRA proteins is the difference observed between their theoretical molecular weight, calculated from the amino-acid sequence, and the molecular weight of the native protein, as detected on SDS-PAGE of tachyzoite lysates. This suggests potential post-translational modifications (for review, see Mercier et al., 2005). Although numerous N- and O-glycosylation sites are predicted within the GRA amino-acid sequences, only a few GRA proteins were shown to be O-glycosylated: GRA2 (Zinecker et al., 1998), GRA4, (Achbarou et al., 1991b), and GRA6, (Travier et al., unpublished). The relative richness of GRA proteins in charged amino acids and in proline residues could also account for the differences between the theoretical and apparent molecular weights. Cv-18, present within DGs as well as within the PV (High et al., 1994), through its potential peptidylprolylcisisomerase activity, might help in the folding of proline-rich proteins like GRA4 and GRA8, for better trafficking (Carey et al., 2000).

The second group comprises a few soluble dense-granule proteins with known function as cyclophilins (Carey et al., 2000), nucleotide triphosphatases (NTPases) (Bermudes et al., 1994; Asai et al., 1995), and serine protease inhibitors of the Kazal family (TgPIs) (Pszenny et al., 2000, 2002; Morris et al., 2002; Morris and Carruthers, 2003). Two NTPase isoforms, TgNTPase-I and TgNTPase-II (E.C. 3.6.1.3), slightly different at the genetic level (97 percent of identity), were described in Toxoplasma tachyzoites, and one in Neospora caninum (Asai et al., 1998). The gene encoding TgNTPase-II is found in all strains of T. gondii, while the gene encoding TgNTPase-I is confined to virulent strains (Bermudes et al., 1994; Asai et al., 1995). In vitro activity of these enzymes has been extensively characterized, but their vacuolar function remains unclear (see below).

11.6.2 Biogenesis of dense-granule organelles: dual features of both constitutive and regulated secretory pathways

Dense granules are homogeneous spherical electrondense vesicles, ~200 nm in diameter and enclosed by a unit membrane. The existence of subpopulations of dense granules storing specific GRAs was examined by double or triple labeling with specific antibodies. All dense granules exhibited multiple labeling, showing localization of different GRAs within the same granules (Sibley et al., 1995; Ferguson et al., 1999a; Labruyere et al., 1999). The number of DGs varies between the different infectious stages. The largest numbers (~15) have been observed in the tachyzoites and sporozoites, with intermediate numbers (8-10) in the bradyzoites and few (3-6) in the merozoites. This may correlate with the number of GRAs expressed and the type of PV formed (see below).

There are still several unresolved questions regarding both dense-granule formation and the sorting of GRA proteins into these organelles. In particular, whether dense granules are functionally analogous to constitutive versus regulated secretory vesicles is not fully established. In other eukaryotic cells, sorting of proteins to constitutive versus regulated secretory pathways occurs in the trans-Golgi network (TGN). Proteins destined for regulated secretion aggregate and are packaged into immature secretory granules (ISGs). Clathrincoated vesicles bud from these ISGs and recycle proteins back to the TGN or endosomes, resulting in further concentration of the secretory proteins. The specific coat proteins, identified at the TGN, include both the adaptor proteins, AP1 and AP3, and adaptor-related proteins, GGAs (for review, see Arvan and Castle, 1998).

In *Toxoplasma*, immature dense granules have never been observed. Moreover, transient and stable expression of several soluble reporter proteins in *Toxoplasma* showed that any soluble protein, provided it possesses a signal peptide, is delivered to dense granules and later secreted into the PV. By contrast, addition of a GPI signal anchor targets the same reporter protein to the plasma membrane through transport vesicles (Karsten *et al.*, 1998). Conversely, the TgSAG1 protein for which the GPI signal anchor domain has been deleted is routed to the PV via the dense granules (Striepen *et al.*, 1998). Thus, dense granules constitute the default constitutive pathway for soluble proteins in *Toxoplasma*.

However, morphologically, dense granules resemble the dense core granules involved in regulated secretion in mammalian cells, indicating that retention and condensation of secretory products may occur during dense granule formation. The prevailing model of sorting by retention in higher organisms is the selective aggregation of regulated, but not constitutive, secretory proteins, which limits the ability of the former to escape from maturing granules during the process of constitutive vesicle budding (Arvan and Castle, 1998). This condensation may be due to an inherent property of the regulated secretory proteins to aggregate via subtle changes in the forming granule, while trafficking through the different compartments of the secretory pathway. These changes include mild acidification or an increasing concentration of bivalent cations such as calcium (Chanat and Huttner, 1991).

Most of the GRA proteins are not intrinsically soluble, and are predicted as type 1 transmembrane proteins. Indeed, they become membrane associated following their secretion into the PV (Lecordier *et al.*, 1999). Within the dense granules, the GRAs are essentially found as both soluble and aggregated forms (Labruyere *et al.*, 1999; Lecordier *et al.*, 1999). The mechanism by which these proteins remain excluded from the endomembranous system of the parasite is unknown.

Are there signals that mediate GRA targeting and/or aggregation into the dense granules? Since dense granules have never been reported to constitute an acidic compartment, protein condensation is unlikely to result from a substantial decrease in pH. An AP-1 ortholog was localized at the *trans*most cisternae of the Golgi apparatus (Ngo *et al.*, 2003) and, despite the fact that YXX ϕ motifs were localized in the cytoplasmic tails of two GRA proteins (GRA4 and GRA7), these were not recognized by the *Toxoplasma* AP-I (Ngo *et al.*, 2003). Whether delivery of the GRAs to dense granules depends on a peculiarity of their transmembrane domain (TMD) (Karsten *et al.*, 2004) or on protein– protein interactions is not yet fully demonstrated (Gendrin, Braun and Cesbron-Delauw, unpublished). Given that GRA1, the most abundant dense-granule product, is a calcium-binding protein (Cesbron-Delauw *et al.*, 1989), a role for Ca²⁺ in regulating aggregation remains a possibility.

11.6.3 Exocytosis of dense granules

The secretion of the dense granules has been difficult to capture: fusion of the dense-granule membrane with the parasite plasma membrane (PPM) takes place sub-apically, at supposed gaps between the plates forming the inner membrane complex (Leriche and Dubremetz, 1990; Dubremetz *et al.*, 1993). Dense-granule secretion appears to respond to signals associated with both constitutive and regulated pathways of secretion.

In favor of constitutive secretion, dense-granule fusion with the target PPM is assisted by small GTPases of the Rab family and by soluble accessory factors (N-ethylmaleimide Soluble Factor (NSF), Soluble NSF-Associated Protein REceptor/Soluble NSF Associated Protein machinery (SNARE/SNAP)) (Chaturvedi *et al.*, 1999). Also, an increase in intracellular Ca²⁺, which usually triggers fusion of mammalian dense core granules with the plasmalemma, has no effect on dense-granule exocytosis (Chaturvedi *et al.*, 1999; Liendo and Joiner, 2000) but does result in secretion of the micronemes (Carruthers *et al.*, 1999a).

In contrast, several features are consistent with regulated secretion including:

- the burst of dense granule secretion into the PV occurring shortly after its formation (Dubremetz *et al.*, 1993)
- the fact that brefeldin A has no effect on the release of pre-stored GRAs (Coppens *et al.*, 1999), and
- dense granule secretion was shown to be quantitatively and specifically induced by heat-inactivated serum (Coppens *et al.*, 1999).

Two mechanisms driving dense-granule secretion are thus hypothesized. Since two distinct populations of dense granules have never been observed, the type of secretion might be related to the compaction stage of dense granules.

11.6.4 Post-secretory trafficking of GRAs within the parasitophorous vacuole

The involvement of GRAs in the maturation of the PV has only been examined in detail during tachyzoite development. Shortly after invasion, the burst of dense-granule secretion into the PV coincides with specific structural changes of the PV that are characteristic of tachyzoite development. At this stage, only GRA1, TgPIs, and NTPases remain primarily in the lumen of the vacuole (Sibley *et al.*, 1994, 1995; Pszenny et al., 2002; Morris and Carruthers, 2003). By immuno-electron microscopy, most of the GRAs are detected associated with the two membranous system of the PV: the MNN and the PVM. Hence, a fraction of the GRA1, GRA3, GRA7, and NTPase pools, as well as a major fraction of the GRA2, GRA4, GRA6, and GRA9 populations, are found associated with the MNN (Charif et al., 1990; Dubremetz et al., 1993; Lecordier et al., 1995; Bonhomme et al., 1998; Labruyere et al., 1999; Adjogble et al., 2004). In these membranes, GRA2, GRA4, and GRA6 are components of a multimeric protein complex (Labruyere et al., 1999). By contrast, GRA3, GRA5, GRA7, and GRA8 are preferentially detected at both the PVM and its membranous extensions within the host cell cytoplasm (Achbarou et al., 1991b; Dubremetz et al., 1993; Lecordier et al., 1993; Sinai et al., 1997; Bonhomme et al., 1998; Carey et al., 2000).

The GRAs exhibit various types of membrane association. While both GRA1 and NTPases exhibit a very loose association with the MNN (Sibley *et al.*, 1994, 1995), GRA4 is only displaced by urea treatment (Labruyere *et al.*, 1999), suggesting an association based mainly on hydrogen bonds. In contrast, GRA2, GRA3, and GRA5-9 are only quantitatively displaced from their respective membranes by non-ionic detergents, indicating membranespanning domains stabilized by hydrophobic interactions. For both GRA2 (Mercier *et al.*, 1998a) and GRA5 (Lecordier *et al.*, 1999), the respective putative

membrane domains (the GRA2 amphipathic α -helices and the GRA5 transmembrane domain) were shown to be responsible for membrane association. Furthermore, GRA5 was determined to be a type 1 transmembrane protein in the PVM (Lecordier *et al.*, 1999). This mechanism of post-translational membrane insertion is unconventional, and remains to be elucidated at both the molecular and structural levels. Moreover, the mechanisms that control selective targeting of GRAs to the MNN or PVM are also unknown.

11.6.5 Dense-granule protein function: more hypotheses than functions

As mentioned above, to date the function of most dense-granule proteins remains unknown. At the tachyzoite stage, their burst of secretion into the PV following host-cell invasion and their selective targeting within the PV compartment suggest that they might contribute significantly to the structural organization of this new compartment and/ or have important functions in PV metabolism (Dubremetz *et al.*, 1993; Cesbron-Delauw *et al.*, 1994; Carruthers and Sibley, 1997).

11.6.5.1 GRAs

BLAST searches performed on GRA sequences did not reveal any significant similarity between the GRAs and other proteins in the databases. However, motif searches indicated some potential biochemical properties: two EF-Hands predicted in GRA1 (aa 149–180 and 197–223) suggested calcium-binding activity, which was subsequently demonstrated (Cesbron-Delauw *et al.*, 1989), an ATP/GTP binding site in GRA4 (aa 307–314), and a RGD adhesion motif in GRA7 (aa 170–172) (Mercier *et al.*, 2005).

The function of several GRAs was examined by the construction of genetic knockout (KO) parasites. Analysis of GRA2, GRA6, or GRA2–GRA6 KOs showed that these proteins contribute to the formation of the MNN (Mercier *et al.*, 2002). GRA2, via its amphipathic alpha-helices, induces tubulation of the vesicular material observed at the posterior end of the parasite shortly after invasion. GRA6 further

stabilizes these preformed membranous tubules. However, despite such a dramatic disruption of the MNN architecture, both the GRA2 and GRA6 KO parasites displayed normal *in vitro* growth rates (Mercier *et al.*, 1998b), indicating that the network organization is dispensable for parasite intracellular replication. However, *in vivo*, both the GRA2 and GRA6 KO parasites are less virulent than their parental RH strain, allowing mice to survive acute infection and to develop brain cyst-like structures (Mercier *et al.*, 1998b; Mercier *et al.*, unpublished). Whether this avirulent phenotype is linked to the absence of intravacuolar tubular structures remains to be investigated.

To date, no key information on the function of PVM-associated GRAs is available since a TgGRA5 KO did not lead to any obvious phenotypic changes (Mercier et al., 2001). Finally, despite numerous attempts, it appears impossible to knock out TgGRA1 or TgGRA4, suggesting that some GRAs may be essential for tachyzoite intracellular survival (Braun, Travier, Mercier and Cesbron-Delauw, unpublished). TgGRA1 is the most abundant EST (2 percent of the total EST derived from the RH strain, http://toxodb.org/News/Item-3.0newESTdata-Mf.shtml), and its Ca2+-binding properties suggest an important role in the calcium homeostasis of the PV, or in assisting the packaging of other GRA proteins (as proposed earlier). Further investigations of the function of these GRA proteins would require the construction of conditional KO mutants.

11.6.5.2 Other dense-granule proteins

Despite their homology with well-characterized proteins, the function of the other dense-granule proteins is not clearly established. The abundant NTPases are essential, and display apyrase activity (Asai *et al.*, 1995; Nakaar *et al.*, 1999). Hence, in a primary model, as *Toxoplasma* is an auxotroph for purines, it was postulated that the presence of vacuolar NTPases, as well as of a 5' nucleotidase, would allow stepwise degradation of ATP into ADP, AMP, and adenosine, with the latter being eventually transported across the parasite plasma membrane via a low affinity adenosine transporter (Stedman

and Joiner, 1999). However, it was also demonstrated that, *in vitro*, maximal activation of NTPases requires dithiols (Asai *et al.*, 1983; Bermudes *et al.*, 1994), and this enzyme is minimally active in the PV (Silverman *et al.*, 1998). Moreover, no 5'-nucleotidase activity was detected on the parasite surface, suggesting that the parasite is incapable of converting AMP to adenosine for transport across the parasite plasma membrane (Ngo *et al.*, 2000). Thus, it appears that the initial hypothesis that NTPases are involved in purine salvage was incorrect.

An alternative role for NTPases in parasite egress has been proposed. With the PVM becoming more permeable as parasites develop, intravacuolar activation of NTPases would occur just before parasite egress (Stommel *et al.*, 1997; Silverman *et al.*, 1998), so that the ATP pumped from the host cell could be degraded and provide the energy necessary for parasite motility. According to this model, and given their abundance, NTPase activity would be tightly regulated to avoid rapid depletion of the ATP vacuolar stock, which would trigger premature egress of the parasite from the vacuole (Silverman *et al.*, 1998).

Among the serine protein inhibitors detected within the PV, TgPI-1 is a broad-spectrum inhibitor that is capable of neutralizing trypsin, chymotrypsin, and elastase *in vitro*, whereas TgPI-2 appears to be specific for trypsin (Morris *et al.*, 2002; Morris and Carruthers, 2003). The intravacuolar function of these inhibitors is unclear. Because both are active against digestive enzymes, TgPI-1 and TgPI-2, possibly released into the extracellular environment during the host cell lysis, may protect the parasite from proteases as it traverses the gastrointestinal tract (Morris *et al.*, 2002).

Cyclophilins (CyPs) are highly conserved proteins associated with an *in vitro* peptidyl– prolyl *cis/trans* isomerase (PPIase or rotamase) activity, and are implicated more broadly in mediating protein–protein interactions within large protein complexes. As such, TgCyP-18 could be involved in regulating the assembly of protein complexes within the dense granules and/or the PV. In particular, GRA4 and/or GRA8, which are rich in proline residues, could be potential substrates (Carey *et al.*, 2000).

11.6.6 Stage-specific expression of dense-granule proteins

Based on the few studies available, bradyzoites and sporozoites express nearly the full tachyzoite repertoire of dense-granule proteins (Ferguson, 2004; Mercier *et al.*, 2005). This is consistent with the tachyzoite-fate of both bradyzoites and sporozoites in the host. The dense-granule repertoire would be required to adapt the PV for optimal tachyzoite development and facilitate parasite proliferation in many cell types. In contrast, merozoites, which undergo limited proliferation and rapidly differentiate into the sexual stages in the enterocytes of the cat small intestine, express a very limited repertoire of dense-granule proteins (Ferguson, 2004).

11.6.6.1 Bradyzoite/tissue cyst

Bradyzoites are quiescent parasites formed in intracellular tissue cysts found within muscle cells and within cells of the central nervous system predominantly neurons. The bradyzoite PV is limited by a unit membrane with numerous shallow invaginations (see Chapter 13). An underlying layer of moderately electron-dense fine granular material contributes to the wall of the tissue cyst (Ferguson and Hutchison, 1987). At the bradyzoite stage, the dense granules contain all the GRAs identified in the tachyzoite, although there is evidence for reduced expression of NTPase (Nakaar et al., 1998; Ferguson et al., 1999a, 1999b). Furthermore, GRA4, GRA8, and NTPases were not detected within the cyst wall, which may be a consequence of their degradation or modification during encystment. All the other GRAs were shown to be present in the cyst wall, with a location reminiscent of that observed within the tachyzoite PV (Torpier et al., 1993; Ferguson, 2004). Whether dense-granule proteins are involved in the structural formation of the cyst wall or in communication with the host cell remains to be investigated. Evidence for dense-granule proteins trafficking beyond the cyst wall has only been reported for GRA7 (Fischer et al., 1998; Ferguson et al., 1999a).

11.6.6.2 Merozoite

During the coccidian development of *T. gondii* in the enterocytes of the cat, the ultrastructural appearance of the PV is very different from that observed for the tachyzoite PV (Ferguson, 2004). The parasites are located in a tightly fitting PV limited by a thickened membrane with a laminated appearance, consisting of three closely applied unit membranes. The PV lacks the MNN and there is no association of the host-cell rough ER or mitochondria (Ferguson, 2004). At this stage, only two dense-granule proteins, GRA7 and TgNTPase, are detected within the dense granules. Both are released into the PV shortly after invasion, but their level of staining drops as the parasites mature (Ferguson *et al.*, 1999a, 1999b).

11.6.6.3 Sporozoite

The dense granules of the sporozoite appear to contain GRA1–8, with the exception of TgGRA3 and TgNTPase (Tilley *et al.*, 1997). *In vitro*, sporozoites entering into a host cell form an unusual large vacuole (PV1) devoid of dense-granule proteins (Tilley *et al.*, 1997), but leave this compartment to enter a new vacuole (PV2) that more closely resembles a tachyzoite PV. Formation of PV2 is correlated with the secretion of the tachyzoite repertoire of dense-granule proteins (Tilley *et al.*, 1997). In fact, GRA3, GRA5, and NTPases are the first to be detected, followed by GRA1, GRA2, GRA4, and GRA6 (Tilley *et al.*, 1997).

11.7 CONCLUSIONS

Toxoplasma pathogenesis is intimately associated with the parasite's ability to invade host cells – an active process that has no counterpart outside the Apicomplexa phylum. The mechanisms involved are likely to be similar throughout the phylum, as first exemplified by the conservation of a family of microneme proteins containing thrombospondinrelated domains first identified in *Plasmodium* spp. The development of modern genetic analysis in *T. gondii* (complete genetic map, efficient genetic transformation and knockout or knockdown of essential genes) has greatly facilitated improvement in knowledge regarding the invasion process of *T. gondii*, and has helped in identifying the key molecular actors of the invasion machinery of *Plasmodium* – a parasite that is less amenable to genetic manipulation. Molecules conserved across the phylum have been recently extended to the motor complex, proteases of the rhomboid family, and proteins translocated at the moving junction. This has highlighted a number of novel putative targets for therapeutic intervention to treat major apicomplexan diseases of humans and livestock.

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12

Alterations in Host-Cell Biology due to *Toxoplasma gondii*

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12.1 Introduction

- 12.2 Observed changes in host-cell biology
- 12.3 Mediators of alterations in host-cell biology

12.4 Conclusions Acknowledgements References

12.1 INTRODUCTION

Host cells infected with *Toxoplasma gondii* undergo profound physiological and morphological changes. These changes result from a combination of the host cell's responses to the parasite, and the parasite's attempts to convert the host cell into a suitable growth environment. In this chapter, we discuss the known *Toxoplasma*-induced alterations in host-cell biology, the scarce data on their mechanisms, and their potential mediators. Delineating how *Toxoplasma* modifies the host cell and how the modifications contribute to parasite survival will increase our understanding not only of the relationship between *Toxoplasma* and its host but also of host processes that otherwise might not ever have been revealed.

In order to establish the cell-biological context in which host-cell modifications occur, we must first describe the formation and architecture of *Toxoplasma*'s intracellular residence, the parasitophorous vacuole (PV). *Toxoplasma* forms the PV concomitantly with invasion (Mordue and Sibley, 1997). Invasion is actively mediated by the parasite, and does not require host phagocytic machinery or an intact host cytoskeleton (Dobrowolski and Sibley, 1996; Dobrowolski *et al.*, 1997). As it penetrates the host cell, the *Toxoplasma* induces an invagination of the host plasma membrane that envelopes the parasite and ultimately becomes the PV membrane (PVM) (Suss-Toby *et al.*, 1996).

At the site of penetration, host and parasite plasma membranes are brought into apposition by a transient protein complex called the moving junction. A recent study providing the first identification of moving junction components reveals that this structure comprises proteins secreted from both the rhoptries and the micronemes (Alexander *et al.*, 2005). Due to a molecular sieving process occurring at the moving junction, the PVM contains a limited set of host proteins (Mordue *et al.*, 1999b; Charron and Sibley, 2004). This sieving process presumably contributes to the non-fusogenic state of the PV by excluding host vesicle fusion machinery (Mordue and Sibley, 1997; Mordue *et al.*, 1999a).

As the vacuole forms, host mitochondria and endoplasmic reticulum (ER) migrate to the PVM (de Melo *et al.*, 1992; Magno *et al.*, 2005a). The mitochondrial and ER membranes form unusually tight associations with the PVM, despite the vacuole's non-fusogenic state (Sinai *et al.*, 1997). The vacuole is also surrounded by host cytoskeletal components: intermediate filaments are reorganized upon invasion to form a cage around the vacuole (Halonen and Weidner, 1994), and microtubules have recently been observed in association with the vacuole (Sehgal *et al.*, 2005; Coppens *et al.*, 2006).

12.2 OBSERVED CHANGES IN HOST-CELL BIOLOGY

This section reviews the characterized changes in host-cell biology resulting from *Toxoplasma* infection and focuses on alterations that appear to be actively mediated by the parasite to promote its intracellular replication and eventual dissemination. More specifically, these alterations facilitate parasite nutrient scavenging, enhance host-cell survival, and titrate the immune response to allow an infection that does not overwhelm the host.

12.2.1 Diversion of host resources

The PV does not fuse with host endocytic/exocytic vesicles (Mordue *et al.*, 1999a). This isolation protects the parasite from the destructive environment of endolysosomes, but also limits the parasite's access to host resources. Mechanisms for acquiring metabolites from the extracellular environment or from intracellular stores must circumvent parasite isolation while not undermining the

protection it confers. Determining how *Toxoplasma* meets its metabolic needs provides context for understanding parasite-induced changes in the host cell, such as manipulation of signaling pathways and recruitment of host organelles to the PV.

Here we briefly review the known auxotrophies and nutritive requirements of *Toxoplasma* and then cover characterizations of nutrient scavenging by the parasite and proposed scavenging mechanisms. Finally, we discuss the potential effects on the host cell of *Toxoplasma*-induced manipulations to facilitate nutrient acquisition.

12.2.1.1 Metabolic requirements

Because *Toxoplasma* is an obligate intracellular parasite that develops within metabolically active cells, distinguishing between the metabolic requirements of the parasite and its host cell is difficult. Studies of mutant cell lines and of labeled metabolic precursors, in conjunction with analyses of available *Toxoplasma* genome sequences, have contributed to the list of metabolites that the parasite must acquire from its host cell. The upcoming release of the annotated *Toxoplasma* genome will facilitate a more complete elucidation of *Toxoplasma* nutrient requirements; the following is a summary of the major metabolic deficiencies currently known.

The tryptophan auxotrophy of *Toxoplasma* was demonstrated by studies on host antiparasitic defenses. In response to γ -interferon, infected human fibroblasts inhibit *Toxoplasma* replication by degrading tryptophan (Pfefferkorn, 1984; Pfefferkorn *et al.*, 1986; Dai *et al.*, 1994). *Toxoplasma* cannot synthesize arginine. Depletion of host arginine arrests parasite replication and, surprisingly, induces conversion to the metabolically quiescent bradyzoite stage (Fox *et al.*, 2004).

Toxoplasma can either synthesize or salvage pyrimidine nucleosides (O'Sullivan *et al.*, 1981; Schwartzman and Pfefferkorn, 1981; Asai *et al.*, 1983; Iltzsch, 1993). The observation that abrogation of the *de novo* pathway significantly decreases parasite virulence indicates that the salvage pathway is not sufficient for *Toxoplasma* development (Fox and Bzik, 2002). Purines are obtained exclusively via salvage pathways (Schwartzman and Pfefferkorn, 1982; Krug *et al.*, 1989; Chaudhary *et al.*, 2004).

Inhibition of host ornithine decarboxylase inhibits parasite growth and reveals *Toxoplasma* requirements for polyamines (Seabra *et al.*, 2004). *Toxoplasma* appears mainly to use glucose as an energy source, and must acquire it from the host (Fulton and Spooner, 1960).

Host cholesterol is necessary for *Toxoplasma* replication and invasion (Coppens *et al.*, 2000; Coppens and Joiner, 2003). The parasite can synthesize sphingolipids (Azzouz *et al.*, 2002), whereas phospholipids and fatty acids are either synthesized or scavenged (Charron and Sibley, 2002).

Iron, a cofactor of metabolic enzymes, must also be obtained from the milieu of the host. Cultured rat enterocytes activated by γ -interferon appear to inhibit *Toxoplasma* replication by iron sequestration (Dimier and Bout, 1998). Similarly, treatment of infected fibroblasts with the iron chelator deferoxamine, which depletes intracellular iron pools, reduces *Toxoplasma* growth (Gail *et al.*, 2004).

12.2.1.2 Acquisition of nutrients

Small metabolites A study on the permeability of the PVM revealed that Toxoplasma does have limited access to the host cytosol. Subsequent to microinjection into infected host cells, fluorescent probes up to 1300 Da entered the vacuole in an energy- and temperature-independent manner, while probes greater than 1900 Da were excluded (Schwab et al., 1994). Because the probes were derivatives of peptides, sugars, and dyes, the authors propose the existence of a channel that enables the indiscriminate exchange of molecules up to 1300-1900 Da between the vacuole and the host cytosol. This channel would allow ions and small metabolites, such as amino acids, sugars, nucleosides, nucleotides, and polyamines, to diffuse passively into the vacuole and then to be taken up by the parasite via substrate-specific transporters in the parasite plasma membrane or via endocytosis.

Data on the mechanisms of *Toxoplasma* nutrient import are limited. Transporters for hexose and for nucleosides have been identified and characterized in the Toxoplasma plasma membrane (Schwab et al., 1995; Chiang 1999; Joet et al., 2002; de Koning et al., 2003). Extracellular parasites can import the polyamine putrescine, but the process has not been characterized (Seabra et al., 2004). Toxoplasma does express at least one protein known to mediate endocytosis in other eukaryotes: TgRab5, a homolog of the small GTPase Rab5 that regulates endosome fusion in yeast and mammalian cells, localizes to vesicular structures near the Toxoplasma Golgi that might be endosomes (Robibaro et al., 2002). However, other components of the endocytic machinery await identification, and the extent to which Toxoplasma utilizes endocytosis for nutrient acquisition is unknown (for review, see Robibaro et al., 2001).

Cholesterol and lipids Toxoplasma tachyzoites obtain cholesterol primarily from host-endocytosed low-density lipoprotein (LDL) particles rather than from endogenously synthesized host stores. Parasite replication is decreased when infected cells are cultured in lipoprotein-deficient media regardless of the competence of host cells to synthesize cholesterol, and compounds that block host uptake of LDL or disrupt lysosome function prevent cholesterol delivery to the parasite (Coppens *et al.*, 2000). Analyses of the post-endocytic trafficking of cholesterol to the parasite suggest the involvement of an unusual cell biological pathway.

Inhibition of cholesterol egress from lysosomes reduces cholesterol accumulation by intracellular parasites, while treatment of infected host cells with N-ethylmaleimide (NEM), an inhibitor of vesicle fusion, does not (Coppens et al., 2000; Sehgal et al., 2005). Moreover, extracellular parasites and parasites within purified, intact vacuoles accumulate free cholesterol via a proteasesensitive mechanism (Sehgal et al., 2005). In accordance with the non-fusogenic state of the PVM, these observations suggest a delivery mechanism that does not involve host endolysosomes. Conditions that reduce vesicle trafficking, however, do inhibit parasite accumulation of cholesterol. Sehgal and colleagues incubated infected host cells briefly with LDL particles

containing fluorescently labeled cholesterol to allow loading of endosomes and then tracked the increase of PV fluorescence in the presence and absence of microtubule disruptors. The PV displayed reduced fluorescence in the presence of the microtubule disruptors as compared with their absence (Sehgal *et al.*, 2005)

A model proposed in a recent study on Toxoplasma nutrient acquisition resolves the apparent contradiction in the data regarding a requirement for vesicle trafficking in delivery of host-endocytosed cholesterol (Coppens et al., 2006). According to this lysosome trap model (Figure 12.1), host late endosomes/lysosomes traffic along microtubules into invaginations of the PVM. A protein collar, which may include the dense-granule protein GRA7, constricts the invagination and traps the host vesicle inside. The invagination then pinches off the PVM to generate a double-membraned structure comprising a host lysosome surrounded by a membrane of PVM origin. The contents of the trapped host lysosome enter the lumen between the inner and outer membranes via transporters in the lysosomal membrane. Transporters in the PVM-derived membrane and/or the PVM pore allow entry into the PV lumen. Once in the PV lumen, molecules enter the parasite via membrane transporters or endocytosis. Clearly there are many steps in this model where the underlying mechanisms have yet to be determined, but it forms an important starting point for direct experimentation.

Data supporting the lysosome trap model include immunofluorescence assay images revealing an accumulation of host microtubules and endolysosomes around the PV, and immunostained electron micrographs showing that within the PV lumen are vesicles that contain the lysosomal marker lysosomal-associated membrane protein (LAMP) 1 (Sehgal *et al.*, 2005; Coppens *et al.*, 2006). In electron micrographs of infected host cells incubated with gold-labeled LDL and transferrin, gold particles are present in vesicles within the PVM invaginations and also in doublemembraned vesicles in the PV lumen (Coppens *et al.*, 2006). Moreover, a GRA7-deficient mutant grown under nutrient-limited conditions displays



FIGURE 12.1 Toxoplasma might scavenge nutrients such as cholesterol and iron via a mechanism involving the sequestration of host lysosomes. A host endolysosome/lysosome translocates along host microtubules toward the parasitophorous vacuole (PV) (1), and enters microtubule-containing invaginations of the PV membrane (PVM) (2). As the lysosome reaches the terminus of the microtubule (3), Toxoplasma proteins associate with the invagination and form collar-like structures that constrict the invagination and trap the host lysosome (4). The invagination pinches off the PVM to generate a double-membraned vesicle comprising a lysosome surrounded by an outer membrane of PVM origin (5). The contents of the trapped host lysosome enter the space between endolysosome and outer membranes via transporters in the lysosomal membrane. Macromolecule transporters in the PVM-derived membrane and/or the PVM pore allow entry into the PV lumen (6). Once in the PV lumen, molecules enter the parasite via membrane transporters or endocytosis (7) See text for data supporting this lysosome trap model. This figure is reproduced in color in the color plate section.

pronounced growth and morphological defects, as compared with wild-type parasites (Coppens *et al.*, 2006).

The data on cholesterol transport fit nicely into the lysosome trap model. Host microtubules are necessary for recruitment and capture of the cholesterol-containing lysosomes. Cholesterol efflux is required to make the cholesterol available for transporter mechanisms in the fragment of the PVM surrounding the trapped lysosome and the parasite plasma membrane. The delivery does not involve vesicle fusion, and thus is NEMinsensitive. The lysosome trap model provides a general means by which Toxoplasma can selectively access nutrients abundant in the lysosomes while maintaining the non-fusogenic state of the parasitophorous vacuole and avoiding exposure to the acidic conditions of the lysosomal lumen. Indeed, the model suggests a potential mechanism for iron scavenging by the parasite (see below).

Studies involving fluorescently labeled fatty acid and phospholipid probes indicate that Toxoplasma readily scavenges these lipids from the host (Charron and Sibley, 2002). Interestingly, adding the fluorescent probes to host cells prior to infection resulted in a peripheral staining of parasites, whereas probe addition subsequent to infection resulted in more extensive internal parasite staining (Charron and Sibley, 2002). The mechanism by which the parasite acquires lipids from the host is unknown. The lipids might be obtained via the lysosome trap model. An alternative mechanism invokes lipid exchange between the PVM and closely apposed host mitochondrial and endoplasmic reticulum membranes (de Melo et al., 1992; Sinai et al., 1997).

Iron Free iron can be toxic to cells because it leads to the formation of oxygen radicals. Intracellular iron not incorporated into enzyme cofactors is stored complexed with ferritin, and extracellular iron is bound to either transferrin or lactoferrin. Iron sequestration is also utilized as a defense mechanism by the host. Consequently, free iron is exceedingly rare in the host, and is not readily available for use by pathogens. To overcome the limited iron availability, intracellular pathogens employ strategies such as the secretion of iron-binding proteins called siderophores, the degradation of iron-containing prosthetic groups, the expression of membrane-localized receptors for transferrin and lactoferrin, or the interception of endosomes containing iron-transferrin complexes bound to the transferrin receptor (TfR) (Andrews *et al.*, 2003; Schaible and Kaufmann, 2004).

Little is known regarding *Toxoplasma* ironscavenging. No evidence suggests that the parasite secretes siderophores or degrades iron-containing prosthetic groups. Recent studies demonstrate that the surface of extracellular tachyzoites binds bovine transferrin, bovine lactoferrin, and human lactoferrin, but does not bind human transferrin (Tanaka *et al.*, 2003; Dziadek *et al.*, 2005). The biological significance of these results is not apparent, because neither internalization of the iron-binding proteins nor delivery of the proteinbound iron to the parasite was assessed. Moreover, the accessibility of these proteins to intracellular parasites was not examined.

Given the established isolation of the parasitophorous vacuole from the host endocytic cascade, and given the observed absence of the transferrin receptor from the vacuole (Mordue et al., 1999a), the acquisition of iron via interception of endosomes containing iron-transferrin-TfR complexes seems unlikely. However, recent evidence suggests that this possibility deserves further exploration. Electron micrographs of infected host cells incubated with gold-adsorbed TfR for 15 minutes show gold particles within double-membraned vesicles inside the PV (Coppens et al., 2006). According to the lysosome trap model (discussed in detail above), the doublemembraned vesicles result from the sequestration and subsequent envelopment of host lysosomes by the PVM. If the host lysosomes are trapped before iron dissociates from transferrin and effluxes from the lysosomal lumen, then the parasite should have first access to the recently endocytosed iron. Toxoplasma does induce increased expression of TfR in human fibroblasts, and this increase in TfR levels seems unlikely to be a host response to iron depletion because it can be

caused by a soluble parasite factor and does not require invasion (Gail *et al.*, 2004). Thus, the parasite might override the host cell's iron-homeostasis network to promote TfR expression and consequently to increase the concentration of iron within the sequestered host lysosomes.

According to this model, iron transporters in the membrane of the sequestered host lysosome would pump free iron out of the lysosomal lumen and into the space between the two membranes. The PVM pore, if present in the PVM-derived portion of the double membrane, would then allow free diffusion of the iron into the PV lumen. Iron transporters in the parasite plasma membrane could import the iron into the parasite from the PV lumen. Expression of an iron transporter at the parasite plasma membrane has not been observed, but the *Toxoplasma* genome does encode a homolog of the iron transporter natural resistance-associated macrophage protein (NRAMP) 1 (http://www.ToxoDB.org).

Consistent with the lack of binding of human transferrin by the parasite surface, this model does not involve interactions between transferrin and the parasite PM. The apparent contradiction between the presence of transferrin and absence of TfR within the PV most likely results from differences in sensitivity of the imaging techniques employed. Indirect immunofluorescence staining was used to detect TfR, while transferrin was visualized by transmission electron microscopy of infected host cells incubated with gold-labeled transferrin (Mordue *et al.*, 1999a; Coppens *et al.*, 2006).

12.2.1.3 Effects of nutrient-scavenging on host cells

The effects on the host cell of *Toxoplasma* nutrientscavenging are poorly understood. Changes could result from manipulations performed by the parasite to facilitate nutrient acquisition or could simply be a response by the host to depletion of metabolites. Distinguishing between the two possibilities is not trivial because of the complex interconnections among metabolic, stress response, and immune signaling pathways. Moreover, homeostatic mechanisms in the host might mask parasite-induced perturbations.

Toxoplasma appears to alter the nucleotide permeability of its host cell. The parasite expresses an unusual purine transporter (TgAT1) in its plasma membrane (Schwab *et al.*, 1995; Chiang *et al.*, 1999). In contrast to host nucleoside transporters, TgAT1 is non-stereo-selective and insensitive to nitrobenzylthioinosine (Al Safarjalani *et al.*, 2003). Surprisingly, host cells infected with *Toxoplasma* display the same purine transport properties as TgAT1. This suggests the intriguing possibility that the parasite somehow introduces a transporter (conceivably, even TgAT1) onto the host-cell surface, but no direct data for such a mechanism have been obtained.

Although *Toxoplasma* acquires polyamines from the host cell, the parasite does not increase the activities of ornithine decarboxylase or arginine decarboxylase – enzymes involved in polyamine biosynthesis (Seabra *et al.*, 2004). The steady-state levels of polyamines appear to be sufficient to support parasite growth, and the parasite does not appear substantially to drain host polyamine reserves.

Toxoplasma alters the free calcium level of the host-cell cytosol. A study using the fluorescent calcium probe indo-1 determined the concentration of free calcium in the cytosol of infected host cells 48 hours post-infection to be 39 nanomolar, as compared with 53 nanomolar for non-infected host cells (Pingret *et al.*, 1996). This decrease in host cytosolic calcium could result from parasite scavenging of this important intracellular signal mediator.

DNA microarrays allow genome-wide monitoring of transcript levels, and thus have been useful tools for studying parasite-mediated changes in host metabolic pathways. Microarray analysis of the response of human foreskin fibroblasts to tachyzoite infection reveals an increase in abundance of transcripts encoding enzymes involved in cholesterol synthesis in infected cells as compared with non-infected host cells (Blader *et al.*, 2001). At 24 hours post-infection, the transcript levels of HMG-CoA reductase (the ratelimiting enzyme of cholesterol biosynthesis) was

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five times greater in infected cells. However, assays of HMG-CoA reductase enzymatic activity at the same time point indicate no increased activity of the enzyme in infected cells (Coppens *et al.*, 2000). How the host cell increases the transcript levels without increasing the amount of active enzyme is not known, but an increase in cholesterol synthesis by the host is not, *a priori*, an expected event, because the parasite efficiently scavenges endocytosed cholesterol (Coppens *et al.*, 2000).

Microarray analyses also led to the observation that the host transcription factor hypoxiainducible factor 1 (HIF1), which regulates the transcription of genes involved in cell growth, cell survival, iron metabolism, and glucose metabolism, is activated by the parasite and is necessary for parasite replication under physiologicallyrelevant oxygen levels (Spear et al., 2006). Interestingly, HIF1 activation requires neither parasite attachment nor invasion and thus appears to be mediated by secreted parasite factors (Spear et al., 2006). The upregulation of transcripts encoding TfR in response to Toxoplasma infection suggests that Toxoplasma might activate HIF1 to facilitate iron acquisition (Blader et al., 2001; Gail et al., 2001). However, the parasite can also increase host TfR transcript levels independently of HIF1. The binding of iron regulatory protein 1 (IRP1) to TfR mRNA stabilizes the transcript (Hentze et al., 1989; Koeller et al., 1989), and a soluble parasite factor does stimulate IRP1 to bind TfR transcripts (Gail et al., 2004). Current data are insufficient to predict which HIF1regulated genes are critical for parasite survival.

The soluble parasite factor(s) that activate HIF1 and promote IRP1 binding of TfR mRNA await identification. Because HIF1 and IRP1 both respond to reactive oxygen species (ROS) (Pantopoulos and Hentze, 1995, 1998; Pantopoulos *et al.*, 1997; Chandel *et al.*, 1998, 2000), it is possible that their induction is a response to ROS generated by *Toxoplasma* and/or by a parasite-stimulated host cell. Regardless of the mechanism(s) involved, the parasite may be able to modify potential host cells prior to entry to prepare an environment conducive to its growth and development.

12.2.2 Effects on host-cell survival

Increasing evidence suggests that the activity of *Toxoplasma* extends beyond a simple parasitic relationship in which the tachyzoite scavenges its host for nutrients to enable intracellular survival and replication. The parasite also displays a high level of sophistication in its ability to manipulate host responses and their underlying signal transduction cascades (Figure 12.2). One major activity of *Toxoplasma* is to render host cells resistant to inducers of apoptosis (Nash *et al.*, 1998; Goebel *et al.*, 1999).

Apoptosis is a complex process that can be mediated through multiple pathways (Gavrilescu and Denkers, 2003). The exogenous pathway involves ligation of surface receptors such as CD95 (Fas/Apo1) or CD120 (tumor necrosis factor receptor) (Wallach et al., 1999). Apoptosis may also be induced by a cell intrinsic pathway that involves changes in mitochondrial membrane potential, resulting in cytochrome c translocation from the inner mitochondrial membrane to the cytoplasm (Green and Reed, 1998). Loss of mitochondrial membrane potential can result from several stimuli, including excess reactive nitrogen and oxygen intermediates, growth factor deprivation, and UV or gamma irradiation. Regardless of whether intrinsic or extrinsic pathways are triggered, execution of apoptosis centers on proteolytic activation of a family of cysteine proteases called caspases (Nicholson, 1999). The so-called 'initiator' caspases 8 and 9 are triggered through extrinsic and intrinsic pathways, respectively. These proteases activate caspase 3, known as the executioner caspase. Caspase 3 targets an array of proteins involved in cell survival, including cell-cycle control, DNA repair, and nuclear and cytoskeletal assembly, as well as additional caspase family members. Thus, activation of the caspase family is the signature event in the programmed cell-death pathway.

Toxoplasma-induced blockade in apoptosis has been reported during *in vitro* infection of several cell types, including fibroblasts and macrophages, as well as HL-60 (a human promyelocytic leukemia cell line) and U937 (a human



FIGURE 12.2 *Toxoplasma* infection activates diverse signaling modules in macrophages. Infection with *T. gondii* elicits anti-inflammatory and survival responses in macrophages. Signaling through an as yet unidentified G-protein coupled receptor (GPCR), the parasite triggers phosphatidylinositol-3-kinase (PI-3K). PI-3K, via its phosphoinositide and protein kinase activity, ultimately leads to recruitment and phosphorylation of protein kinase B (PKB) and MKK1/2, respectively. PKB promotes survival by inactivating Bad and caspase 9 cascades as well as FKHR1, a pro-apoptotic transcription factor. Infection with *Toxoplasma* renders macrophages unresponsive to LPS triggering and blocks NF- κ B nuclear translocation. Inefficient translocation of this transcription factor inhibits early IL-12 and TNF- α production. Infection also activates the STAT3 pathway to inhibit LPS responses. All three MAP kinases (ERK1/2, p38, SAPK/JNK) are transiently activated by infection. Erk1/2 activation plays a role in anti-apoptotic response, while p38 is required for IL-12 induction by the parasite after long-term infection.

This figure is reproduced in color in the color plate section.

histiocytic lymphoma). Caspase-mediated proteolytic degradation of poly(ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair, cell survival, and proliferation, is strongly inhibited in cells infected with *Toxoplasma* (Goebel *et al.*, 2001). Mitochondrial release of cytochrome *c* in response to apoptotic signals is also blocked by *T. gondii*. Perhaps most significantly, activation of caspases 8, 9, and 3 is strongly inhibited by the parasite (Goebel *et al.*, 2001; Payne *et al.*, 2003).

The ability of the parasite to overcome triggers of host-cell apoptosis, including cytolytic T cellinduced death, treatment with TNF- α , growth factor withdrawal, and incubation with drugs such as staurosporine and beauvericin suggests that *T. gondii* may target multiple steps in the programmed cell death pathway (Nash *et al.*, 1998). Kim and Denkers have reported an anti-apoptotic mechanism that requires host phosphatidylinositol 3-kinase (PI3K) activation through G-protein coupled receptor signaling (Kim and Denkers, 2006).

PI3K activation in turn leads to activation of protein kinase B (PKB) and ERK1/2 MAPK. Both have anti-apoptotic effects. PKB phosphorylates and inactivates the pro-apoptotic proteins Bad and caspase 9 (del Peso *et al.*, 1997; Blume-Jensen *et al.*, 1998; Cardone *et al.*, 1998). In addition, PKB inactivates the Forkhead family transcription factor FKHR1, which regulates apoptosis-inducing genes (Brunet *et al.*, 1999). ERK1/2 activation leads to CREB phosphorylation and NF-κB nuclear translocation, two transcription factors controlling the expression of anti-apoptotic Bcl-2 proteins (Alexaki *et al.*, 2006). Nevertheless, chemical inhibition of ERK1/2 activity appears to have no effect on the anti-apoptotic properties of *Toxoplasma* in macrophages (Kim and Denkers, unpublished results).

Although it is not clear whether this relates to the $G_{\alpha i}$ -PI3K-PKB/Akt pathway, *Toxoplasma* has also been reported to block apoptosis in a manner dependent upon NF- κ B signaling (Payne *et al.*, 2003). Nevertheless, activation of NF- κ B would be consistent with the known ability of this signaling family to activate anti-apoptotic genes (Karin and Lin, 2002). In addition, *Theileria parva*, another intracellular apicomplexan, exploits NF- κ B signaling to prevent apoptosis of host cells (Machado *et al.*, 2000).

Dependence upon NF- κ B signaling implies that new host-cell protein synthesis is involved in inhibition of apoptosis. However, evidence that the parasite blocks apoptosis induced by both actinomycin D and cycloheximide suggests lack of a strict requirement for *de novo* RNA and protein synthesis (Goebel *et al.*, 2001). In fact, the degree to which *Toxoplasma* activates NF- κ B during infection is an area of controversy at present (see section 12.2.3.2).

The question of why T. gondii would block apoptotic death in the cells it infects has yet to be answered. One appealing possibility is based upon the observation that in vivo infections often elicit extremely robust amounts of proinflammatory cytokines. Levels of these proinflammatory mediators can reach pathologic levels in some cases. Certain mouse strains, such as C57BL/6, undergo severe tissue destruction in the small intestine, along with apoptotic death of T lymphocytes in the Peyer's patches, during oral Toxoplasma infection (Liesenfeld et al., 1996, 1997). This is caused by excessive production of cytokines including TNF-α and IFN-y, as well as proinflammatory mediators such as nitric oxide (Khan et al., 1997; Liesenfeld et al., 1999). Mice undergoing systemic infections, in particular with the virulent type I RH strain, also display overproduction of Th1 cytokines and extensive splenic apoptosis (Gavrilescu and Denkers, 2001; Mordue et al., 2001). Insofar as these proinflammatory mediators can deliver potent apoptotic death signals, it is possible that suppressing these signals is a parasite strategy to maintain host-cell viability in an overwhelmingly pro-apoptotic cytokine milieu.

12.2.3 Manipulation of host proinflammatory signaling pathways

In addition to modulating cell-survival responses in host cells, Toxoplasma exerts effects on signaling cascades involved in proinflammatory cytokine induction. This is particularly evident during infection of macrophages – major cells of innate immunity that are also targets of infection by the parasite. Although macrophages are a major source of proinflammatory cytokines in innate immunity, early production of TNF- α , IL-12, and many other proinflammatory mediators is suppressed when these cells are infected with T. gondii in vitro (Lee et al., 2006). While IL-12 production is eventually initiated after a delay of 18–24 hours, TNF- α synthesis remains suppressed. The following sections examine three molecular signaling cassettes that are important in proinflammatory signaling and that are initiated by Toxoplasma infection. These are the mitogen activated protein kinase (MAPK) transduction pathways, the NF-kB signaling cascade, and the cytokine transducing signal transducer and activator of transcription (STAT) 3. In each case, the parasite targets these pathways for manipulation.

12.2.3.1 Activation and deactivation of MAPK

The MAPK family comprises three modules: the stress-activated and Jun N-terminal kinases (SAPK/JNK), the extracellular signal-regulated kinases (ERK) 1 and 2, and p38 MAPK (Dong *et al.*, 2002). Activation of MAPK begins at the plasma membrane when cells are stimulated by ligation of any number of receptors, including inflammatory cytokine and Toll-like receptors (TLR). For most TLR, signaling requires the adapter protein MyD88, and subsequent recruitment of TNF receptor-associated-factor (TRAF)6 and IL-1 receptor-associated kinases (IRAK) 1 and 4 (Takeda and Akira, 2005). This is followed by assembly of a complex containing transforming growth factor- β -activated kinase (TAK) 1, and TAK1-binding proteins TAB1 and TAB2. Subsequently, TAK1, a MAPK kinase kinase (M3K), is activated, and the signaling cascade bifurcates at this point: TAK1 initiates the MAPK cascade as well as acting to phosphorylate I κ B kinase (IKK), leading to the activation of NF- κ B (see section 12.2.3.2).

When bone-marrow derived macrophages are subjected to Toxoplasma infection, all three MAPK signaling modules (ERK1/2, p38, SAPK/ JNK) are rapidly activated (Valere et al., 2003; Kim et al., 2004). Activation of the MAPK is shortlived, however, and phosphorylation returns to basal levels within 2 hours post-infection. When infected macrophages are subsequently stimulated with lipopolysaccharide (LPS), MAPK rephosphorylation is defective. This result resembles the endotoxin tolerance phenomenon, in which cells become refractory to secondary LPS triggering following an initial stimulation (Dobrovolskaia and Vogel, 2002). However, the T. gondii-infected macrophage is not simply a reiteration of an LPS-tolerized macrophage. This is because during endotoxin tolerance, LPS pretriggering and subsequent LPS exposure fail to rephosphorylate MKK3 and 6, major kinases that activate p38 MAPK. In contrast, Toxoplasma induces sustained phosphorylation of MKK3 while failing to activate MKK6 (Kim et al., 2004). LPS-induced downregulation of its cell surface receptor, TLR4, is another proposed mechanism of LPS tolerance. However, Toxoplasma actually upregulates TLR4 during infection (Kim et al., 2004).

Phosphorylation of p38 MAPK is essential for IL-12 production in both LPS-treated and parasite-infected cells. To delineate this pathway in parasite-infected cells, Kim and colleagues used active site-directed inhibitors and macrophages deficient in upstream kinases (Kim *et al.*, 2005). This series of experiments demonstrated that *Toxoplasma* infection activates p38 MAPK by stimulating autophosphorylation, rather than through activation of upstream p38-directed MKK. Autophosphorylation of p38 is a recently recognized mechanism for activation of this particular MAPK (Ge *et al.*, 2002, 2003).

12.2.3.2 NF-кB activation and nuclear translocation

NF-KB is an essential factor for transcriptional regulation of several proinflammatory cytokines, including IL-12 and TNF-α (Karin and Ben-Neriah, 2000). Its activity is controlled in major part by IkB family molecules that bind cytoplasmic NF-KB, masking nuclear localization sequences (NLS). Phosphorylation of upstream IkB kinases (IKK) by molecules such as TAK1 results in turn in IkB phosphorylation. This serves as a signal for ubiquitinylation-dependent IkB degradation, revealing NF-kB NLS. Several laboratories have demonstrated that Toxoplasma infection stimulates rapid phosphorylation and degradation of IkB, but what happens as a consequence is a matter of some debate. NF-KB accumulation in the nucleus has been reported in several studies to be deficient in infected cells, and it is actively suppressed when infected macrophages are subsequently stimulated with endotoxin (Butcher et al., 2001; Shapira et al., 2002). In addition, inhibition is only accomplished by active invasion (Butcher and Denkers, 2002). Using an inhibitor of nuclear export, Shapira and colleagues recently provided evidence that NF-kB is translocated to the nuclei of infected cells, but that it subsequently undergoes rapid export (Shapira et al., 2005). When macrophages are subjected to longer infections (6-18 hours) there is still no apparent accumulation of NF-kB in the nuclei of infected macrophages. However, when infected cells are stimulated with endotoxin at the later time points, the inhibition is lifted and NF-kB displays nuclear translocation.

In contrast to these studies, it has been found that NF- κ B translocation occurs in *Toxoplasma*infected fibroblasts (Molestina *et al.*, 2003). Recent work suggests the appearance of parasite-derived I κ B kinase (IKK) at the parasitophorous vacuole, which is coincident with parasite replication (Molestina and Sinai, 2005). It is argued that parasite IKK activity, in association with host IKK, is responsible for activation of NF- κ B and its subsequent transcriptional activity. What accounts for these discrepant results is not clear. Lack of NF- κ B nuclear accumulation is reported during infection of both macrophages and fibroblasts (Shapira *et al.*, 2005), arguing against differences in host-cell responses. The studies in each of these cases employed type I RH tachyzoites, arguing against parasite strain-specific effects (but see section 12.2.4). Clearly, this is an area in need of resolution.

Although NF- κ B controlled cytokines can readily be measured in sera of *Toxoplasma*-infected mice, *in vitro* studies have demonstrated that infected macrophages are not a primary source of early proinflammatory cytokines. An intriguing report by Courret and colleagues demonstrates that CD11c+ CD11b+/– dendritic cells are among the first cells to be infected, and that these cells facilitate parasite dissemination to the mesenteric lymph nodes (Courret *et al.*, 2006). Later, CD11b+ monocytes harbor the parasite and provide transportation to the brain. In addition, neutrophils releasing preformed IL-12 may provide the earliest cytokine response (Bliss *et al.*, 1999).

12.2.3.3 MAPK and NF-κB initiation signals

The parasite signals for initiating MAPK and NF-KB activation in the context of Toxoplasma infection are currently unknown. The Toxoplasma molecule cyclophilin-18 is reported to trigger dendritic cell IL-12 release by signaling through chemokine receptor CCR5 (Aliberti et al., 2000). However, in macrophages this molecular interaction does not appear to be required for MAPK activation. Nevertheless, parasite-induced activation of ERK1 and 2, but not other MAPK, while independent of CCR5 requires G_i-protein coupled receptor signaling (Kim and Denkers, 2006). Recently, a parasite molecule purified from soluble tachyzoite extracts, profilin, was shown to interact with TLR11 on splenic dendritic cells to stimulate IL-12 production (Yarovinsky et al., 2005). It seems likely that parasite profilin will prove to activate proinflammatory signaling in cells of the innate immune system. TLR2 is also implicated in promoting resistance during Toxoplasma infection, but in this case parasite ligands have yet to be identified (Mun *et al.*, 2003; Del Rio *et al.*, 2004). Communication between *Toxoplasma* and its host cell begins at the moment of contact, and continues through invasion, the establishment of the parasitophorous vacuole, and subsequent replication of the parasite. At each stage, it is likely that the host encounters a different subset of parasite molecules and the consequences of exposure will depend upon whether the signal is generated at the cell surface or from within the vacuole.

12.2.3.4 Hijacking STAT3 signaling by Toxoplasma

Inhibition of proinflammatory responses seems a perfect strategy for *T. gondii* to gain purchase within the macrophage. *Toxoplasma* does not invade silently, but activates host MAPK pathways and sets into motion, perhaps unproductively, the NF- κ B pathway. The parasite actively inhibits endotoxin responses, and does so by usurping the very pathways leading to inflammatory responses, directing them to different ends.

A striking example of this is with regard to the IL-10 signaling pathway. IL-10 is an anti-inflammatory modulator that suppresses many proinflammatory cytokines, including IL-12 and TNF- α . IL-10 requires the intracellular signaling molecule STAT3 to suppress cytokine production (Robben *et al.*, 2004). Mice that have tissue-targeted deletion of STAT3 succumb to overwhelming enterocolitis associated with a dramatic hyperinflammatory response. This phenotype is virtually identical to that of IL-10 knockout mice (Takeda *et al.*, 1999).

The Janus kinase (JAK)/STAT signaling pathway commences upon cytokine receptor ligation and subsequent phosphorylation of JAK molecules (Shuai and Liu, 2003). STAT proteins are then recruited to the receptor/JAK complex via its SH2 domain, whereupon JAK phosphorylates STAT. Upon dimerization of phosphorylated STAT (p-STAT) and translocation to the nucleus, the p-STAT dimer is further phosphorylated by MAPK. The phosphorylated dimer then binds to target DNA to regulate STAT-sensitive gene transcription.

In vitro Toxoplasma infection of bone-marrow macrophages reveals rapid and sustained STAT3 phosphorylation and subsequent translocation to the nucleus (Butcher et al., 2005). When STAT3 null macrophages are infected with Toxoplasma and subjected to endotoxin triggering, inhibition of IL-12 and TNF- α is severely compromised, suggesting that the parasite exploits an established host-cell immunosuppressive activity. It is not clear at present how STAT3 is activated by the parasite. Experiments in MyD88-deficient macrophages show that this common adapter of TLR signaling is not involved in T. gondii-induced STAT3 activation (Butcher and Denkers, unpublished results). While the response requires live parasites and either host-cell contact or invasion itself, the identity of host JAK molecules activated by the parasite is unknown. Indeed, it seems possible that the parasite itself may directly activate STAT3 during its interaction with the host cell. Regardless, these data are the first example of a microbial pathogen that directly manipulates STAT3 signaling in host cells.

12.2.4 *Toxoplasma* strain-specific effects

There are currently almost no studies that have directly compared Toxoplasma strain types for their effects on infected host cells. A recent review by Saeij and colleagues summarizes the known similarities and differences (Saeij et al., 2005). During in vivo infections, it is clear that strain type can exert an important influence on immunity and pathology. Type I strain infections in mice are uniformly lethal, and this is associated with induction of an excessive proinflammatory cytokine response (Gavrilescu and Denkers, 2001; Mordue et al., 2001). Parasite strain-specific differences in development of toxoplasmic encephalitis during murine infection have also been reported (Suzuki et al., 1989; Ferguson et al., 1994). In human infection, most cases of reactivating toxoplasmosis in AIDS patients result from type II infections (Howe and Sibley, 1995). Whether these effects can be traced to differences in host-cell responses during intracellular infection remains to be determined. Nevertheless, it was recently reported that the ability to induce IL-12 during *in vitro* macrophage infection depended upon parasite strain type. In particular, type II strain infection elicited higher IL-12 levels compared with type I strain infection (Robben *et al.*, 2004). Clearly, this is an area to be investigated further in the future.

12.2.5 *Toxoplasma* stage-specific effects

Essentially, all of what has been written above concerns the tachyzoite stage of the parasite. This rapidly growing form is clearly responsible for much of the disease associated with acute infection, but, as extensively described elsewhere in this volume, Toxoplasma can also exist as an encysted bradyzoite. This stage, which dominates during the chronic phase of infection, is semiquiescent, with relatively slow growth and reduced metabolism. It is believed that bradyzoites' primary function is to serve as the infectious form during transmission between intermediate hosts and/or between an intermediate host and the definitive host, members of the family Felidae. In rare instances, they can also serve as a reservoir for reactivation when a chronically infected host becomes immunocompromised. Bradyzoites, therefore, are central to the entire life cycle of the parasite.

Studies of bradyzoites have been substantially hampered by the fact that they are difficult to grow *in vitro* and they are relatively rare *in vivo*, especially compared with the large numbers of tachyzoites during the acute stages of the infection. The cyst in which they reside further complicates their analysis, as it makes them harder to purify away from the host cells and it might limit uptake of reagents for radio-labeling, for example, although this has not been looked at systematically.

Although tissue cysts can be very large, containing upwards of 500–1000 bradyzoites each, available data indicate that they are at least sometimes inside an individual host cell. This begs the question of how they interact with what is now almost a 'nurse' cell, given the length of time that they may be associated (perhaps as long as months). Certainly, it would be expected that there would be substantial differences compared with the much more aggressive relationship of a tachyzoite with its host cell (a relationship that may last only days and which typically ends with destruction of the host cell). Because of the difficulties of working with bradyzoites there is not a lot of information in the literature, but we have recently explored this question through microarray analysis, comparing the human foreskin fibroblasts infected with tachyzoites vs. bradyzoites (Fouts and Boothroyd, 2006). This work was limited to examination of in vitro cultures about 48 hours after infection, which is very early in the life of a bradyzoiteinfected cell. Interestingly, there were no dramatic differences between tachyzoites and bradyzoites in how they impacted host mRNA abundance, although several genes involved in cytokine- and chemokine-signaling were substantially less induced by the bradyzoite infection than was seen with tachyzoites. This supports the notion that the bradyzoites are more in a 'stealth' mode, attempting to perturb the host cell as little as possible and thus escape immune clearance. Tachyzoites may be long gone by the time the immune response arrives in full force, and thus may not need to be as subtle as bradyzoites.

12.3 MEDIATORS OF ALTERATIONS IN HOST-CELL BIOLOGY

The mechanisms underlying *Toxoplasma*-induced alterations in the host cell are poorly understood. Given that the PV forms the interface between parasite and host, it follows that proteins secreted into the PV are likely to be key mediators of these alterations. Although such roles have long been predicted for proteins localized to the PV, the majority of identified PV proteins have little, if any, homology to characterized proteins, and their specific functions remain unknown.

Toxoplasma possesses three specialized secretory organelles: rhoptries, dense granules, and micronemes. Proteins secreted from the micronemes have not been localized to the PV, and appear to function exclusively in attachment to and invasion of the host cell. The PV contains numerous proteins secreted from rhoptries and dense granules; thus in this section we focus on the rhoptry and dense-granule proteins that may have a role in altering host-cell biology.

First, the proteins secreted into the PV are discussed, and activities that have been localized to the vacuole but whose protein mediators remain anonymous are described. We also speculate about the processes occurring in PV subcompartments, before discussing how recent proteomic analyses have identified new candidates for protein mediators of host modification. Finally, an emerging aspect of *Toxoplasma*-host interactions – the trafficking of proteins beyond the PV into various host compartments – is covered.

12.3.1 Proteins of the parasitophorous vacuole

Toxoplasma extensively modifies the PV with rhoptry and dense-granule proteins. Rhoptry proteins are delivered to the PVM and PV lumen via secretion into the forming vacuole and via trafficking through the host cytosol within evacuoles - vesiclelike structures comprising membranes and rhoptry proteins (Hakansson et al., 2001). Immediately prior to parasite entry these evacuoles are secreted directly into the host cytosol, where they then move along host microtubules and eventually fuse with the PVM (Hakansson et al., 2001). Secretion from the rhoptries appears to occur only during host-cell invasion, whereas dense granules secrete constitutively. Dense-granule proteins localize to the PVM, the PV lumen, and the intravacuolar network (IVN) - a collection of membranous tubules within the PV lumen (for a review on dense granules, see Mercier et al., 2005). These locations can be considered distinct subcompartments of the PV because they contain only partially overlapping sets of proteins.

12.3.1.1 The parasitophorous vacuolar membrane

As the delimiting membrane of the vacuole, the PVM provides a physical interface between the

parasite and the host cytosol (Sinai and Joiner, 1997; Lingelbach and Joiner, 1998). Consequently, *Toxoplasma* PVM proteins are well positioned to have significant roles in host–parasite interactions. Proteins in the PVM have been implicated in recruitment of host organelles (Sinai and Joiner, 2001), nutrient acquisition (Schwab *et al.*, 1994; Sehgal *et al.*, 2005), and activation of immune signaling pathways (Molestina *et al.*, 2003; Molestina and Sinai, 2005).

The rhoptry protein ROP2 has been implicated in recruitment of the host ER and mitochondria to the vacuole. ROP2 associates with the PVM in a manner that exposes its amino-terminus to the host cytosol (Beckers et al., 1994). The processed amino-terminus of ROP2 contains an amphipathic helix reminiscent of mitochondrial import sequences. When expressed as a recombinant protein in CHO cells, a soluble fragment of the ROP2 amino-terminus associates with mitochondria and ER, and GFP is targeted to host mitochondria when expressed as a fusion with the ROP2 amino-terminus (Sinai and Joiner, 2001). Host mitochondria might thus be tethered to the PVM by a stalled attempt to import ROP2. Toxoplasma depleted of ROP2 by an antisense-RNA-mediated process do exhibit decreased mitochondrial, but not ER, recruitment (Nakaar et al., 2003). However, a specific role for ROP2 was not confirmed because the ROP2-deficient parasites also display severe defects in rhoptry biogenesis and parasite replication, and the effects of ROP2 depletion on the secretion of other rhoptry proteins into the vacuole were not assessed. Moreover, Toxoplasma possesses ROP2 homologs, for example ROP4 and ROP8, that could function in conjunction with and/or in the absence of ROP2 (Herion et al., 1993; Beckers et al., 1997). A role for rhoptry proteins in recruitment of host organelles is supported by the observation that evacuoles display the same tight association with host mitochondria and ER as does the PVM (Hakansson et al., 2001). Evacuoles do contain ROP2, but the complete set of evacuole proteins is unknown.

The mechanism by which host organelles first migrate to the PVM remains unclear but appears to utilize host microtubules. Pre-treatment of host cells with nocodazole, a microtubule-depolymerizing agent, reduces the fraction of PVM surface area associated with mitochondria by roughly 50 percent while not affecting the fraction of PVM surface area associated with host ER (Sinai *et al.*, 1997). The observations that evacuoles form tight associations with host ER and mitochondria and that evacuoles traffic along host microtubules to the PVM suggest that they might have a role in organellar recruitment, but this possibility has yet to be directly tested (Hakansson *et al.*, 2001).

The PVM regulates the exchange of material between the PV lumen and the host cytosol. Initially impermeable, the mature PVM allows molecules up to 1300-1900 Da to diffuse freely into and out of the vacuole (Schwab et al., 1994). Presumably, rhoptry or dense-granule proteins form this non-specific channel. Interestingly, Toxoplasma sporozoites secrete a decreased number of proteins into the first PV (PV1) they form, and this vacuole does not display a pore activity (Tilley et al., 1997). Proteomic comparisons of the contents of sporozoite PV1 and the tachyzoite PV will be instrumental in establishing a shortlist of protein candidates for the channel. Recent evidence suggests that the PVM contains specific transporters for macromolecules. Parasites within purified, intact PVs incorporate cholesterol from the medium via a protease-sensitive mechanism (Sehgal et al., 2005).

A PVM protein appears be involved in modulation of host signaling pathways. *Toxoplasma* induces an apoptotic-resistant state in infected host cells partially via activation of the host transcription factor NF- κ B (Payne *et al.*, 2003). As discussed above, a kinase activity that activates NF- κ B co-purifies with a PVM fraction (Molestina and Sinai, 2005; see also section 12.2.3.2).

The PVM-targeted dense-granule proteins GRA3 and GRA7 are also present in strands extending from the PVM into the host cytoplasm (Dubremetz *et al.*, 1993; Coppens *et al.*, 2006). Rhoptry proteins have not been localized to these strands. This lack of rhoptry proteins is consistent with the presence of a protein-sorting mechanism that might be linked to the formation of these strands, which have been observed extending from vacuole to vacuole in host cells harboring multiple vacuoles (Dubremetz *et al.*, 1993; Kim and Boothroyd, unpublished results). Whether these connected vacuoles result from multiple invasion events or partitioning of a single vacuole is not known. The PV extrusions might play a role in nutrient acquisition by serving as conduits for nutrient transport or by simply increasing the surface area of the PVM and thus increasing the sites of interaction between host and parasite. Recent evidence suggests these extrusions might have a role in hijacking host centrosomes (Coppens *et al.*, 2006). Another exciting possibility is that these extensions deliver parasite proteins to locations in the host cell (see section 12.3.3).

12.3.1.2 The intravacuolar network

Dense-granule proteins assemble the IVN from multilamellar vesicles discharged from the posterior end of the parasite 10-20 minutes after invasion (Sibley et al., 1986, 1995). Conversion of the vesicles to nanotubules requires GRA2, and complete formation and maintenance of the IVN requires GRA6, which is recruited by GRA2 (Labruyere et al., 1999; Mercier et al., 2002). After IVN maturation, tubules extend inward from invaginations at the PVM and terminate with closed ends near the parasite plasma membrane (Sibley et al., 1995). As with the strands extruding from the PVM, a role in nutrient acquisition has been proposed for the IVN. Electron micrographs suggest that the cores of some of the IVN tubules are contiguous with host cytosol; thus, the IVN might facilitate increased sites of exchange between the PV lumen and host cytosol by expanding the surface area of the PVM. The authors of a recent electron-micrograph study of infected host cells hypothesize that the IVN has a structural role in the arrangement of parasites within the PV (Magno et al., 2005b). The relationship between the PV extrusions and the IVN is not clear, but they appear to be different entities. Connections between the IVN and the PV extensions have not been seen, and the networks contain only a partially overlapping set of proteins (reviewed in Mercier et al., 2005). PVM invaginations, which

appear to be distinct from the IVN, have also been observed (Magno *et al.*, 2005b; Coppens *et al.*, 2006).

12.3.1.3 The parasitophorous vacuolar lumen

Soluble proteins in the PV lumen may be involved in the capture, transport, and/or processing of metabolites that cross the PVM. The demonstrated ability of GRA1 to bind Ca2+ and its loose association with the IVN have led to speculation that GRA1 delivers Ca2+ to stores in the IVN. The most extensively characterized proteins of the PV lumen are the abundant nucleoside triphosphate hydrolases (NTPases) that catalyze the step-wise dephosphorylation of nucleoside triphosphates to nucleoside monophosphates (Bermudes et al., 1994). A role for the NTPases in nucleoside scavenging has been proposed. According to the model, host nucleoside triphosphates diffuse into the vacuolar lumen through the PVM pore and are then converted to nucleosides, which enter the parasite via transporters in the parasite plasma membrane. However, the NTPases cannot hydrolyze the α -phosphate, and no 5'-nucleotidase has been detected in the vacuole (Ngo et al., 2000). NTPase activity is regulated by the oxido-reductive state of the enzyme. Because addition of the reducing agent dithiothreitol to infected host cells induces parasite egress, and because NTPase activity is maximal under reducing conditions, a link between NTPase activity and parasite egress has been hypothesized (Stommel et al., 1997; Silverman et al., 1998). Depletion of host ATP catalyzed by over-active NTPase might be sensed by Toxoplasma as a signal to exit a cell no longer suitable for parasite replication.

The PV lumen contains a *Toxoplasma* cyclophilin homolog, C-18, that functions as an agonist of chemokine receptor CCR5 (High *et al.*, 1994; Aliberti *et al.*, 2003). Signaling through CCR5 leads to immune activation that prevents the host from succumbing to infection prior to parasite spread (Aliberti *et al.*, 2000). Because dense-granule proteins are constitutively secreted by intracellular as well as extracellular parasites, it is not clear whether the C-18 in the PV lumen has a function other than as a peptidylproline *cis*-trans-isomerase. However, recent evidence suggests that host CCR5 is present in the PVM (Charron and Sibley, 2004). Incorporation of CCR5 into the PVM would allow continued immune activation by intracellular parasites instead of limiting the immune signaling to the brief time that *Toxoplasma* is extracellular.

Also present in the PV lumen are *Toxoplasma* homologs of serine protease inhibitors, (Morris *et al.*, 2002; Morris and Carruthers, 2003). As with C-18, these inhibitors may be important for survival of extracellular parasites and may only be in the PV lumen as a consequence of constitutive secretion through the dense granules. Alternatively, they could be important for inactivating proteases released from the lumen of the host lysosomes sequestered by the parasite within the PV (Coppens *et al.*, 2006).

12.3.2 Proteomic analyses identify potential mediators

Recent proteomic analyses of Toxoplasma secretory organelles have expanded the list of candidates for mediators of alterations in host biology. Prior to these analyses only a limited number of secretory proteins had been identified, and their discovery was largely contingent on the abundance and the antigenicity of the proteins. Given the importance of these secretory organelles in invasion and the establishment of a productive infection, and given the number of observed effects on host cells mediated by unknown parasite factors, proteomic analyses of purified rhoptries and of the excreted/secreted antigens (comprising dense-granule and microneme proteins released into the medium by extracellular parasites) were undertaken to increase understanding of host-parasite interactions (Bradley et al., 2005; Zhou et al., 2005).

Similar to the previously known secretory proteins, a significant portion of proteins identified in these proteomic analyses either lacked homology to characterized proteins or shared homology only with proteins of other Apicomplexan parasites such as *Plasmodium* species – the causative agents of malaria. The large number of novel proteins emphasizes the divergence of the Apicomplexa and the specialization of the phylum for intracellular parasitism. Furthermore, the proteins unique to either *Toxoplasma* or *Plasmodium* reflect the different challenges to invasion and to intracellular parasitic development presented by the preferred host cell of each parasite: *Toxoplasma* invades metabolically active cells, while *Plasmodium* species infect metabolically inert erythrocytes.

The set of newly identified rhoptry proteins includes putative kinases, phosphatases, and proteases (Bradley et al., 2005). The proteases might participate in rhoptry-protein processing, either pre- or post-secretion, or possibly in the inactivation of host signaling proteins or metabolic enzymes. The kinases and phosphatases might regulate rhoptry biogenesis or secretion. They could have roles in regulating the activities of parasite proteins in the PV or even host proteins. ROP2, ROP4, GRA2, GRA6, and GRA7 are phosphorylated post-secretion (Labruyere et al., 1999; Carey et al., 2004; Mercier et al., 2005; Dunn and Boothroyd, unpublished results), and a parasite kinase is capable of phosphorylating IkB, the inhibitor of host NF-kB (Molestina and Sinai, 2005). Provocatively, some of these kinases and phosphatases contain nuclear localization sequences that could target them to the host nucleus. Because the phosphorylation state is a key regulator of transcription factors, a parasite kinase or phosphatase in the host nucleus could have dramatic effects on the host transcriptional response to infection. Indeed, preliminary results indicate that at least two rhoptry proteins do ultimately find their way to the host nucleus (Bradley et al., unpublished results; Gilbert et al., 2006).

The proteins identified in the analysis of excreted/secreted antigens include proteases, adhesins, and a perforin-like protein (Zhou *et al.*, 2005). Perforins are pore-forming proteins that insert into membranes and oligomerize to form channels. *Plasmodium* species employ perforin-like proteins to disseminate within their hosts

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(Kadota *et al.*, 2004; Kaiser *et al.*, 2004). The *Toxoplasma* perforin could function in invasion by inducing the transient membrane break (Suss-Toby *et al.*, 1996), could form the pore in the PVM that allows exchange between the host cytosol and the PV lumen (Schwab *et al.*, 1994), or could be responsible for the permeabilization of the host plasma membrane during ionophore-induced egress (Black *et al.*, 2000).

12.3.3 Proteins trafficked beyond the parasitophorous vacuole

Recent evidence suggests that Toxoplasmainduced host-cell manipulations may be mediated by proteins localized beyond the confines of the parasitophorous vacuole. In addition to the rhoptry proteome and evacuole data described above, GRA7 has been detected on the host surface by FACS analysis (Neudeck et al., 2002), and immunostained electron micrographs appear to show SAG1 within vesicles in the host cytosol (Grimwood and Smith, 1996). Moreover, host cells infected with parasites engineered to secrete recombinant ovalbumin are killed by cytotoxic T cells that specifically recognize an ovalbumin peptide presented by major histocompatibility complex (MHC) class I molecules (Gubbels et al., 2005). MHC class I molecules present peptides derived from proteins in the host cytosol; thus, the recombinant ovalbumin secreted by the parasites appears to be exiting the PV (intact or as peptides) and entering the host cytosol. The same study includes a second example of parasite proteins outside the PVM: credependent DNA recombination occurred in host cells infected with parasites engineered to secrete a cre recombinase with a nuclear targeting signal (Gubbels et al., 2005). However, it was not possible to distinguish between specific targeting of the reporter constructs into the host and low-level leakage of a limited number of proteins from the vacuole.

The export of *Toxoplasma* proteins into the host cell raises the interesting cell-biological question of delivery mechanism(s). Two non-exclusive models are secretion into the host cytosol prior to

vacuole formation, and secretion from within the developed vacuole (Figure 12.3). The former model is based in part on the observation that Toxoplasma secretes evacuoles into the host cell prior to vacuole formation (Hakansson et al., 2001). Evacuoles associate with host cytoskeletal elements and fuse with the PVM. They contain a subset of rhoptry proteins, and have been proposed to have a role in delivering rhoptry proteins and membranes to the developing PV. As yet, it is not clear whether evacuoles are membrane-limited; however, if they are, rupturing or some other process would be needed to allow proteins they might contain to get out and become free within the host cytosol. Even if evacuoles are not used to transport proteins into the host cytosol, they demonstrate that parasite material can be delivered outside the vacuole, and this might include soluble proteins in addition to the evacuoles. Electrophysiology studies have demonstrated a transient break in the host plasma membrane immediately prior to invasion; this could be the hole through which Toxoplasma secretes evacuoles and other proteins into the host cell (Suss-Toby et al., 1996).

In the second model, Toxoplasma trafficks proteins, perhaps via the PVM extrusions, into the host cell subsequent to vacuole formation. No protein translocation machinery in the PVM has been identified, but a definitive analysis of PVM proteins has not been performed. Likewise, no protein translocator has been seen in Plasmodium species, which are known to target numerous proteins to the erythrocyte surface and cytosol. After these erythrocyte-bound proteins are secreted into the PV, they undergo a sorting process and are then translocated into the host cell by an undefined mechanism. The amino-acid motif that targets Plasmodium proteins for translocation beyond the PV has recently been determined (Hiller et al., 2004; Marti et al., 2004). Currently there is no evidence to indicate that a similar signal functions in Toxoplasma; more proteins targeted to the host cell must be identified before the presence of a targeting motif can be assessed.



FIGURE 12.3 Delivery of Toxoplasma proteins beyond the parasitophorous vacuole. Toxoplasma proteins have been localized to compartments of the host cell outside the parasitophorous vacuole (PV): the densegranule protein GRA7 trafficks to the host plasma membrane, while recently identified rhoptry proteins traffic to the host nucleus. Microneme proteins are secreted during invasion, but have not been localized inside the host cell. The mechanisms of transport are unknown. Two possible, non-exclusive models are direct secretion into the host cell during invasion, and translocation into the host from within the PV. During invasion, Toxoplasma secretes evacuoles, proteinaceous membrane structures, directly into the host cytoplasm. Although evacuoles comprise proteins and membranes, it is not clear whether they are true membranelimited vesicles. Evacuoles deliver rhoptry proteins (ROPs) to the PV membrane (PVM) and might be involved with recruitment of host mitochondria to the PVM. Proteins destined for localization outside the PV might be secreted as constituents of evacuoles or concomitantly with evacuoles (1). After invasion, dense-granule proteins (GRAs) secreted into the PV might be entering the host cytoplasm via protein translocators in the PVM and/or via inclusion in vesicles budding from the PVM (2). Translocation out of the vacuole may involve the PV extrusions. Once in the host cytoplasm, Toxoplasma proteins might utilize host transport systems. For example, ROPs possessing putative nuclear localization signals have been observed in host nuclei. This figure is reproduced in color in the color plate section.

12.4 CONCLUSIONS

It is clear that *Toxoplasma* has evolved complex mechanisms for very intricate cross-talk with the host cells in which it resides. While we are beginning to understand the outcomes of these conversations – i.e. which host genes and proteins are

modulated and how the modulations impact parasite survival – we remain largely ignorant of the underlying mechanisms. Delineating the mechanisms whereby *Toxoplasma* alters its host cells will be a challenge in the coming decade, but the powerful tools for genome-wide analysis, genetics, proteomics and the like should make for rapid progress. Ultimately, this information will have obvious benefit in opening up new potential avenues for intervention in disease. For example, knowing that the parasite upregulates a pathway in the host cell will identify drugs that downregulate that pathway as potential inhibitors of *Toxoplasma* growth. For instances where the parasite and host cell each contribute to an essential process, combination therapy might be highly synergistic.

Less obviously, we may also learn much about the basic biological processes of non-infected human or other mammalian host cells. That is, *Toxoplasma* is a powerful probe that, as it perturbs a human cell, reveals how such a host cell normally functions. Hence, as we observe the coordinated cascades of changes in gene expression in infected cells and find the host molecules that mediate these cascades, we are likely to find entirely new pathways that were previously 'known' only by the parasite.

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13

Bradyzoite Development

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13.1 INTRODUCTION

Toxoplasma gondii is a ubiquitous protozoan parasite of mammals and birds. It belongs to the phylum Apicomplexa, which includes other important pathogens such as *Plasmodium*, *Eimeria*, *Cyclospora*, *Babesia*, and *Cryptosporidium* (Luft and Remington, 1988; McLeod *et al.*, 1991; Wong and Remington, 1993). The Apicomplexa are defined by the presence of a complex of apical secretory organelles (micronemes, rhoptries, and dense granules). *Toxoplasma gondii* has three infectious stages: tachyzoites (asexual), bradyzoites (in tissue cysts, asexual), and sporozoites (in oocysts, sexual reproduction). Infection is typically acquired either by ingestion of undercooked meat harboring tissue cysts (containing bradyzoites) or by the ingestion of food contaminated with oocysts (containing sporozoites). Upon ingestion, sporozoites or bradyzoites will invade the intestinal epithelium, differentiate into the rapidly growing tachyzoite form, and disseminate throughout the body. After oral ingestion, in the intestine of definitive hosts (i.e. cats) the parasites can continue to differentiate into merozoites, and sexual reproduction can occur with development of oocysts containing sporozoites. Oocysts are only produced in cat intestine, and are present in cat feces.

In both definitive and intermediate hosts, tachyzoites disseminate and then differentiate into bradyzoites that remain latent. The number of tissue cysts formed in mouse brain appears to regulated by the class I gene L^d (Brown et al., 1995). More tissue cysts are produced in mice that become mildly ill from infection than in those that become highly symptomatic. Tissue cyst persistence may vary with both the strain of T. gondii and the strain of murine host (Ferguson et al., 1994). Mice that are resistant to acute infection can be very susceptible to chronic infection with encephalitis, suggesting that control of chronic infection is not linked to the loci that control susceptibility to acute infection (Brown and McLeod, 1990; Brown et al., 1994; McLeod et al., 1989, 1993). Bradyzoites contained in cysts are refractory to most chemotherapeutic agents used for treatment of toxoplasmosis, and tissue cysts are produced in any animal capable of being infected with T. gondii. The persistence and reactivation of bradyzoite forms is a major cause of disease in humans.

In most individuals, acute infection with T. gondii is asymptomatic or causes mild symptoms similar to a self-limited mononucleosis-like syndrome. If an immunologically naïve (i.e. seronegative) pregnant woman is infected, transmission of this parasite to the fetus can occur with the development of a congenital infection that can result in a fetopathy and a relapsing chorioretinitis (Remington et al., 1995; Wong and Remington, 1993). Although overwhelming disseminated toxoplasmosis has been reported in immunocompromised hosts, such as patients with advanced HIV infection, the predilection of this parasite for the central nervous system, causing necrotizing encephalitis, constitutes its major threat. The incidence of clinically apparent toxoplasmosis has waned with the development of highly active antiretroviral treatment (HAART). Although less prevalent in AIDS patients, T. gondii has been implicated in waterborne outbreaks and deaths in marine wildlife due to water pollution, resulting in its listing as a Category B agent (http:// www3.niaid.nih.gov/Biodefense/bandc_priority. htm) (Bowie et al., 1997; Aramini et al., 1999; Miller et al., 2002).

The development of *Toxoplasma* encephalitis as well as relapsing chorioretinitis in congenital

infection is believed to be due to the transition of bradyzoites in tissue cysts into the active and rapidly replicating tachyzoite stage. It is hypothesized that in chronic toxoplasmosis bradyzoites regularly transform to tachyzoites, but that the tachyzoites are controlled by the immune system. In mice, new tissue cysts have been demonstrated to be formed during chronic infection (van der Waaij, 1959; McLeod et al., 1991; Gross et al., 1992; Ferguson et al., 1994). Such a dynamic equilibrium between encysted and replicating forms would lead to recurrent antigenic stimulation, possibly accounting for the lifelong persistence of antibody titers found in chronically infected hosts (Frenkel and Escajadillo, 1987; Ferguson et al., 1989). In addition to rodents (mice, hamsters, and rats), tissue cyst rupture has been demonstrated to occur in the primate Aortus lemurinus (Frenkel and Escajadillo, 1987). Due to its central importance in disease pathogenesis, the biology of stage differentiation has been an active area of research. While progress has been made, bradyzoite-totachyzoite interconversion is not well understood. Many initial seminal observations have been reviewed elsewhere (Weiss and Kim, 2000).

13.2 BRADYZOITE AND TISSUE CYST MORPHOLOGY AND BIOLOGY

Tachyzoites (*tachos* = fast) are the rapidly growing life stage of *T. gondii* that have also been called endozoites or trophozoites. Bradyzoites (*brady* = slow), also called cystozoites, are the life stage found in the tissue cyst and are believed to replicate slowly. Both stages replicate within a parasitophorous vacuole within the host cell, which is modified by the particular life stage into either a tachyzoite- or a bradyzoite- (tissue cyst) specific vacuole. Tissue cysts are intracellular structures in which the bradyzoites divide by endodyogeny (in the same replicative mechanism as tachyzoites).

Recent data suggest that in mature cysts bradyzoites may enter a G_0 stage of the cell cycle, essentially becoming a non-replicative differentiated organism with a DNA content of 1N

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(Radke et al., 2003). In mature cysts, occasional degenerating bradyzoites are seen (Pavesio et al., 1992); in vitro missegregation and loss of apicoplasts in mature bradyzoites has also been demonstrated (Dzierszinski et al., 2004). Immature tissue cysts may be as small as 5 µm, containing only two organisms; suggesting that the commitment to bradyzoite differentiation is an early event in the establishment of a parasitophorous vacuole. Such immature cysts increase in size, and thus it is clear that T. gondii expressing bradyzoite-specific markers can replicate during the maturation of tissue cysts (van der Waaij, 1959). The size of a tissue cyst is variable, but on average a mature cyst in the brain is spherical and 50-70 µm in diameter, containing approximately 1000 crescent-shaped $7 \times 1.5 \,\mu$ m bradyzoites. In muscle such tissue cysts are more elongated, and may be up to 100 µm in length (Dubey et al., 1998).

Tissue cyst size is dependent on cyst age, the host cell parasitized, the strain of T. gondii, and the cytological method used for measurement. Young and old cysts can be distinguished readily by their ultrastructural features. (Scholytyseck et al., 1974; Sims et al., 1989; Fortier et al., 1996; Dubey et al., 1998). While tissue cysts can develop in any visceral organ (e.g. lungs, liver, and kidneys), they are more common in neural (e.g. brain and eyes) or muscle (e.g. skeletal and cardiac) tissue (Dubey et al., 1998). In the central nervous system, cysts have been reported in neurons, astrocytes, and microglia (Ferguson and Hutchison, 1987a, 1987b; Ferguson et al., 1989); however, it is not known which is the preferred or predominant cell in which cysts form, and whether host cells influence cyst formation. In tissue culture, both astrocytes and neurons have been demonstrated to support cyst formation (Halonen et al., 1996, 1998a; Fagard et al., 1999) (Figure 13.1). In vitro bradyzoites have been demonstrated to exit an existing cyst (vacuole) and establish new cysts (vacuoles) in the same cell or adjacent cells (Dzierszinski et al., 2004). This may account for the observation of clusters of cysts seen in vivo in the central nervous system.

The tissue cyst wall or bradyzoite parasitophorous vacuole membrane is elastic, thin (< 0.5 μ m thick),



FIGURE 13.1 Ultrastructure of a *T. gondii* tissue cyst *in vitro*. This electron micrograph demonstrates multiple bradyzoites within a cyst. The cyst is within a murine astrocyte grown in *in vitro* tissue culture. The clear vacuoles are consistent with amylopectin granules seen in bradyzoites. The cyst wall is formed from the parasitophorous vacuole membrane. Bar = $25 \mu m$. Image courtesy of Dr S. Halonen, MSU.

faintly PAS positive, and argyrophic (although this depends on the silver staining method used) (Sims et al., 1988). The tissue cyst wall is phase-lucent by phase-contrast microscopy, and the vacuole often contains an odd number of organisms (asynchronous division) that are club-shaped (Weiss et al., 1995; Dzierszinski et al., 2004). Pale blue autofluorescence of the tissue cyst wall can be observed using UV light at 330–385 nm (Lei et al., 2005). The cyst wall appears to be composed of both hostand parasite-derived materials and lined by granular material, which also fills up the space between the bradyzoites (particularly in mature cysts) (Ferguson and Hutchison, 1987a, 1987b; Sims et al., 1988). During development in astrocytes, the bradyzoite parasitophorous vacuole is surrounded by a layer of host-cell intermediate filaments (glial fibrillary acidic protein) that limits the contact of the vacuole with host ER and

mitochondria (Halonen *et al.*, 1998b); however, this material is not incorporated into the cyst wall. Tissue cysts have a specific gravity of 1.056, and have been successfully purified from brain tissue using isopycnic centrifugation in a Percoll gradient (Cornelissen *et al.*, 1981), with discontinuous 25–30 percent Percoll gradients (Blewett *et al.*, 1983; Pettersen, 1988) and 20 percent dextran (Freyre, 1995) (see Chapter 15 for protocol).

Bradyzoites differ ultrastructurally from tachyzoites in that they have a posteriorly located nucleus, solid rhoptries which are often looped back on themselves, numerous micronemes, and polysaccharide (amylopectin) granules (Ferguson and Hutchison, 1987b; Dubey, 1997). Lipid bodies are not seen in bradyzoites, but are numerous in sporozoites and are occasionally seen in tachyzoites. The contents of rhoptries in mature bradyzoites are electron-dense, in contrast to the labyrinthine rhoptries seen in tachyzoites and in immature bradyzoites (Dubey et al., 1998). Bradyzoites stain red with periodic acid-Schiff (PAS) due to the presence of amylopectin granules, whereas tachyzoites are usually PAS negative (Dubey et al., 1998). In general, bradyzoites are more slender than tachyzoites. Bradyzoites are more resistant to acid pepsin (1- to 2-hour survival in pepsin-HCl) than tachyzoites (10-minute survival) (Jacobs and Remington, 1960; Popiel et al., 1996). The prepatent period (time to oocyst shedding) in cats following feeding of bradyzoites is shorter (3-7 days) than that following feeding of tachyzoites (over 14 days). This is the most sensitive biologic marker of mature functional tissue cysts (Dubey, 1997; Dubey et al., 1997).

13.3 THE DEVELOPMENT OF TISSUE CYSTS AND BRADYZOITES IN VITRO

The development of tissue cysts *in vitro* was reported over 40 years ago (Hogan *et al.*, 1960; Matsukayashi and Akao, 1963); however, the morphologic similarity of bradyzoites and tachyzoites by light microscopy made it difficult to study these differentiation events until the development of antibodies to bradyzoite-specific antigens. In 1977, Hoff demonstrated that in vitro-produced cysts led to oocyte excretion in cats with a prepatent period consistent with that of tissue cysts (i.e. mature bradyzoites) (Hoff et al., 1977). Using TEM, it has been demonstrated that while cyst-like structures were present within 3 days of infecting host cells in tissue culture, by bioassay (in cats) mature cysts were not present until 6 days post-infection of host cells in vitro (Dubey, 1997). Prolonged passage of T. gondii or other Apicomplexa in vitro may lead to the loss of their ability to differentiate into other stages. For example, prolonged passage of Besnoitia jellisoni in vitro leads to a loss of its ability to form tissue cysts in mice, and many type II isolates (e.g. PLK) of T. gondii cannot form oocysts in cats (Frenkel et al., 1976).

Bradyzoite-specific monoclonal antibodies (see Table 13.1) have greatly facilitated studies of bradyzoite development *in vitro* and the recognition of techniques for the induction of differentiation. Recently, parasite lines have also been developed that use GFP under the control of various stage-specific promoters (Singh *et al.*, 2002; Ma *et al.*, 2004). Using parasites containing chloramphenicol acetyltransferase (CAT) expressed constitutively from the α -tubulin promoter (TUB1), and β -galactosidase (β GAL) expressed from a bradyzoite-specific promoter (BAG1), it is possible to measure both the growth rate and degree of bradyzoite differentiation (Eaton *et al.*, 2005).

In tissue-culture studies, it is evident that bradyzoites spontaneously convert to tachyzoites and that tachyzoites spontaneously convert to bradyzoites (Jones et al., 1986; Lindsay et al., 1991, 1993a, 1993b; Bohne et al., 1993, 1994; McHugh et al., 1993; Soete et al., 1993, 1994; Popiel et al., 1994, 1996; Weiss et al., 1994; Parmley et al., 1995; Lane et al., 1996; Soete and Dubremetz, 1996; De Champs et al., 1997). It was noted by Matsukavashi in 1963, and has been confirmed by several groups using bradyzoite-specific monoclonal antibodies, that T. gondii strains with a slower rate of replication were more likely to develop cysts in vitro, and that slowing the replication of virulent strains would allow tissue cysts of virulent stains to develop in vitro (Matsukayashi and Akao, 1963). Lowvirulence strains are high cyst-forming strains in

Name of antigen	Monoclonal antibody	Size on immunoblot (kDa)	Location by IFA	Proposed function	Cloned
BAG1/hsp30 aka BAG5	7E5 74.1.8	28	Cytoplasm	Small heat- shock protein	Yes
BSR4 (p36)	T84A12	36	Surface	SAG1 family antigen, also in sporozoites	Yes
SAG4A (p18)	T83B1	18	Surface	Surface antigen	Yes
None	DC11	Not reactive	Surface	?	One of SAG family?
p21	T84G11	21	Surface	Surface antigen	One of SAG family?
p34	T82C2	34	Surface	?	One of SAG family?
SRS9	Murine polyclonal only	43	Surface	Surface antigen, SAG related sequence	Yes
MAG1	None	65	Matrix		Yes, revised studies indicate also expressed in tachyzoites
None	E7B2	29	Matrix		No
None	93.2	Not reactive	Matrix		No
None	1.23.29	19	Matrix		No
CST1	73.18; also recognized by DBA lectin	116	Cyst wall		No
CST1?	CC2	115 (bradyzoite) 40 (tachyzoite)	Cyst wall	Same as CST1?	No
LDH2	Polyclonal sera weakly cross- reacts to LDH1	35 kDa (33 kDa for LDH1)	Cytosolic	Glycolysis	Yes, tachyzoite isoform is LDH1
ENO1	Polyclonal sera to ENO2 and ENO1 do not cross-react		Nuclear and cytosolic	Glycolysis	Yes, tachyzoite isoform ENO2

 TABLE 13.1
 Common bradyzoite markers

mice (e.g. type II, such as ME49 or Pru, or type III strains 76K or VEG), and have a higher spontaneous rate of cyst formation in culture than do virulent type I strains such as RH (Soete *et al.*, 1994). This biological phenotype also correlates with the patterns of gene expression observed (Radke *et al.* 2005). Additionally, we have observed that the efficiency of cyst production by avirulent *T. gondii* isolates decreases with prolonged *in vitro* passage (this correlates with the development of a more rapid growth phenotype for these strains in prolonged tissue culture).

Stress conditions are associated with an induction of bradyzoite development - i.e. there are more bradyzoites under these conditions than would be expected from the simple inhibition of tachyzoite replication. Conditions that induce bradyzoite formation within host cells are temperature stress (43°C; Soete et al., 1994), pH stress (pH 6.6-6.8 or 8.0-8.2; Soete et al., 1994; Weiss et al., 1995; Soete and Dubremetz, 1996), chemical stress (Na arsenite; Soete et al., 1994), and nutrient stress (arginine starvation; Fox et al., 2004). In murine macrophage lines derived from bone marrow, IFN-γ increases bradyzoite antigen expression (Bohne et al., 1994). This appears to be due to the production of nitric oxide (NO), as bradyzoite differentiation was inhibited by treating macrophages with NMMA (NG-monomethyl-L-arginine, an inducible nitric-oxide synthase inhibitor) (Bohne et al., 1994). Bradyzoite differentiation is also enhanced by sodium nitroprusside (SNP), an exogenous NO donor (Bohne et al., 1994; Weiss et al., 1996; Kirkman et al., 2001). Similarly, both oligomycin (an inhibitor of mitochondrial ATP synthetase function) and antimycin A (an inhibitor of the electron transport of the respiratory chain) increase bradyzoite antigen expression (Bohne et al., 1994; Tomavo and Boothroyd, 1995). pH stress is one of the most commonly used methods to induce bradyzoite differentiation in the laboratory (Figure 13.2).

The contribution of the host cell to stage conversion remains to be elucidated. Two groups have reported (Weiss *et al.*, 1998; Yahiaoui *et al.*, 1999) that exposure of extracellular tachyzoites to stress conditions (pH 8.1) will result in an increase in bradyzoite differentiation, consistent with a direct effect of stress on the parasite. Nonetheless, most of the agents that induce differentiation have profound effects upon host cells, and it is likely that alterations in host cell signaling also have significant impact upon bradyzoite differentiation. It has recently been demonstrated that the parasite-specific PKG inhibitor compound 1 (Donald and Liberator, 2002; Donald *et al.*, 2002; Gurnett *et al.*, 2002; Nare *et al.*, 2002) requires host-cell protein synthesis in order to induce bradyzoite formation, suggesting that its effect on differentiation is mediated through a perturbation of the host cell rather than directly on the parasite (Radke *et al.*, 2006).

When cells are infected by bradyzoites from tissue cysts, differentiation to tachyzoites and the appearance of tachyzoite-specific antigens (SAG1) occurs within 15 hours and before cell division has occurred (Soete and Dubremetz, 1996). Vacuoles containing organisms expressing only tachyzoite antigens are clearly evident within 48 hours of infection. When bradyzoite differentiation occurs in cell culture following infection with tachyzoites, all of the currently available markers for bradyzoite formation, with the exception of p21 (mAb T84G10), can be detected within 24 hours of infection (Gross et al., 1996; Lane et al., 1996). This includes markers of bradyzoite surface antigens, as well as those related to cyst-wall formation. Conversion between these two stages is a rapid event, and the commitment to differentiation may be occurring at the time of or shortly after invasion and formation of the parasitophorous vacuole.

By 3 days after exposure to conditions that induce bradyzoite development, vacuoles are present in tissue culture with the electron microscopic characteristics of cysts; however, reactivity to mAb T84G10 (p21) does not appear until after 5 days. As assessed by bioassay in cats, mature/functional cysts are not formed until at least 6 days in culture. Additional markers of mature functional cysts are needed to facilitate *in vitro* studies on cyst biology. There may be differences in antigen expression and replication in early bradyzoites and late bradyzoites, but a detailed chronology of marker expression has not yet been developed.



FIGURE 13.2 Development of T. gondii life stages in vitro.

Primary murine astrocytes (Figures 13.2A, 13.2B) and human foreskin fibroblasts (Figures 13.2C, 13.2D) infected with *T. gondii* (ME49/PLK strain). Human fibroblasts infected with *T. gondii* (ME49/PLK strain) were maintained at pH 8.0 (Figure 13.2C) or pH 7.2 (Figure 13.2D) for 3 days.

(A) Phase micrograph demonstrating an *in vitro* tissue cyst.

- (B) IFA using anti-CST1 (mAb 73.18) demonstrating staining of the cyst wall of the bradyzoite parasitophorous vacuole (the corresponding phase microscopy is shown in Figure 13.2A).
- (C) IFA using anti-BAG1 (mAb 74.1.8) demonstrating bradyzoite development in several parasitophorous vacuoles. Staining occurs throughout the cytoplasm of the bradyzoites.
- (D) IFA using anti-SAG1 (mAb DG52) demonstrating tachyzoite development and rosette formation. Staining is localized to the surface of each tachyzoite.

This figure is reproduced in color in the color plate section.

13.4 THE CELL CYCLE AND BRADYZOITE DEVELOPMENT

It is probable that bradyzoite differentiation from tachyzoites is a programmed response related to a slowing of replication and lengthening of the cell cycle (Jerome *et al.*, 1998), similar to the programmed expansion and differentiation reported in other coccidia. The cell cycle in tachyzoites is characterized by major G1 and S phases and a relatively short G2+M. As *T. gondii* replication slows, there is an increase in duration of the G1 phase of the cell cycle. It is not known whether the checkpoints within this cell cycle differ from those observed in yeast and mammalian cells, but preliminary studies suggest some differences (Radke *et al.*, 2001;

Khan et al., 2002). When T. gondii sporozoites (VEG strain) infect human fibroblasts in vitro, they transform to rapidly dividing tachyzoites having a half-life of 6 hours. After 20 divisions (approximately 5 days in culture), these tachyzoites shift to a slower growth rate with a half-life of 15 hours (Radke et al., 2001). Bradyzoite differentiation, as defined by the expression of bradyzoite-specific antigens, occurs spontaneously when the population shifts to a slower growth rate $(t_{1/2} \ 16 \ h)$, but is not seen in the rapidly dividing $(t_{1/2} 6 h)$ organisms. Analysis of gene expression with SAGE shows that bradyzoite mRNA levels correlate well with what has been observed in studies of bradyzoite antigen expression (Radke et al., 2005). These data are consistent with observations that spontaneous bradyzoite differentiation occurs less readily in rapidly dividing strains of T. gondii such as RH, and that stress conditions that slow growth induce bradyzoite differentiation (Bohne et al., 1994; Jerome et al., 1998; Weiss and Kim, 2000).

Bradyzoites can undergo asynchronous division in vacuoles, resulting in vacuoles with odd numbers of organisms instead of the usual multiples of two seen in tachyzoite vacuoles (Dzierszinski *et al.*, 2004). Bradyzoite differentiation cannot be uncoupled from slowing of the cell cycle, and may be a stochastic event that occurs at a specific point in the cell cycle when replication has slowed sufficiently. It appears that all conditions that slow (but do not arrest) the cell cycle result in bradyzoite differentiation, whereas conditions that block cell-cycle progression do not result in appreciable differentiation (Fox and Bzik, 2002; Khan *et al.*, 2002).

When bradyzoites liberated from tissue cysts are used to infect host cells, tachyzoite-specific antigens (e.g. SAG1) are expressed within 15 hours of infection prior to any significant cell division by the infecting bradyzoite (Soete and Dubremetz, 1996). By 48 hours p.i., vacuoles containing organisms expressing only tachyzoite antigens are evident. In a similar fashion, tachyzoites used for infection with stress exposure (pH 8.1) express bradyzoite antigens at 24 hours, and vacuoles containing one or two organisms can be found that have bradyzoite antigens. Conversion between these developmental stages is therefore a rapid event, and commitment to differentiation may be occurring at the time of or shortly after invasion.

Although a small proportion of replicating parasites (< 10 percent) have 1.8 to 2N DNA content (i.e. are in a G2 premitotic state), parasites that coexpress bradyzoite marker BAG1 and tachyzoite marker SAG1 are much more likely to have a G2 premitotic DNA content - approximately 50 percent of these parasites (Radke et al., 2003). Interestingly, flow cytometry measurement of DNA content of mature bradyzoites isolated from tissue cysts demonstrates that this stage has a 1N DNA content, consistent with their being in a quiescent G_o state (Radke et al., 2003). Overall, it appears that commitment to bradyzoite differentiation probably occurs at a particular point in the cell cycle and that transit through the cell cycle is required for differentiation. These early 'prebradyzoites' can continue to replicate, but at some point in the development and maturation of the tissue cyst the fully mature bradyzoites enter a quiescent Go state.

13.5 THE STRESS RESPONSE AND BRADYZOITES

While differentiation is a programmed response, bradyzoite differentiation is also a stress-related response of T. gondii to environmental conditions for example, the inflammatory response of the host. Many different classical stress-responseinducing conditions, such as temperature, pH, and mitochondrial inhibitors, are associated with bradyzoite development in vitro. Bradyzoite differentiation probably shares features common to other stress-induced differentiation systems, such as glucose starvation and hyphae formation in fungi or spore formation in Dicvostelium (Soderbom and Loomis, 1998; Thomason et al., 1999). These systems have demonstrated unique proteins related to specific differentiation structures in each organism, as well as the utilization of phylogenetically conserved pathways. Many of these signaling pathways involve cvclic nucleotides and kinases as part of the regulatory system in differentiation. There is a significant body of evidence relating heat-shock proteins with differentiation in various phyla (Heikkila, 1993a, 1993b).

BAG1 (also known as BAG5) is one of the most abundant bradyzoite-specific genes found in the *T. gondii* bradyzoite ESTs, representing about 3 percent of all bradyzoite-specific clones. This gene has homology to small heat-shock proteins, and therefore has also been called *hsp30* (Bohne *et al.*, 1995; Parmley *et al.*, 1995). Both *BAG1* mRNA and protein (a 28-kDa cytoplasmic antigen) are upregulated during bradyzoite formation, suggesting transcriptional regulation of its expression. *Toxoplasma gondii* expressing BAG1 are seen within 24 hours of exposure to pH 8.0 or other stress conditions.

The carboxyl-terminal region of BAG1 has a small heat-shock motif most similar to the small heat-shock proteins of plants, and near the N-terminus is a synapsin Ia-like domain that may be involved in the association of this small heatshock protein with proteins during development. Yeast two-hybrid screening with both the N-terminus and C-terminus domains has identified potential BAG1 interacting proteins that are currently being confirmed (Weiss and Ma, unpublished data). BAG1 appears to be present mainly as a monomer, and the carboxy-terminal region of BAG1 did not react with itself in yeast two-hybrid screening (Weiss and Ma, unpublished data). Four other small heat-shock proteins are present in T. gondii; of these, hsp20, hsp21, and hsp29 are expressed in both tachyzoites and bradyzoites, and hsp28 is specific for tachyzoites (de Miguel, 2005). None of these other small heat-shock proteins is associated with bradyzoite differentiation, and all are present as multimeric forms in the cytosol of T. gondii.

A homolog of heat-shock protein 70 (hsp70) is induced during the transition both from tachyzoite to bradyzoite and from bradyzoite to tachyzoite (Lyons and Johnson, 1995, 1998; Silva *et al.*, 1998; Weiss *et al.*, 1998; Miller *et al.*, 1999). Induction of hsp70 can be demonstrated at both the protein and RNA levels. Quercetin, an inhibitor of hsp synthesis, can suppress hsp70 and decrease the ability of pH shock to induce bradyzoite formation (Weiss et al., 1996, 1998). Extracellular T. gondii treated with 1-hour exposure to pH 8.1 versus pH 7.1 express a 72-kDa inducible hsp70 (detected with mAb C92F3A-5; Stressgen) (Weiss et al., 1998), and this extracellular treatment induces bradyzoite formation. T. gondii-infected cultures treated with pH 8.1 show four-fold induction of the hsp70 levels compared to T. gondii grown in pH 7.1-treated cells (Weiss et al., 1996, 1998), which is similar to the magnitude of the hsp70 response demonstrated in Trypanosoma cruzi, Theileria annulata, and Plasmodium falciparum (Shiels et al., 1997). A similar increase in hsp70 is seen with in vivo cysts during reactivation in a murine model induced by anti-\gamma-interferon (Silva et al., 1998). The relative level of expression of hsp70 by T. gondii is also associated with virulence, and RH strain has four copies of a sevenamino-acid repeat unit (GGMPGGM) at the C-terminus of its hsp70, compared with five copies in the ME49 strain (Lyons and Johnson, 1998).

In addition to hsp70, hsp90 mRNA and protein levels also increase during bradyzoite differentiation (Echeverria *et al.*, 2005). Fluorescence microscopy demonstrated that in tachyzoites hsp90 is in the cytosol, whereas in mature bradyzoites hsp90 is present in both the nucleus and cytosol. In *T. gondii* mutants that are unable to differentiate, hsp90 is only found in the cytoplasm. Geldanamycin, a benzoquinone ansamycin antibiotic capable of binding and disrupting the function of hsp90, blocks conversion both from tachyzoite to bradyzoite and bradyzoite to tachyzoite (Echeverria *et al.*, 2005).

The expression of reporter genes driven by the hsp70 promoter is also responsive to conditions that induce bradyzoite formation (Ma *et al.*, 2004). The pH regulated *cis*-element of the hsp70 promoter maps to the region –420 through –340 from the initial ATG of the hsp70 gene (Ma *et al.*, 2004). At –650 bp from the initial ATG is the sequence AGAGACG, which has been described as a *cis*-acting element that acts as an enhancer in the transcription of several *T. gondii* genes (Mercier *et al.*, 1996). There is a series of nGAAn repeats –385 from the initial ATG, which have similarity to the heat-shock element (HSE) described in other

eukaryotes (Morimot *et al.*, 1994). A CCGGGG located next to this HSE is similar to the sp1-hsp70 site in the human hsp70 promoter (Morgan, 1989).

In addition, the hsp70 promoter contains several AGGGG or CCCCT regions which are similar to the core region of the STRE (stress-response element) described in many eukaryotic genes (Estruch, 2000). Similar STRE and HSE elements are seen in the promoter region of enolase 1, a bradyzoite-specific isoform of enolase (Kibe et al., 2005). In yeast, enolase is also known as hsp48, as it is a stress-related heat shock protein (Iida and Yahara, 1985). The STRE-binding activity detected in nuclear extract from stress-induced bradyzoites is significantly higher than that from non-stressed tachyzoites (Kibe et al., 2005). Transcription factors responsible for regulation of hsp70 and enolase 1 have not vet been identified, although electromobility shift assays (EMSA) suggest that there are specific proteins that recognize the STRE and HSE elements of these genes (Ma et al., 2004; Kibe et al., 2005). Although there is an area of similarity between the BAG1 promoter region and that of hsp70 promoter region, oligonucleotides from this BAG1 upstream region do not compete in EMSA (Ma et al., 2004). (See Chapter 16 for further discussion of regulation of gene expression).

In eukaryotes, the cellular stress response is associated with phosphorylation of the alpha subunit of eIF2 (eukaryotic initiation factor-2) enhancing the translational expression of bZIP proteins such as GCN4 in yeast and ATF4 in mammals (Hinnebusch and Natarajan, 2002). A novel eIF2 protein kinase, designated TgIF2K-A (Toxoplasma gondii initiation factor-2 kinase) has been identified, and eIF2 phosphorylation is enhanced by conditions known to induce bradyzoite differentiation. Analysis of the T. gondii genome demonstrates homologs of genes involved in this stress response pathway, such as GCN1 and GCN20 (Sullivan et al., 2004), as well as the histone acetyltransferae GCN5 (Hettmann and Soldati, 1999; Sullivan and Smith, 2000) and an SWI/SNF homolog (Sullivan et al., 2003). Both GCN5 and SWI/SNF are known to be recruited by GCN4 in yeast, resulting in changes in gene expression as a consequence of stress conditions (Rodrigues-Pousada et al., 2004).

Epigenetic events, such as histone modification, are probably key factors in the differentiation process resulting in bradyzoite formation. TgSRCAP (Toxoplasma gondii Snf2-related CBP activator protein) is an SWI2/SNF2 family chromatin remodeler whose expression increases during cyst development (Nallani and Sullivan, 2005). As in other organisms, histone acetylation and methylation patterns correlate with gene activation or repression in T. gondii (Saksouk et al, 2005). As bradyzoite differentiation proceeds, gene activation markers are associated with bradyzoite promoters (Saksouk et al, 2005). Epigenetically regulated changes in gene expression and changes in chromatin modifications typically require transit through S phase, as has been observed for induction of expression of bradyzoite markers (Radke et al., 2003). Genome-wide analyses of epigenetic changes that occur during stage differentiation are underway (Gissot and Kim, unpublished). It is interesting to note that in *Dictyostelium*, a GCN2 ortholog is involved in the development of the fruiting body in response to nutrient stress (Fang et al., 2003).

13.6 SIGNALING PATHWAYS AND BRADYZOITE FORMATION

Although tachyzoites and bradyzoites are well defined morphologically, little is known about the signal(s) that mediate this transformation. Studies of other microorganisms, including the fungi and other protozoa, suggest that differentiation involves conserved signaling pathways, such as cyclic nucleotides, which are also involved in the response to stress or to nutrient starvation. The effect of cyclic nucleotides on bradyzoite differentiation has been assessed using non-metabolized analogs of cAMP and cGMP as well as forskolin (to stimulate a short pulse of cAMP). Bradyzoite induction is measured using either IFA techniques employing a cyst-wall lectin (DBA) or a bradyzoite promoter reporter parasite (Kirkman et al., 2001; Singh et al., 2002; Eaton et al., 2005). These studies demonstrate that cGMP or forskolin can induce bradyzoite formation. Using extracellular T. gondii tachyzoites, conditions such as pH 8.1, or forskolin, or SNP exposure induces bradyzoite formation and results in a transient three- to four-fold elevation in cAMP levels that within 30 minutes returns to the cAMP levels comparable to those seen in control parasites incubated in pH 7.1 media. No reproducible changes in cGMP are observed.

Most of the effects of cAMP within cells can be attributed to regulation of cAMP-dependent protein kinase A activity (PKA). Effects of cGMP can be attributed to stimulation of a cGMPdependent kinase (PKG), effects upon phosphodiesterases or other signaling molecules. Several kinases that are potentially involved in differentiation have been cloned. These include T. gondii PKA1, PKA2, and PKA3 (Eaton, Gissot, Tang and Kim, unpublished data), a glycogen synthase kinase (GSK-3) homolog (Qin et al., 1998), and a unique apicomplexan PKG (Donald and Liberator, 2002; Donald et al., 2002; Gurnett et al., 2002; Nare et al., 2002). Overexpression of T. gondii PKA1 or PKA2 appears to have opposing effects upon parasite proliferation (Gissot, Eaton and Kim, in preparation). Inhibitors of PKA or apicomplexan PKG inhibit replication and induce differentiation (Nare et al., 2002; Eaton et al., 2005).

It is likely that there are both inhibitory and stimulatory pathways affected by cyclic nucleotide signaling. PKA has effects upon metabolism, gene expression, and cell cycle. The dissection of exact signaling cascades is complicated by the presence of more than one PKA isoform and multiple phosphodiesterases that are likely to modulate signaling. Further, the inhibitors of PKA or PKG may have off-target effects upon other kinases, and will also have profound effects upon host-cell signaling.

Recently, yeast two-hybrid analysis has been used to identify proteins, some of which are metabolic enzymes, that interact with *T. gondii* PKA1 and PKA2 (Gao and Kim, unpublished). An SRCAP homolog, which in other eukaryotes is involved in the regulation of CREB (cAMP response element binding protein), has been identified in *T. gondii* (Sullivan *et al.*, 2003). Using yeast two-hybrid analysis, TgSRCAP was found to interact with several proteins that could be transcription regulators, including TgLZTR (Nallani and Sullivan, 2005). Detailed analysis of these signaling pathways will be required in order to understand the regulatory network triggered during bradyzoite formation.

As protein phosphorylation has proven to be a major mechanism of regulation of gene expression and integration and amplification of extracellular signals, the presence of highly conserved signaling molecules suggests that many of the pathways identified in other eukaryotes are likely to be preserved in *T. gondii*. A novel mitogen-activated protein kinase (TgMAPK-1) related to p38 (a human MAPK involved in the stress response) is also increased during bradyzoite formation (Brumlik *et al.*, 2004). At least two MAPK orthologs are present in the *T. gondii* genome (www.toxodb.org), and one of these MAPK may play a role in the signaling processes involved in bradyzoite differentiation.

13.7 THE IDENTIFICATION OF BRADYZOITE-SPECIFIC GENES

Several bradyzoite-related genes, most related to antigens identified by monoclonal antibodies, have been cloned and characterized, including BAG1, MAG1, BSR4 (p36), LDH2, and SAG4A (p18) (Parmley et al., 1994, 1995; Bohne et al., 1995; Yang and Parmley, 1995, 1997; Odberg-Ferragut et al., 1996; Knoll and Boothroyd, 1998a, 1998b). Random sequencing of cDNA libraries (EST projects) from bradyzoites and tachyzoites has resulted in the identification of numerous stage-specific genes. Lists of bradyzoite-specific ESTs are available at http://www.cbil.upenn.edu/apidots/ and http:// cmgm.stanford.edu/micro/boothroyd/toxo1.html. Genes that are induced on bradyzoite differentiation or unique to the bradyzoite libraries have also been identified, but the majority of these encode proteins of unknown function (Ajioka, 1998; Ajioka et al., 1998; Manger et al., 1998a). Additional efforts with sporozoite as well as other tachyzoite and bradyzoite libraries are underway, and are accessible at http://toxodb.org (Li et al., 2003), along with a 12X coverage of the T. gondii genome.

Bradyzoite-specific genes have also been identified by using a subtractive cDNA library approach (Yahiaoui *et al.*, 1999). Sixty-five cDNA clones were analyzed from a bradyzoite subtractive cDNA library; of these, many were identified that were exclusively or preferentially transcribed in bradyzoites. This included homologs of chaperones (mitochondria heat-shock protein 60 and T complex protein 1), nitrogen fixation protein, DNA damage repair protein, KE2 protein, phophatidylinosoitol synthase, glucose-6-phosphate isomerase, and enolase. Differential display also confirms differences in gene expression between bradyzoites and tachyzoites (Roos unpublished; Knoll and Boothroyd, 1998a).

Serial analysis of gene expression (SAGE) has been used to understand the progression of gene expression during bradyzoite formation (Radke et al. 2005). A 15-day time course of genes induced during the programmed bradyzoite development from sporozoite infected cultures, as well as genes induced by pH stress, is available from the SAGE tag analysis (http://vmbmod10.msu.montana.edu/vmb/ white-lab/newsage1.htm). Overall, the SAGE data are suggestive that T. gondii undergoes a programmed differentiation response similar to Plasmodium falciparum, with the coordinate 'just-in-time' regulation of groups of genes during this developmental program (Radke et al. 2005). Unique stagespecific mRNAs mark the phenotypic transitions seen in parasite development. Further, type II and III strains, which are more capable of bradyzoite differentiation, contain bradyzoite SAGE tags, suggesting that there is priming of developmental gene expression.

Microarrays enriched in bradyzoite-specific cDNAs obtained from sequencing a bradyzoite library have been used to identify groups of genes that are also coordinately regulated during bradyzoite differentiation (Manger *et al.*, 1998a; Cleary *et al.*, 2002). These analyses have confirmed the induction of previously described bradyzoite genes, i.e. BAG1, LDH2, and SAG4A, and identified other potential bradyzoite-specific genes. In addition, genes not known to be regulated during differentiation were shown to have altered mRNA expression (Cleary *et al.*, 2002). The data from these studies and genetic studies suggest that bradyzoite differentiation is a complex pathway. Despite the identification of many bradyzoitespecific genes, as well as *T. gondii* mutants that are unable to differentiate, a unified model for bradyzoite differentiation has not yet been developed. Several themes, however, have emerged from the available data:

- 1. Tachyzoites and bradyzoites express related genes encoding structural homologs in a mutually exclusive way.
- 2. Metabolic genes that are stage-specific exist, suggesting these stages are metabolically distinct.
- 3. Stress-related differentiation pathways and stress proteins are associated with these stage transitions.
- 4. It is likely that coordinate regulation of a developmental program exists, and some of the control elements of this program involve chromatin remodeling.

13.8 CYST WALL AND MATRIX ANTIGENS

The development of the tissue cyst wall and matrix are early events in the process of bradyzoite differentiation. Cyst-wall proteins are detected at the same time as other bradyzoite-specific antigens such as BAG1 (Gross et al., 1996; Zhang et al., 2001). An important function of the cyst wall and matrix is to protect bradyzoites from harsh environmental conditions such as dehydration. In addition, these structures provide a physical barrier to host immune defenses. Much of this may be due to carbohydrates present in the cyst wall. The cyst wall is a modification of the bradyzoite parasitophorous vacuole membrane formed by the parasite which is enclosed in host-cell membrane i.e. tissue cysts are intracellular (Scholytyseck et al., 1974; Ferguson and Hutchison, 1987b). On electron microscopy, the membrane of the bradyzoite parasitophorous vacuole has a ruffled appearance and is associated with a precipitation of underlying material creating the cyst wall (Figure 13.3).

The cyst wall is periodic acid-Schiff (PAS) positive, stains with some silver stains, and binds the lectin



FIGURE 13.3 Electron microscopy of the cyst wall of *T. gondii* isolated from murine brain.

- (A) Transmission electron microscopy of cyst wall.
- (B) Immunoelectron microscopy of cyst stained with mAb 73.18 showing labeling of the cyst wall matrix (20 nm gold). Bar = 1 μ m.
- (C) Immunoelectron microscopy of cysts stained with *Dolichos biflorus* lectin demonstrating labeling of the cyst wall matrix (10 nm gold). Bar = 1 μm.

Figure reproduced with permission from Zhang et al., 2001.

of *Dolichos biflorus* (DBA) and the lectin succinylated-wheat germ agglutinin (S-WGA), suggesting that polysaccharides are present in this structure (Sims *et al.*, 1988; Boothroyd *et al.*, 1997; Dubey *et al.*, 1998; Guimaraes *et al.*, 2003). The binding of these lectins to tissue cysts can be inhibited by competition by their specific sugar haptens – N-acetylgalactosamine (GalNAc) for DBA and N-acetylglucosamine (GlcNAc) for S-WGA (Boothroyd *et al.*, 1997). Treatment with chitinase disrupts the cyst wall and eliminates S-WGA binding, suggesting that chitin or a similar polysaccharide may be present in this structure (Boothroyd *et al.*, 1997).

Binding of the lectin DBA is a marker of cyst-wall formation *in vitro*. CST1 is a 116-kDa glycoprotein recognized by DBA as well as the

monoclonal antibody 73.18 (Weiss et al., 1992; Zhang et al., 2001) (Figure 13.4). It appears to be identical to an antigen recognized by the serum of animals with chronic infection (Smith, 1993; Zhang and Smith, 1995; Smith et al., 1996) and by a rat monoclonal antibody CC2 (Gross et al., 1995). Lectin overlay experiments of two-dimensional gels suggest that the lectins DBA and S-WGA recognize different antigens, with S-WGA recognizing a 48-kDa antigen (Zhang et al., 2001). CST1 localizes to the granular material in the cyst wall under the limiting membrane of the bradyzoite parasitophorous vacuole (Zhang et al., 2001; Ferguson, 2004). Protocols for purification of the cyst wall have now been developed, and should facilitate identification of component proteins



FIGURE 13.4 Two-dimensional electrophoresis of *Toxoplasma gondii* (R5 strain) grown in human fibroblasts pH 8.1.

- (A) Silver-stained 2D gel. The band indicated by a '<' at PI 5.7 and 116 kDa corresponds to CST1, as defined by reactivity to mAb 73.18.
- (B) Immunoblot of a 2D gel with mAb 73.18 demonstrating that this antibody reacts with a 116-kDa band at PI 5.7.
- (C) Lectin overlay of a 2D gel using *Dolichos biflorus* lectin. The identified band is at PI 5.7 and 116 kDa. This band can be superimposed on the immunostained band identified by mAb 73.18.
- (D) Lectin overlay using S-WGA. This demonstrates that S-WGA localizes to a protein distinct from that identified by DBA and mAb 73.18.

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using a proteomic approach (Zhang and Weiss, unpublished; Zhang *et al.*, 2001). Several glycosyl transferase genes including a polypeptide N-acetylgalactosaminyltransferase that may be involved in cyst wall formation have been identified, expressed, and characterized in *T. gondii* (Wojczyk *et al.*, 2003; Stwora-Wojczyk *et al.*, 2004a, 2004b, 2004c).

Several dense-granule proteins (GRA1–8) are known to localize to the parasitophorous vacuolar membrane, the matrix of the vacuole, and the tubular structures within the parasitophorous vacuole of *T. gondii* (Ferguson, 2004) (Figure 13.5). GRA5 (Lecordier *et al.*, 1993) is found in both tachyzoites and bradyzoites. By immunohistochemistry, GRA5 is localized primarily to the cystwall membrane and not to the granular material under this membrane (Lane *et al.*, 1996; Ferguson, 2004). Less intense staining of the cyst-wall membrane is demonstrated by antibodies to GRA1, GRA3, and GRA6 (Torpier *et al.*, 1993; Ferguson, 2004). At present no other proteins that are present in the cyst wall have been identified, but, given the documented localization of a



FIGURE 13.5 Distribution of dense-granule proteins (GRA) in bradyzoite parasitophorous vacuoles. Representative sections from the lung of an acutely infected mouse containing tachyzoites (Figure 13.5A) and the brain of a chronically infected mouse containing tissue cysts (Figures 13.5B–J). All of the sections were stained for dense-granule proteins using the peroxidase technique and corresponding antisera specific to each GRA protein. Bars = 5 μ m.

(Å) Section stained with anti-GRA8 showing strong labeling of the parasitophorous vacuole containing tachyzoites.

(B)–(J) Tissue cysts demonstrate relatively uniform staining of the dense granules within the bradyzoites (B–I). There was variable staining of the cyst wall with positive staining for GRA1 (B), GRA3 (D), GRA5

(F), GRA6 (G), and GRA7 (H) but little staining with GRA2 (C), GRA4 (E), GRA8 (I) or NTPase (J).

Courtesy of D.J.P. Ferguson, reprinted with permission from Ferguson (2004). This figure is reproduced in color in the color plate section.

number of dense-granule proteins to the parasitophorous vacuole, it seems possible that many of the protein components of the cyst wall will be known dense-granule proteins with new carbohydrate modifications or bradyzoite-specific glycoproteins secreted from dense granules. In support of this idea is that rat monoclonal antibody CC2 reacts with a 115-kDa antigen similar (if not identical) to CST1 in bradyzoites, but recognizes a 40-kDa protein in tachyzoites (Gross *et al.*, 1995).

MAG1 was originally identified as a 65-kDa protein expressed in the cyst matrix that was not expressed in tachyzoite (Parmley et al., 1994). RT-PCR data indicate that mRNA for MAG1 is present in both tachyzoites and bradyzoites (Parmley et al., 1994). It has now been demonstrated that MAG1 is also expressed in tachyzoites and secreted into the parasitophorous vacuole, albeit less abundantly than in bradyzoites (Ferguson and Parmley, 2002). Antibody to recombinant MAG1 reacts with extracellular material in the cyst matrix, and to a lesser extent with the cyst wall, but not with the surface or cytoplasm of bradyzoites. The dense-granule proteins GRA1, GRA2, GRA3, GRA5, GRA6, and GRA7 are present in the matrix of both tachyzoites and bradyzoites, but GRA4 and GRA8 appear to be more specific for tachyzoites.

Recently, a bradyzoite-specific rhoptry protein, BRP1, was described which is secreted into the parasitophorous vacuole on invasion (Schwarz *et al.*, 2005). Knockout of the BRP1 gene did not affect the ability of the parasite to invade or to form cysts or bradyzoites (Schwarz *et al.*, 2005). Monoclonal antibodies E7B2 (Lane *et al.*, 1996), 93.2 (Weiss, unpublished) and 1.23.29 (Weiss, unpublished) also recognize matrix antigens, but the corresponding genes have not been identified (see Table 13.1).

13.9 SURFACE ANTIGENS

Most *T. gondii* surface antigens are members of a gene family with similar structure to SAG1 (Boothroyd *et al.*, 1998; Manger *et al.*, 1998b; Lekutis *et al.*, 2001). It is not clear why so many

family members exist (at least 150 genes, although some are pseudogenes), since antigenic variation as described for trypanosomes or malaria has not been described. All of these SAGs appear to be attached to the plasma membrane by a similar glycolipid anchor. While SAG3 and other antigens are found in all life stages, several of these surface antigens appear to be stage-specific. SAG1 SRS1-SRS3, SAG2A, and SAG2B are expressed in tachyzoites, and SAG2C, SAG2D, SAG4A, SAG5A (SAG 5.1), and SRS9 in bradyzoites (Jung et al., 2004; Kim and Boothroyd, 2005). These antigens may be involved in persistence of tissue cysts in their hosts and the relative lack of an immune response to tissue cysts (Kim and Boothroyd, 2005). Both SAG3 (p43) and SAG1 (p30) have been implicated in adhesion to host cells. Disruption of SAG3 leads to two-fold decreased adhesion of parasites (Dzierszinski et al., 2000), but disruption of SAG1 in RH strain, but not in PLK, results in parasites that are more invasive (Mineo et al., 1993). Either the SAG1 or SAG3 disruption in the RH strain results in a decrease in virulence (Dzierszinski et al., 2000).

Surface antigens similar to SAG1 with a conserved placement of 12 cysteines include SAG1, SAG3 (p43), BSR4 (p36) and SRS 1–4 (SAG-related sequences), SAG5, SAG5.1, and SAG5.2. A second family of surface antigens related to SAG1 forms a group with a less consistent conservation of cysteine spacing. This group includes SAG2A (SAG2 or p22), SAG2B, SAG2C, and SAG2D. SAG2C and SAG2D are only detected in *in vivo* bradyzoites not *in vitro* bradyzoites, suggesting they may be expressed later in bradyzoite differentiation and could be markers of cyst maturation (Lekutis *et al.*, 2001).

SAG4 (p18; now SAG4A) is an 18-kDa surface protein of bradyzoites (Odberg-Ferragut *et al.*, 1996). A SAG4 homolog SAG4.2 (AF015715; SAG4B) has also been identified, and these genes are distinct from the SAG1-related genes. *SAG4A*, similar to *BAG1*, appears to be transcriptionally regulated during bradyzoite development.

BSR4/p36, or a cross-reacting antigen, is a surface protein on bradyzoites that reacts with mAb

T84A12 (Knoll and Boothroyd, 1998b). It was initially believed to be bradyzoite-specific, but has also been reported in merozoites (Ferguson, 2004). BSR4 was isolated from pH 8.0-treated *T. gondii* cultures in human fibroblasts using a promoter trap strategy. This gene demonstrates a restriction fragment length polymorphism between ME49 (PLK; type II) and CEP (type III) strains, which correlates with the lack of mAb T84A12 binding to the CEP strain. RNA levels of BSR4 are similar in tachyzoites and bradyzoites, suggesting that post-transcriptional regulation of BSR4/p36 occurs via some unknown mechanism. Disruption of BSR4 did not result in any phenotype – i.e there was no effect on bradyzoites or cysts (Knoll and Boothroyd, 1998b).

13.10 METABOLIC DIFFERENCES BETWEEN BRADYZOITES AND TACHYZOITES

It is probable that the energy metabolism of bradyzoites is different from that of tachyzoites, given the location of bradyzoites in a thick-walled vacuole and their slower growth rate. An unusual and unexplained feature of T. gondii differentiation is the presence of stage-specific differences in the activity and isoforms of several glycolytic enzymes. It is known that tachyzoites utilize the glycolytic pathway, with the production of lactate as their major source of energy. Functional mitochondria with a TCA cycle exist in tachyzoites, and are thought to contribute to energy production. While both tachyzoites and bradyzoites utilize the glycolytic pathway for energy, data suggest that bradyzoites lack a functional TCA cycle and respiratory chain (Denton et al., 1996).

Overall, the regulation and activation of glycolysis appear to be different in *T. gondii* than in many other eukaryotes (Denton *et al.*, 1996; Saito *et al.*, 2002; Maeda *et al.*, 2003). Lactate dehydrogenase (LDH) and pyruvate kinase activity are higher in bradyzoites than in tachyzoites, while PP_i-phosphofructokinase activity is higher in tachyzoites than in bradyzoites (Denton *et al.*, 1996). These data suggest that bradyzoite energy metabolism may be dependent on the catabolism of amylopectin (which is present in bradyzoites and essentially absent in tachyzoites) to lactate. The bradyzoitespecific glycolytic isoenzymes are resistant to acidic pH, suggesting that bradyzoites are resistant to the acidification resulting from the accumulation of the glycolytic products of amylopectin catabolism to lactate.

Lactate dehydrogenase 2 (LDH2) is a 35-kDa cytoplasmic antigen with a PI of 7.0 that is expressed in bradyzoites but not in tachyzoites (Yang and Parmley, 1995, 1997). Its expression appears to be transcriptionally regulated, because LDH2 mRNA is detectable by RT-PCR only in bradyzoites. The tachyzoite isoform, LDH1, has also been cloned. LDH1 and LDH2 are 71.4 percent identical. The LDH activities of tachyzoite and bradyzoite extracts are different; however, there are no significant differences in the activity of recombinant enzyme activities of LDH1 or LDH2 (Yang and Parmley, 1995, 1997). Despite its apparent stagespecificity, attempts to disrupt LDH2 have failed (Singh et al., 2002); however, knockdown of LDH2 has been achieved using dsRNA (Al-Anouti et al., 2004). When LDH2 expression was downregulated, bradyzoite differentiation and growth were impaired (Al-Anouti et al., 2004).

Two stage-specific enolases have also been cloned and characterized, consistent with the hypothesis that utilization of the glycolytic pathway is different in tachyzoites compared to bradyzoites. Enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate (Manger et al., 1998a; Yahiaoui et al., 1999). In yeast, enolase is known to be a stress-response protein, i.e. hsp48 (Iida and Yahara, 1985). ENO2, the tachyzoite form, has three-fold higher specific activity than ENO1, the bradyzoite enzyme, but both have similar Michaelis constants (K_m) (Dzierszinski et al., 2001). Surprisingly, polyclonal antisera to each isoform do not cross-react despite the similarity of these two isoforms (Dzierszinski et al., 1999, 2001). Both isoforms are found localized to the nucleus in dividing cells, but in late bradyzoites, which are quiescent, ENO1 is cytoplasmic (Ferguson and Parmley, 2002; Ferguson et al., 2002). The significance of these observations is unknown, but it is possible that some glycolytic enzymes may have alternate regulatory functions that are not yet fully understood.

An obvious difference between tachyzoites and bradyzoites is the presence of cytosolic granules of amylopectin that are composed of glucose polymers (Guerardel et al., 2005). Structural studies of amylopectin have revealed it to be a plant-like amylopectin with predominantly $(\alpha 1-4)$ linkages, which is most similar to the semicrystalline floridean starch accumulated by red algae (Coppin et al., 2005). Amylopectin granules disappear from bradyzoites when they transform into tachyzoites during cell culture (Coppin et al., 2003). While not seen in tachyzoites, amylopectin is present in the sexual cycle in the cat intestine in macrogametes, persists during oocyst formation, and is present in sporozoites. Merozoites lack amylopectin. Amylopectin is believed to be a carboyhydrate store for the bradyzoite or sporozoite during long periods of quiescence and nutrient deprivation. Candidate genes for enzymes involved in amylopectin breakdown and synthesis have been identified (Coppin et al., 2003).

A bradyzoite-specific P-type ATPase whose mRNA and protein are preferentially expressed in bradyzoites has also been characterized and localizes in punctate pattern to the region of the plasma membrane (Holpert et al., 2001). A second P-type ATPase also may be preferentially expressed in bradyzoites as judged by RT-PCR of steady-state mRNA, although mRNA can also be detected in tachyzoites. TgSRCAP (Snf2-related CBP activator protein) is a SWI2/SNF2, the expression of which increases during cyst development (Nallani and Sullivan, 2005). This protein, by yeast two-hybrid studies, also binds to several metabolic enzymes (Nallani and Sullivan, 2005), further underscoring that metabolic regulation that is probably different in tachyzoites and bradyzoites.

It is not known whether the metabolic changes between bradyzoites and tachyzoites cause differentiation, or are a consequence of the differentiation process. One hypothesis is that monitoring for nutrient deprivation, a type of stress response, might serve as the sensor for differentiation in *T. gondii*. It should be noted, however, that no sensors that respond to any environmental change have yet been identified in *T. gondii*.

13.11 GENETIC STUDIES ON BRADYZOITE BIOLOGY

Bradyzoite- and tachyzoite-specific promoter regions have been utilized to create reagents for the study of differentiation in vitro (Gross, 1996). Reporter genes such as chloramphenicol acetyltranferase (CAT), β-galactosidase, and luciferase are useful for mapping promoter activity and the definition of minimal promoter sequences. Studies using these markers have confirmed the stage-specific expression of SAG1 as a tachyzoite marker, and LDH2 and BAG1 as bradyzoite markers. Stage-specific transcription factors or other regulatory molecules have yet to be identified (Bohne et al., 1997; Yang and Parmley, 1997; Ma et al., 2004; Eaton et al., 2005). Stage-specific reporter constructs have been used in both transient and stable transfection assays. Another reporter gene that has proven useful for the identification of bradyzoite differentiation mutants by FACS is green fluorescent protein (GFP) (Matrajt et al., 2002; Singh et al., 2002).

It might be expected that the knockout of a bradyzoite-specific gene would be feasible, as growth should occur in the tachyzoite stage even if bradyzoite development is prevented. This strategy has been applied to the bradyzoite-specific gene BAG1/hsp30 (BAG5) (Bohne et al., 1998; Zhang et al., 1999). A bag1 knockout was created using HGXPRT as a selectable marker in an HGXPRT^{neg} PLK strain of T. gondii (Bohne et al., 1998). Another bag1 knockout was performed using CAT as a selectable marker in a clone of PLK strain that had been passaged through mice to ensure it made cysts at the start of the study (Zhang et al., 1999). Cyst formation in vitro and in vivo occurred in both knockouts, indicating that BAG1 is not essential for cyst formation. Zhang et al. (1999), however, found that the number of cysts formed in vivo in CD1 mice was reduced in the *bag1* knockouts. Complementation resulted in the production of similar numbers of

cysts *in vivo* as in the wild type PLK strain (Zhang *et al.*, 1999). When parasites were grown in sodium nitroprusside (SNP), the *bag1* knockout grew faster than PLK. This may be a result of a difference in transition rate from the rapidly growing tachyzoite to the slowly growing bradyzoite stage in this *bag1* knockout. The decrease in cyst formation is a relatively subtle phenotype, which was not observed when *BAG1* was disrupted in the HGXPRT^{neg} PLK strain background (not passaged in mice prior to the knockout) and cyst formation was tested in highly susceptible C57BL/6 mice (Bohne *et al.*, 1998).

The capacity to convert from tachyzoite to bradyzoite is a key feature for *T. gondii* persistence in the host, and thus it is likely that multiple genes with redundant functions are involved in this process. Small heat-shock proteins, such as BAG1, have been postulated to act as specialized chaperones for enzymes such as glutathione reductase during differentiation, but the exact function of such small heat-shock proteins is unknown. It is possible that the other small heat-shock proteins in *T. gondii* can partially compensate for a lack of BAG1 (de Miguel, 2005).

Promoter trapping has been an effective technique for the identification of genes induced during bradyzoite differentiation. A promoterless hypoxanthine-xanthine-guanine phosphoribosyltranferase (HGXPRT) gene with 6-thoxanthine (6-TX) or 8-azaguanine (8-AzaH) as negative selection and mycophenolic acid with xanthine (MPA-X) as a positive selection has been utilized by several groups (Bohne et al., 1997; Knoll and Boothroyd, 1998a, 1998b). Selection works by growing transfected parasites at pH 7.0 in the presence of 6-TX, which kills all organisms that have the HGXPRT gene on a constitutive or tachyzoite promoter. To confirm bradyzoite specificity this population of organisms is then exposed to pH 8.0 and MPA-X, and only parasites which express HGXPRT (i.e. those with a bradyzoite or constitutive promoter in front of the HGXPRT gene) will survive. It should be noted that this approach can be 'leaky', depending on the concentrations of 6-TX and MPA-X used. Nonetheless, when the 6-TX and MPA-X selections are repeated several times,

one will enrich the population for organisms with HGXPRT under the control of bradyzoite-specific gene promoters. Using this approach, additional bradyzoite-specific promoters (Donald and Roos, unpublished; cited in Matrajt *et al.*, 2002) and eight bradyzoite-specific recombinant (BSR) strains were obtained (Knoll and Boothroyd, 1998b). One of these, BSR4, was later found to be the bradyzoite surface antigen p36, which had been previously identified by a monoclonal antibody (Knoll and Boothroyd, 1998b).

Two additional genetic strategies have been developed to identify mutants unable to undergo bradyzoite differentiation (Matrajt *et al.*, 2002; Singh *et al.*, 2002). Singh *et al.* (2002) generated point mutants in an *LDH2-GFP* Prugnaiud (type II) background, which expresses GFP under the control of the bradyzoitespecific promoter LDH2, to obtain mutants with an altered ability to transform into bradyzoites (Singh *et al.*, 2002). Parasites unable to differentiate were identified by FACS enrichment of GFP-negative parasites when these organisms were exposed to bradyzoite-inducing conditions.

Matrajt et al. (2002) also utilized insertional mutagenesis of an engineered stable line expressing a bradyzoite-specific pT7-HGXPRT cassette in UPRT-deficient RH (type I) parasites (Matrajt et al., 2002). Earlier studies had demonstrated that RH UPRT disruptants differentiate into bradyzoites under conditions of CO2 starvation (Bohne and Roos, 1997). The pT7-HGXPRT stable line was obtained by rounds of negative and positive selection, alternating 6-TX (tachyzoite conditions) with MPA-X (bradyzoite conditions) selection. The result was a cell line where HGXPRT was highly regulated by differentiation conditions. Insertional mutagenesis was then performed in this pT7-HGXPRT line using DHFR cassettes that earlier were shown to have a high frequency of non-homologous insertion (Donald and Roos, 1993). An inability to differentiate into bradyzoites was detected by the inability of the disruptant to express HGXPRT.

Both groups were able to demonstrate that these differentiation mutants were unable to make bradyzoites at the same degree of efficiency as the parental strains, and both groups demonstrated global defects in expression of previously characterized markers as determined by immunofluorescence and microarray analysis. Due to technical difficulties, the exact mutations could not be identified. The insertional mutants identified by Matrajt et al. (2002) were similar to the bag1 knockout described by Zhang et al. (1999), and had more rapid growth under bradyzoite-inducing conditions than seen in wild-type parasites (Matrajt et al., 2002). Microarray analysis of these mutants identified classes of genes, including a 14-3-3 homolog, a PISTLRE kinase, and a probable vacuolar ATPase, whose expression was decreased in the differentiation mutants (Matrajt et al., 2002; Singh et al., 2002). Microarray study of these mutants suggested that there was a hierocracy of gene expression during bradyzoite differentiation (Singh et al., 2002), which is consistent with the developmental program seen in Plasmodium.

Vanchinathan *et al.* (2005), using an insertional mutagenesis FACS strategy similar to that of Singh *et al.* (2002) and Matrajt *et al.* (2002), identified two differentiation mutants – TBD-5 and TBD-6 – that switch to bradyzoites at 10 and 50 percent of wild-type levels respectively (Vanchinathan *et al.*, 2005). TBD-5 had multiple insertions and was not characterized further, but TDG-6 had a single insertion 164 bp upstream of the transcription start site of a gene encoding a zinc finger protein (ZFP1). ZFP1 is targeted to the parasite nucleolus by CCHC motifs and significantly altered expression levels appear to be toxic to *T. gondii*. The phenotype of decreased bradyzoite development could be replicated by directed integration into the same upstream region.

Knoll and colleagues have made libraries of signature-tagged mutants (Knoll *et al.*, 2001; Mordue and Knoll, unpublished) that have been screened *in vivo* and *in vitro*. Several of these mutants have defects in cyst formation, whereas others have defects in intracellular growth. (Knoll *et al.*, unpublished). The characterization and rescue of these mutant genes is an ongoing project (Knoll *et al.*, unpublished).

13.12 SUMMARY

Investigations into bradyzoite biology and the differentiation of tachyzoites into bradyzoites

have been accelerated by the development of *in vitro* techniques to study and produce bradyzoites, as well as by the genetic tools that exist for the manipulation of *T. gondii*. The recent completion of the *Toxoplasma* genome and the development and integration of the SAGE expression database with the genome database provide additional important tools for the study of bradyzoite development.

Unfortunately, the genetic triggers and sensors for the differentiation response have yet to be identified. Many of the features of this differentiation are reminiscent of epigenetic phenomena described in other systems. Bradyzoite differentiation appears to be coupled with a slowing of the cell cycle. It is likely that many signals can result in appropriate signals that induce bradyzoite formation. The development of bradyzoites appears to be a stress-mediated differentiation response that leads to metabolic adaptations. It is abundantly clear that transcription of a whole set of bradyzoite-specific genes occurs during differentiation. These gene products include metabolic enzymes, surface antigens, secretory antigens (including rhoptry proteins), and cyst-wall components.

Studies of the mechanism(s) by which development is triggered and coordinated should eventually result in the identification of new therapeutic strategies for the control of toxoplasmosis. These advances may ultimately result in the radical cure of infection by eliminating the latent *T. gondii* cyst stage in tissue. Furthermore, genetic strategies that prevent cyst formation may also prove useful in the development of vaccine strains of this pathogenic apicomplexan.

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Development and Application of Classical Genetics in *Toxoplasma gondii*

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14.1 Introduction14.2 Biology of *Toxoplasma*14.3 Establishment of transmission genetics14.4 Development of molecular genetics tools

14.5 Application of genetic mapping 14.6 Future challenges Acknowledgements References

14.1 INTRODUCTION

Apicomplexan parasites have complex life cycles involving both asexual and sexual replication in a variety of invertebrate and vertebrate hosts. The extent to which sexual recombination occurs in different apicomplexan species varies greatly: in organisms like *Plasmodium* it is essential for transmission, while in the case of *Toxoplasma gondii*, asexual replicating stages can also give rise to infection of naïve hosts. Not surprisingly, *T. gondii* exhibits a highly clonal population structure as a consequence of this lack of a requirement for genetic recombination. Nonetheless, the ability to cross strains experimentally has been exploited to develop conventional genetic mapping strategies in *T. gondii*. Genetic mapping provides a forward genetic system for analyzing complex biological traits, including drug resistance, growth, virulence, and induction of host immune responses. Classical genetic analysis offers the most promise when combined with other technologies, such as reverse genetics, comparative genomics, and gene expression studies (reviewed in Chapters 15 and 16 of this volume).

14.2 BIOLOGY OF TOXOPLASMA

14.2.1 Life cycle

Toxoplasma gondii is a member of the phylum Apicomplexa: a diverse group of largely parasitic organisms that contains a number of human (i.e. Plasmodium, Cryptosporidium) and animal (Eimeria, Sarcocystis, Neospora, Theileria, Babesia) pathogens. Toxoplasma gondii has a typical heteroxenous life cycle, alternating between sexual replication in members of the cat family, which serve as the definitive host, and asexual replication in a wide range of warm-blooded vertebrates that serve as intermediate hosts (Dubey, 1977). Similar heteroxenous life cycles are thought to have arisen several independent times in the Coccidia (Barta, 1989), attesting to the advantages of this complex developmental process for transmission. Divergence of apicomplexans appears to follow that of their definitive hosts (Barta, 1989). Consistent with this, molecular phylogeny suggests that Toxoplasma diverged from Neospora approximately 10 million years ago (Su et al., 2003a), closely matching the speciation of cats and dogs - their respective definitive hosts.

During the initial infection, T. gondii grows rapidly as a haploid form called the tachyzoite, which is capable of invading and replicating within virtually any nucleated cell from warmblooded animals. In response to stress brought on by the immune response the parasite converts to a slow-growing form called a bradyzoite, which is encased in a thick-walled cyst. Ingestion of these cysts by cats leads to sexual differentiation within intestinal epithelia cells, and the resulting fusion of gametes to form a diploid zygote (Dubey and Frenkel, 1972; also described more fully below). Following the development of an impervious wall, oocyst stages are shed in the feces and undergo meiosis in the environment to yield eight haploid progeny (Frenkel et al., 1970). Oocysts are longlived, resistant to environmental conditions, and responsible for dissemination due to contamination of food or water (Mead et al., 1999). Chapters 1 and 2 provide micrographs of the various life-cycle forms.

Toxoplasma gondii has an unusual population structure comprised of three widespread lineages that are prevalent in North America and Europe. These lineages are known as I, II, and III, and significant biological differences have been described in them (see below), despite their closely related genotypes. Differences in these lineages were first described by RFLP markers (Sibley and Boothroyd, 1992a) or MLEE studies (Dardé *et al.*, 1992), and these have since been verified by a variety of additional surveys (see Chapter 3). The three lineages are themselves quite closely related and derived from several common ancestral strains that underwent limited genetic recombination in the wild (Grigg *et al.*, 2001a; Su *et al.*, 2003a). Importantly, this highly unusual population structure was not fully appreciated until quite recently, and was completely unknown when the sexual phase of the life cycle was first described and when early genetic crosses were conducted.

14.2.2 Infection and initiation of the sexual cycle

The progression through the sexual cycle in the cat intestinal tract appears to be influenced by the initial infecting stage of the parasite, suggesting that there may be an obligatory pathway to gametogenesis. Experimental evidence suggests that the sexual cycle in the cat can be initiated by all three major forms of T. gondii: bradyzoites (also known as cystozoites) from tissue cysts, tachyzoites (also known as trophozoites) from the acute infection, and sporozoites from sporulated oocysts (Dubey and Frenkel, 1972; Dubey, 2005). However, these forms are associated with different prepatent periods prior to shedding of oocysts: 3–10 days for bradyzoites, > 19 days for tachyzoites and sporozoites (Dubey et al., 1970; Dubey and Frenkel, 1972, 1976; Freyre et al., 1989). Early studies demonstrated that cats fed bradyzoites shed oocysts 3-10 days post-infection with little or no dependence on strain or dose, showing that the sexual cycle is rapidly initiated by this form. In contrast, the prepatent period for both the sporozoite and tachyzoite forms is two to three times longer. Cats fed with the M-7741 (type III) strain derived from mice during the acute infection, both from tissue (< 2 days post-infection) and peritoneal exudates (PEX), shed oocysts with a prepatent period of 19 days or more (Dubey and Frenkel, 1972). In this experiment the parasites were assumed to be tachyzoites, since the mice were still within the acute phase of infection.

However, more recent observations from cats fed with T. gondii-infected mouse PEX showed greater variation in prepatent periods (Dubey, 2005). In this study, VEG (a type III strain maintained through sexual-cycle passage) and a recent livestock isolate TgCkAr23 were used to generate PEX. Of the cats that shed oocysts after being fed with PEX derived from these two strains, more than half shed oocysts in less than 10 days, with the remainder having prepatent periods of 11 days or greater. The early prepatent period observed in this experiment is thought to be due to bradyzoite conversion in the mouse peritoneum, while the later period is due to the predominance of tachyzoites in the material fed to cats. Collectively, these studies are consistent with a requirement that the sexual cycle be initiated by the bradyzoite form, while sporozoites and tachyzoites must first initiate an acute infection in the cat intestinal tract in order to allow bradyzoites to develop. The potential range of prepatent periods during natural infections may afford a greater opportunity to establish a productive co-infection for recombination between strains.

14.2.3 Generation of gametes

The formation of various parasite types leading to gamete formation within the cat intestinal tract has been described from histological studies that relied on fixed tissues examined by conventional light microscopy or electron microscopy. Comparison of the properties of T. gondii from these studies reveals differences and similarities with gametogenesis in related gut-dwelling coccidians (West et al., 2001; Smith et al., 2002). Unlike Eimeria, T. gondii (and closely related Neospora, Isospora, and Hammondia) does not appear to have a fixed number of rounds of merogony, and instead has as many as five morphologically distinct intracellular replicating types prior to gametocyte formation. Despite these differences, Eimeria spp. and T. gondii both have a highly female-biased sex ratios - i.e. a greater number of female or macrogametocytes compared with male or microgametocytes (West et al., 2001).

Studies by Dubey and colleagues originally defined five different morphological types, denoted as types A to E, and they postulated that each type sequentially leads to the next, culminating in the formation of gametocytes (Dubey and Frenkel, 1972; Speer and Dubey 2005). The types are differentiated on the basis of size/shape (both the overall structure and individual parasites), pattern of parasite and vacuole arrangement, mode of division, staining characteristics, and location within the intestine. The proposed sequence is based on temporal frequency of appearance and spatial proximity of each type (summarized in Figure 14.1). Studies by Ferguson and colleagues (Hutchison et al., 1971; Ferguson et al., 1974, 1975) described very similar characteristics for the later schizont types D and E, but types A-C were not described, possibly because histological studies were performed 5-7 days post-infection - a time when these forms are rare or were not seen in the earlier studies by Dubey and colleagues (Dubey and Frenkel, 1972; Speer and Dubey, 2005).

14.2.4 Timeline of gametocyte development in the cat intestine

The type A vacuoles are only seen at 12–18 hours post-infection in jejunum epithelial cells. Type A organisms differ from tachyzoites (also known as trophozoites) in that they are round/ovoid, smaller, and are not usually seen in the lamina propria, where the majority of tachyzoites are found. Type A vacuoles contain only two or three parasites that divide by endodyogeny and do not have PAS staining granules.

The type B vacuole is seen 12–54 hours postinfection in both the jejunum and the ileum. Type B organisms are found in enterocytes primarily in the epithelium, although infected epithelial cells are also found recessed within the lamina propria. Type B vacuoles contain 2–48 ovoid parasites that are somewhat larger than tachyzoites. Like type A, the type B parasites divide by endodyogeny, but have a few lightly staining PAS granules and show bipolar staining with Giemsa. The parasites are contained within a thin (probably singlemembrane) parasitophorous vacuole membrane



FIGURE 14.1 Diagrammatic representation of five replicative stages of *T. gondii* that occur in the cat intestine. Two stages that divide by endodyogeny (binary fission) (A, B) are followed by three rounds of schizogony that divide by endopolygeny (C–E). Trophozoites (tachyzoites) can also develop from these early forms, and they typically disseminate into deep tissues. Parasites released from E schizonts go on to form microgametocytes and macrogametocytes. Reproduced from Dubey and Frenkel (1972), with permission.

(PVM), which, like the tachyzoite-containing vacuole, contains a tubulovesicular membrane network (TMN). Type B parasites are occasionally found as singular cells and multinucleate forms within the same vacuole.

The type C schizont is seen 24–54 hours postinfection in the jejunum and the ileum. Like the type B schizont, they are common in peripheral epithelial cells and in epithelial cells that are displaced in the lamina propria. Occasionally, type C schizonts are also seen in intraepithelial lymphocytes. Type C schizonts divide by endopolygeny, resulting in 12–18 parasites and the formation of a residual body. At this point there is apparently a shift in parasite physiology, as the TMN is absent, although the PVM remains relatively thin. Type C schizonts are the only type with deeply staining PAS granules; the rhoptries now appear electron-dense and amylopectin granules are apparent.

Type D schizonts are seen after 40 hours and up to 15 days post-infection, and these schizonts extend to the colon as well as the jejunum and ileum. Type D schizonts appear to be restricted to the epithelium, where they continue to occupy primarily enterocytes. Type D schizonts divide by endopolygeny and develop a residual body. The parasite physiology continues to change, as the PVM is now thick/electron-dense, with indentations and PAS, and amylopectin granules are lost and a new granular vacuole appears.

Type E schizonts are very similar to type D schizonts except that the parasites appear larger, and several granular bodies replace the granular vacuole. The type E schizonts are thought to be derived from merozoites released by type D schizonts. Type E schizonts multiply by endopolygeny to a final size of 50–80 parasites, which are thought to give rise to the male and female gametocytes because of the physical proximity of these forms in the intestine.

The observation that schizogony occasionally occurs in epithelial cells within the lamina propria (i.e. type C and D schizonts) could be an important pre-adaptation for tissue dissemination. Parasites that enter leukocytes may undergo trafficking to distant sites, rather than remain in the intestine. Once they are in deep tissues, the program for gametocytogenesis may be shut down in favor of development of tissue stages (trophozoites that may replicate rapidly or develop into chronic stages). Ancestral coccidian life cycles do not contain such tissue stages (i.e. *Eimeria, Cryptosporidium*), and the factors that led to this development in parasites like *T. gondii* are unknown.

Both microgametocytes and macrogametocytes develop exclusively within the epithelial enterocytes

and are generally seen in close proximity to type E schizonts (Dubey and Frenkel, 1972; Speer and Dubey, 2005). Like the type D and E schizonts, the gametocytes are surrounded by an electrondense PVM with the characteristic indentations. The microgametocyte divides by endopolygeny, and microgametes bud from a large residual body into the electron-lucent parasitophorous vacuole space. A single microgametocyte will produce about 20-30 microgametes (Dubey and Frenkel, 1972). Each microgamete contains two flagella, a single mitochondrion, and an electron-dense nucleus. The inner-membrane complex is supported by 12 microtubules set against the opposite side of the mitochondria from the nucleus. The macrogametocyte resembles those seen in other coccidians, including a central nucleus, types I and II wall-forming bodies, amylopectin granules, lipid bodies, apicoplast (multiple membrane bound vacuole), endoplasmic reticulum, and Golgi complex. Each macrogametocyte gives rise to a single macrogamete.

Although previous studies have outlined the cell biology of gametogenesis, oocyst formation, and sporogony, sexual differentiation of *T. gondii* could be explained by two hypotheses:

- Individual parasites are committed to a given sex by stimulus in the cat intestine that allows differentiation into only either macro- or microgametocytes
- 2. Individual parasites can differentiate into both micro- and macrogametocytes.

The first hypothesis requires the presence of two genetically committed parasites to form and excrete oocysts, whereas the second hypothesis only requires one form of the parasite that can differentiate into both micro- and macrogametocytes. Following the development of a procedure to clone *T. gondii* (Pfefferkorn and Pfefferkorn, 1976), cats fed with cysts from a single-cell cloned parasite line were able to shed oocysts (Pfefferkorn *et al.*, 1977). This finding demonstrated that the *T. gondii* has the genetic capability to differentiate into both micro- and macrogametocytes from the same progenitor parasite – a result that was independently confirmed by later studies (Cornelissen and Overdulve, 1985). A strict genetic basis for gametocyte differentiation (e.g. sex chromosome) can thus be discounted, although the cellular mechanisms that lead to differentiation into male (micro-) or female (macro-) gametocytes are, as yet, undiscovered.

Studies on the development of potential vaccine strains of T. gondii have also shed light on the development of gametocytes (Frenkel et al., 1991). Isolation of a mutant strain (T-263) that was unable to undergo complete development but could be rescued by co-infection with a wild-type strain demonstrated that strains could become 'reproductively deficient'. This defect is presumably due to the inability to generate either macro- or microgametes, although the exact nature of the mutation was never identified. Similar defects have been described in P. falciparum, where the extreme bias in crossing frequency of the $Dd2 \times HB3$ cross that was used to map chloroquine resistance was traced to a defect in microgamete formation by the Dd2 strain (Guinet et al., 1996). On a practical level, this fragility in the ability of T. gondii strains to undergo transmission through the cat requires that strains used for genetic crosses be maintained at low passages.

The developmental program necessary for completion of transmission through the cat is evidently complex and highly susceptible to disruption. Rapid-passage tachyzoites through mice resulted in the loss of ability to form oocysts after approximately 30 passages (likely fewer than 10^3 cell divisions) (Frenkel *et al.*, 1976). This remarkably high rate of loss implies either that any single mutation in a large number of essential genes can disrupt the process, or a single target that easily becomes inactivated in an irreversible manner. Loss of developmental transformation may not be a process of mutation, as epigenetic phenomena might result in a similar block in gene expression.

14.2.5 Sexual cycle, sporulation, and meiosis

The first description of the *T. gondii* life cycle correlated the presence of the parasite in the feces

of cats that were fed mice infected with T. gondii (Hutchison, 1967). Although this original description incorrectly associated T. gondii with the eggs of the nematode parasite Toxocara cati, subsequent studies confirmed the fecal association and established the coccidian nature of T. gondii (Frenkel et al., 1970; Sheffield and Melton, 1970). Fecal forms (oocysts) of the parasite resembling those of a related coccidian, Isospora bigemina, appeared in the cat feces 3-5 days after eating an infected mouse. Crucially, the oocysts were observed to undergo sporogony 3-4 days after fecal shedding. Oocysts contained an octet of sporozoites equally divided between two sporocysts, and microscopic measurements distinguished these from Isospora oocysts. This and associated studies provided a foundation for identifying the position of meiosis in the sexual cycle, and the development of genetic crossing experiments.

Classic studies by Cornelissen and co-workers established the position of gametogenesis and meiosis in the sexual cycle by analyzing the DNA content of gametes, zygote/oocyst, and sporozoites (Cornelissen et al., 1984a, 1984b). A mathematical model was used to calculate the DNA content of these cells from measurements using a combination of microfluorometry and scanning of microphotographic negatives after Feulgen staining. Within the cat intestinal epithelia, stages referred to as M2 and M3 merozoites have a nominally haploid DNA content. Classifying these merozoites with respect to the typing scheme described by Dubey and colleagues (see above) is difficult because of differences in parasite strain and tissue preparation, but they likely correspond to parasites in type D and E schizonts. These studies also suggested that the commitment to form micro- or macrogametes is made during the preceding rounds of asexual division. Although the M3 merozoites are nominally haploid, they appear to be synthesizing DNA and contained an average of 17 percent above the haploid content. The increased DNA content is thought to correlate with the initiation of replication immediately after the M3 merozoites are formed. The microgametes that are ultimately derived from these merozoites contain 3.3 percent more than haploid DNA that is speculated to be mitochondrial DNA. In contrast, the M2 merozoites have a haploid DNA content, while the macrogametocytes/macrogametes derived from these merozoites show a bimodal distribution of haploid and diploid DNA content. The diploid cells are thought to be fertilized macrogametes/zygotes. Notably, both the fertilized and unfertilized macrogametes appear to contain 11 percent extra DNA, which is thought to be due to amplification of genes whose products are necessary for sporulation. Sporogony occurs after the oocyst is shed into the environment, and sporozoites contain a haploid DNA content. Collectively, these results provide direct evidence that micro- and macrogametes are haploid, that fertilization and zygote/oocyst formation occurs in the cat gut epithelia, and that meiosis must occur during sporogony, which occurs in the environment after shedding.

14.2.6 Oocyst formation and sex ratio bias

Following the initial infection of a naïve cat, millions of oocysts are shed in the feces, each of which is thought to result from the fertilization of a macrogamete by a single microgamete (Dubey and Beattie, 1988). Since both types of gametes develop within independent host cells, the process of fertilization and its contribution to oocyst formation remains a mystery, in part because all infections will have some component of 'selfing'. The result of selfing in oocyst formation cannot differentiate between a conventional mechanism of genetically identical microgamete/macrogamete fertilization and possible alternative mechanisms such as macrogamete parthenogenesis.

Notably, the female/macrogametocytes may outnumber the male/microgametocytes 20-fold or more, and each microgametocyte only produces a limited number of gametes (Dubey and Frenkel, 1972; Ferguson, 2002). This female-biased sex ratio is thought to result from natural selection to maximize transmission in the face of high inbreeding/ selfing (i.e. in completely outbred populations, the fitness contribution would be equal between males and females so the gametocyte sex ratio should be 50/50; Nee et al., 2001; West et al., 2001). Given that each male/microgametocyte can produce a limited number of microgametes, sex allocation theory suggests that the optimal male-to-female sex ratio with inbreeding should be: 1/(1 + c), where c = the number of viable microgametocytes released by a single microgametocyte (West et al., 2001). In T. gondii, inbreeding will be high given that productive co-infections are rare. Since a single microgametocyte can give rise to 20-30 microgametes, compared with a single macrogametocyte per macrogamete, the gametocyte sex ratio should be female biased, so theory and observation are in good agreement. However, as Ferguson points out, this demands nearly complete viability of male/microgametes and 100 percent efficiency in fertilization (Ferguson, 2002). Since oocyst development occurs within a host cell, fertilization is assumed to occur there. The microgametocyte must make a very difficult journey, from budding off the microgametocyte, exiting the parasitophorous vacuole and host cell, swimming through the gut lumen, identifying and penetrating a host cell and parasitophorous vacuole that contains a macrogametocyte, and finally successfully fertilizing the macrogamete. Although this process may be more efficient than it would appear, further investigation is essential for understanding what selective forces have shaped the female-biased sex ratio. Alternative mechanisms such as macrogametocyte parthenogenesis (Ferguson, 2002) have been suggested to explain the high ratio of female gametes, although

Morphological studies of sporulation in *T. gondii* indicate that the initial division into two sporocysts is followed by reduction into four haploid sporozoites each (Speer *et al.*, 1998). Presumably the first division represents the meiotic division, although this has not been confirmed experimentally. Classical tetrad analysis using micro-dissection and single-cell PCR amplification might resolve this, and also provide a rapid means of evaluating the fraction of recombination in future crosses. Techniques for harvesting and sporulating oocysts (Dubey *et al.*, 1972) allow for infections to be

experimental genetic crosses do not support such

a mechanism.

initiated *in vitro* from material collected as the result of naturally or experimentally infected cats. Oocysts can be stored for long periods of time at 4°C (Dubey, 1998), which facilitates the isolation of progeny from experimental crosses (as described below).

14.3 ESTABLISHMENT OF TRANSMISSION GENETICS

14.3.1 Initial genetic crosses

While morphological observations of T. gondii infection in the cat intestinal epithelia and of oocysts in fecal material provided a framework for the sexual cycle, key mechanistic details such as sexual differentiation/determination and recombination required the establishment of transmission genetics. Early work by Pfefferkorn and colleagues laid the foundation for T. gondii as a genetic model organism by generating necessary tools, making key observations, and carrying out the first genetic crosses. Performing genetic crosses in T. gondii is relatively complex and not easily undertaken in most laboratories, in part due to the biological hazard posed by oocysts. These stages are resistant to chemical treatments and disinfectants (i.e. chlorine, aldehyde fixation, strong acids or bases, UV irradiation). Heat inactivation is the only reliable means of disinfecting surfaces that come in contact with T. gondii oocysts (Dubey, 1998). Other stages of T. gondii (tachyzoites and bradyzoites) can be used with typical BL-2 level containment, making these relatively easy to work within the laboratory. Successful completion of a genetic cross requires the production of tissue cysts of two compatible parental strains, typically by chronically infecting mice, co-feeding of tissue cysts to specific pathogen-free kittens, collection of oocysts shed in the feces, sporulation, re-initiation of in vitro cultures, cloning, and phenotyping of progeny. Not surprisingly, the number of crosses that have been conducted has been very limited, although the results have been highly informative.

As with other genetic model organisms, *T. gondii* lines with mutant phenotypes such as drug resistance and temperature sensitivity had to be
generated as tools for genetic crosses. These studies exploited the fact that T. gondii tachyzoites are haploid and can be maintained indefinitely in vitro and cryopreserved, thus aiding in the isolation of defined clones (Pfefferkorn, 1988). In particular, the screen for temperature-sensitive (ts) mutants benefited from this, as temperature sensitivity in other genetic models is usually a recessive trait (Pfefferkorn and Pfefferkorn, 1976). The ts mutants, selected after RH parasites were exposed to N-methyl-N-N'-nitro-N-nitrosoguanidine, were able to form plaques only at the permissive temperature of 33°C and not at 40°C. The ts lines tested in mice were substantially less virulent than the parental strain, the highly virulent RH strain (later shown to be the prototype for the type I lineage), consistent with growth inhibition during a febrile response. Although only one ts line showed obvious reversion, the low rate of recovery of mutants overall (1.4 percent of survivors of the mutagenesis) argues that most of the ts mutants recovered were due to single lesions. The selected ts lines varied in their optimal growth temperature and ability to remain infectious at 40°C, suggesting that the genetic basis for the growth defects were different for each line. By comparison, the screen for resistance to the drug 5-fluorodeoxyuridine (FUDR) (Pfefferkorn and Pfefferkorn, 1977a, 1979) targeted a single gene encoding the salvage enzyme uracil phosphoribosyltransferase, previously characterized from a spontaneous mutant (Pfefferkorn and Pfefferkorn, 1977b; Pfefferkorn, 1978). For the recovery of FUDR-resistant mutants, alkylating agents were found to be the most efficient mutagens (Pfefferkorn and Pfefferkorn, 1979). The results from these studies showed that mutants with selectable phenotypes could be easily recovered, opening up the possibility to use genetic crosses and recombination to study the genetic basis for biologically relevant phenotypes such as drug resistance.

For the first *T. gondii* cross, two mutant lines resistant to adenine arabinoside (AraA) and FUDR were separately selected from the parental C strain (also known as CTG, CEP) after chemical mutagenesis (Pfefferkorn and Pfefferkorn, 1980). The CTG strain was isolated from a naturally infected cat in New Hampshire by Elmer Pfefferkorn (Pfefferkorn et al., 1977), and it was later found to be a member of the type III lineage. Several clones were recovered, and a line resistant to each drug was chosen for testing. Clones resistant to AraA or FUDR were each independently able to pass through the cat and shed oocysts. All 116 oocyst-derived clones recovered from the AraA-resistant line were drug resistant. This confirmed the haploid nature of the parasite and the production of both micro- and macrogametocytes. When roughly equal numbers of AraA-resistant and FUDR-resistant bradyzoites were fed to a cat, approximately 12 percent of the clones recovered were resistant to both drugs. This rate of doubly resistant clones was observed in two separate experiments and is consistent with the expected meiotic recombination yield, given that half of all fertilizations will be self-fertilizations, and of the remaining cross-fertilizations 25 percent of the progeny are expected to be doubly resistant hence 12.5 percent overall. This calculation assumes these markers to be unlinked, something that was later confirmed by linkage mapping studies. These data argue that mating types are not predetermined and that parthenogenesis does not contribute substantially to the output of oocysts during experimental crosses.

14.3.2 Genetic crosses between different lineages

The early experiments with genetic crosses in T. gondii were conducted with different clones of the type III strain CTG, suggesting that similar approaches could be used to perform genetic crosses between strains of different lineages. In fact, the population structure of T. gondii was not appreciated at the time, and the choice of a second strain was based primarily on availability: the ME49 strain, originally isolated from a sheep in California, had been propagated in vitro, subcloned, and passaged through animals (Kasper and Ware, 1985). The ME49 strain was later shown to be representative of the type II lineage. A genetic cross was conducted between the type II strain ME49 and the type III strain CTG to test the feasibility of inter-strain crosses (Sibley et al., 1992).

Prior to conducting this cross, the ME49 strain was passed singly though a cat to obtain a line that was capable of producing oocysts. This line was subsequently cloned to produce clone B7, which was also later used for completion of the whole genome sequence of T. gondii (http://ToxoDB.org). Extensive testing of RFLP markers and sequencing of individual genes from the isolates of ME49, B7, and PTG (an independent clone of ME49; Kasper and Ware, 1985) failed to identify any genetic differences, suggesting that ME49 was effectively already a biological clone. The other parental line used in this cross was a doubly resistant clone of CTG bearing the drug-resistance markers AraA^R and sinefungin (SNF^R). Tissue cysts from the two parental lines were co-fed to susceptible kittens, and oocysts were collected after shedding.

Progeny from the II × III cross were isolated based on their drug-resistance phenotypes, and clones that were singly resistant to either SNF or AraA were retained. By definition, such progeny are recombinants due to the independent segregation of these unlinked markers. The frequency of doubly, singly, and non-resistant parasites was not determined, so it is unclear what the rate of outcrossing vs. selfing was in this cross. A total of 19 progeny were obtained from this cross, and they were cryopreserved in order to make them available for subsequent analyses. Unfortunately the population of recombinants was not retained, and, in hindsight, it was realized that this should be a standard step following genetic crosses in T. gondii. A second cross between the type II and III lineages was performed several years later (ca. 1996) using a similar strategy, and this cross is known as c96. In this cross, the parental strains were carrying the following drug-resistance markers: the type II PTG strain was resistant to aprinocide-N-oxide (ANO) and FUDR, and the type III strain CTG was resistant to SNF, AraA, and diclazuril (DCL) (Khan et al., 2005a). Recombinants from this cross are only available from a pool of parasites that was doubly selected with SNF and ANO, and no information is available about the rates of out-crossing vs. selfing (Khan et al., 2005a).

More recent crosses between other lineages have resulted in very different proportions of

inbreeding vs. out-crossing than reported in the original studies with CTG strain clones. A genetic cross between the virulent type I strain GT-1 (Dubey, 1980) and the non-virulent type III strain CTG (Pfefferkorn et al., 1977) was used to investigate the genetic basis of acute virulence (Su et al., 2002) (discussed further below). The GT-1 strain was tagged using FUDR resistance by chemical mutagenesis and selection as described previously (Pfefferkorn and Pfefferkorn, 1979). The CTG parental strain was previously obtained by crossing a clone resistant to AraA (AraA^R) (Pfefferkorn and Pfefferkorn, 1978) with a clone resistant to sinefungin (SNF^R) (Pfefferkorn and Pfefferkorn, 1980) to generate the doubly resistant clone CTG-AraA^R-SNF^R. Progeny from this cross were initially selected for segregation of these resistance markers to obtain FUDR^R-AraA^R and FUDR^R-SNF^R clones. Subsequent to this, clones were isolated randomly and typed solely by PCR analysis using polymorphic genetic markers (Su et al., 2002). In the absence of any selection, virtually all the clones obtained were recombinants (24 of 25 clones tested), with the exception of a single type III clone. This very high frequency of outcrossing cannot be explained simply by inefficient gamete formation, as each strain readily produced oocysts when fed to cats singly (unpublished data). Analysis of the segregation of the apicoplast genome in this cross may be informative, as studies have demonstrated that this organellar genome is inherited maternally (Ferguson et al., 2005). The observation that such an extreme bias can occur in experimental crosses may have a bearing on the outcome of genetic exchange in the wild. If similar bias exists in natural crosses, this could give rise to a very high proportion of genetic recombinants, thus increasing the genetic diversity of natural populations. Population genetic studies of T. gondii indicate a high level of clonality, which could be maintained by asexual transmission or by a high proportion of selfing. Despite this highly clonal population structure (Dardé et al., 1992; Sibley and Boothroyd, 1992a; Howe and Sibley, 1995; Ajzenberg et al., 2002a), pockets of high genetic diversity also occur in the wild (Ajzenberg et al., 2002a, 2002b), suggesting that locally high

rates of genetic crossing are important in the population structure of *T. gondii*.

14.4 DEVELOPMENT OF MOLECULAR GENETICS TOOLS

14.4.1 Molecular karyotyping

One of the challenges of developing genetics approaches further in *T. gondii* was the initial uncertainty about the size and complexity of the genome. Cytofluorometry measurements indicated that the haploid genome size is approximately 8×10^7 bp (Cornelissen *et al.*, 1984b). While the limited number of genetic markers that had been analyzed in experimental crosses showed evidence of independent segregation (Pfefferkorn and Pfefferkorn, 1980; Pfefferkorn and Kasper, 1983), there were too few markers to provide an adequate estimate of the number of chromosomes. During mitosis the chromosomes of apicomplexans do not condense, limiting the usefulness of cytogenetic approaches.

Pulsed-field gel electrophoresis (PFG) was originally developed to separate chromosomes of yeast, which range from 50 kb to ~1 mB (Schwartz and Cantor, 1984). The architecture of the gel electrodes (and hence the features of the resulting electrical field) was subsequently modified in various designs (i.e. OFAGE, CHEF) that allowed for separation of increasingly larger karyotypes, including Schizosaccharomyces pombe (Chu et al., 1986), Candida albicans (Magee et al., 1988), Trypanosoma brucei (van der Ploeg et al., 1989), and malaria (van der Ploeg et al., 1985). Application of PFG techniques to laboratory strains of T. gondii revealed that its chromosomes were relatively large, necessitating the use of conditions that included special 'chromosome-grade' agarose, high electrical field strengths, and the ability to alter the angle between the electrodes. TAFE (Transverse Alternating Field Electrophoresis apparatus) was used to separate a minimum of ten chromosomes, nine resolvable bands plus material that did not enter the gel (Sibley and Boothroyd, 1992b). The T. gondii chromosomes ranged in size from 2 Mb to greater than 6 Mb, and mapping of 57 different DNA probes to these gel-separated bands by Southern blot hybridization defined the initial linkage groups in T. gondii (Sibley and Boothroyd, 1992b). The total size of all of the resolvable chromosomes in T. gondii was estimated at ~40 Mb, although the accuracy of size standards in this range made this estimate very approximate. Comparison of different strains of T. gondii revealed significant differences in size for chromosomes III and V between the different lineages (Sibley and Boothroyd, 1992b). However, compared to Plasmodium (Wellems et al., 1987; Corcoran et al., 1988), the karyotype of T. gondii appears remarkably stable following repeated laboratory passage (Sibley and Boothroyd, 1992b).

14.4.2 Development of genetic markers

Initial studies on transmission genetics in T. gondii relied on drug-resistance markers introduced by the relatively laborious process of chemical mutagenesis, selection, and cloning by limiting dilution. Typically, these markers require the use of drugs that either selectively target the parasite or affect host processes that are not essential to the parasite (i.e. DNA metabolism) (Pfefferkorn, 1990). In both cases, the pathways must be non-essential or redundant in the parasite. The number of markers that fit these criteria is relatively small, and this is a major limitation to developing linkage analysis. Microsatellite (MS) probes, which differ in the number of simple nucleotide repeats, have been used successfully for developing a high-resolution map for P. falciparum (Su et al., 1999). While MS probes have been used for analyzing genetic diversity of T. gondii strains (Ajzenberg et al., 2002a), they were not sufficiently abundant, or easily accessible prior to the availability of the whole genome sequence, to allow their development for genetic mapping. Efforts to expand the number of genetic markers for T. gondii instead relied on identification of restriction fragment length polymorphisms (RFLP) detected between strains.

Attempts to develop RFLP markers for *T. gondii* were based on the handful of cloned genes available

at the time: cDNA probes were used for Southern blot hybridization against genomic DNAs (gDNAs) from representative clones of the three lineages: RH (type I), ME49 (type II), and CTG (type III) (Sibley et al., 1992). RFLPs were not abundant, and less that 10 percent of single enzyme digests revealed useful polymorphisms (Sibley et al., 1992). Randomly isolated clones from a small insert library had a slightly higher success rate of approximately 15 percent, but this low level of polymorphism was still a major limitation to developing an adequate number of markers. In the end, cosmid clones consisting of genomic inserts of ~30 kB were used to perform Southern blot hybridization against restriction enzyme digested gDNAs from type strains (Sibley et al., 1992). This resulted in a much higher level of polymorphism (~45 percent of single enzyme digests revealed polymorphisms), presumably because a larger region of the genome was scanned in a single blot. While these cosmid probes proved very useful for establishing the initial RFLP linkage maps, this library turned out to have a large percentage of chimeric clones, such that some RFLPs that were mapped genetically to a specific linkage group were found to hybridize to multiple bands on PFG gels. Eventually these probes were all converted to PCR-based markers, eliminating this problem. In some cases, original markers identified by cosmids were replaced with nearby polymorphisms that were more reliable.

One of the early observations from these RFLP mapping studies was the finding that all RFLP markers were essentially biallelic - that is, one strain would invariably be different at a given RFLP while the other two strains shared a second allele (Boothroyd et al., 1993). The frequency of the rare allele was higher in the type I RH strain, although RH also shared many alleles with the non-virulent type II and III lineages. This finding initially came as a surprise due to the fact that the type I lineage had been shown to be highly clonal and genetically distinct from types II and II at several loci (Sibley and Boothroyd, 1992a). Subsequent sequencing of different genes from the different lineages confirmed the observation that there are only two alleles at every locus (Bulow and Boothroyd, 1991;

Parmley *et al.*, 1994; Meisel *et al.*, 1996). These findings culminated in the proposal of a model that the clonal lineages (i.e. I, II, III) arose from several closely related parental strains in the wild – a rare example of natural recombination that led to three very successful lineages (Grigg *et al.*, 2001a; Su *et al.*, 2003a).

14.4.3 Genetic polymorphisms identified through EST sequencing

The advent of high throughput genome sequencing centers opened up new possibilities for exploring the genome of understudied organisms like parasites. The generation of expressed sequence tags (ESTs) provided a rapid means of gene discovery from single-pass sequencing of randomly selected cDNAs (Adams et al., 1992; Hillier et al., 1996; Marra et al., 1999). This approach has been widely used in parasites, where it has led to early discovery of a variety of genes based on BLAST homologies (Reddy et al., 1993; Chakrabarti et al., 1994; Ajioka, 1998; Ajioka et al., 1998; Li et al., 2003). One of the added bonuses of EST sequencing is that the process is naturally redundant, leading to multiple reads from the same gene that can be assembled in silico into a complete coding region. Redundancy has the benefit of offsetting the inherent error rate of Taq polymerase used in the PCR-based sequencing.

Sequencing of ESTs from T. gondii was undertaken to survey different life-cycle stages (tachyzoites, bradyzoites, and sporozoites) from different lineages of the parasite. Generation of cDNA libraries from these different stages and strains ultimately resulted in a large database of sequences (more than 125000 are found in the dbEST section of NCBI) that were subsequently assembled (Li et al., 2003, 2004). Comparison of orthologs expressed by different lineages has been useful in identifying polymorphism defined by single nucleotide polymorphisms (SNPs). Due to the inherent error rate of such sequences, a simple rule requiring two or more complementary reads from two or more strains was used to define putative strain-specific SNPs. These SNP differences occur at about 1 in 100 bp, and are scattered

roughly equally across the genome. As predicted from earlier studies, they occur in a strictly biallelic pattern and the rare allele occurs in roughly equal proportions among the three lineages. A complete summary of the clustered *T. gondii* ESTs, including strain-specific SNPs, can be found in the ApiESTDB (http://www.cbil.upenn.edu/apidots/) (Li *et al.*, 2003, 2004).

14.4.4 PCR-based genotyping

The availability of a large set of sequence-based polymorphisms, combined with reduced costs and increased accuracy of PCR-based methods, led to a shift in typing strategies for T. gondii genetic markers. All of the first-generation makers that required Southern hybridization were first converted to PCR-based markers (Su et al., 2002). Typically, this was done first by comparing them to the EST database to define those where existing sequence data were available for alignment and identification of SNPs. In cases where EST sequences were not available, the DNA region corresponding to the marker was amplified from each of the three lineages and sequenced to determine the particular alleles. Comparison of these sequences was used to verify SNP-based markers. Additionally, a set of new markers was developed based on the whole genome sequence of T. gondii that was conducted by TIGR (http://www.tigr.org/tdb/e2k1/tga1/). Sequence assemblies (http://ToxoDB.org) were initially searched with existing markers to position them on contigs or scaffolds. Regions with low marker coverage were then selected to develop additional markers. These new regions of interest were first screened for EST content to identify potential strain-specific SNPs. In regions where EST densities were too low, specific regions were selected and sequenced from all three lineages to identify SNPs. In both cases, the approximate frequency of SNPs was found to be similar (i.e. ~1 in 100 bp). In total, a set of 250 PCR-based markers spanning the genome was identified through these various strategies (Khan et al., 2005a). A complete description of these markers and the conditions for their analysis can be found at http://ToxoMap.wustl.edu.

The current set of PCR-based markers for T. gondii is designed to be amplified using flanking primers, the resulting product digested with the appropriate restriction enzyme, and products run in agarose gels to visualize RFLPs. The advantage of this method is that it is extremely robust and can be scaled in 96-well format to allow a large number of analyses to be conducted in parallel. Alternative strategies for marker typing have also been developed based on PCR-based priming from the 3' end of an allele-specific oligo (Su et al., 2003b). While this method also works well and can be multiplexed, it was not developed to full capacity. The advent of array-based typing methods is likely to greatly speed up the analysis of genetic polymorphism in T. gondii and replace the traditional method of genotyping on a locus-by-locus basis.

14.5 APPLICATION OF GENETIC MAPPING

14.5.1 Generation of RFLP linkage maps

The generation of RFLP-based linkage maps for T. gondii has proceeded through three phases. Initially, a set of 64 markers was used to analyze segregation from a single genetic cross between types II and III (Sibley et al., 1992). Segregation of these markers in 19 recombinant progeny was used to generate the first-generation linkage map. At this early stage, 11 chromosomes were recognized and the total genetic map distance was less than 150 centiMorgans (cM). While low resolution, this map was sufficient to link several drugresistance markers to specific chromosomes (SNF, AraA) and to establish the basic parameters of meiotic recombination in T. gondii. The next advance in genetic mapping in T. gondii came with the successful analysis of a cross between the virulent type I (GT-1) and the non-virulent type III (CTG) lineages. The total number of markers increased to 112 (identifying 57 unique loci), and a combined linkage map was generated from the analysis of two separate crosses (Su et al., 2002). While the number of chromosomes remained the

same, the linkage map expanded to ~400 cM. The highly different virulence phenotype of the parental clones in the second cross was used to analyze genes that contribute to pathogenesis (see below). Finally, in phase three, a greatly expanded number of genetic markers (250 in all) was used to analyze 71 progeny from genetic crosses between two of the three pair-wise groupings (II × III and I × III) (Khan *et al.*, 2005a). MapMaker EXP 3.0 (Lander *et al.*, 1987) was used to generate linkage maps with a LOD score of >3.0. In some cases, the order of markers was fixed based on physical maps provided by assembly of the whole genome sequence.

The current genetic linkage map of T. gondii consists of 14 linkage groups that collectively comprise 590 cM (Khan et al., 2005a). The chromosomes are largely the same as those identified in earlier studies, with the addition of several new groups that had not been resolved by PFG gels and/or had been missed due to the low density of markers in the earlier studies. The current map places a genetic marker approximately every 300 kb across the genome, providing reasonably complete coverage. Individual linkage maps for each chromosome and their corresponding markers are shown in Figure 14.2. As expected, the relative size of each chromosome, based on PFG separation and physical maps from the genome assembly, shows a roughly linear relationship with genetic size in map units. The rate of crossover in T. gondii averages about 100 kb/cM, although marked differences are seen from a low of 42 kb/cM on chromosome Ia to a high of ~150 kb/cM on chromosome VIIb (Khan et al., 2005a). The average crossover frequency for T. gondii is substantially lower than for P. falciparum (17 kb/cM) (Su et al., 1999), making it more difficult to finely map genetic traits in T. gondii. Interestingly, both T. gondii (Khan et al., 2005a) and P. falciparum (Su et al., 1999) show a high frequency of closely spaced double-crossover events. These occur at much closer distances than would be predicted by crossover frequency, and likely do not represent reciprocal events. This can complicate mapping analyses, as regions between existing markers might also have undergone such

exchanges, potentially altering important phenotypes without resulting in easily detectable genetic changes.

The biallelic nature of genetic markers in T. gondii presents an unusual situation that complicates assembly of the genetic maps. Because, at any given locus, two strains are identical while the third strain is unique, SNP markers are not informative in all pair-wise crosses. Additionally, crosses between types I and II have thus far failed due to technical problems. Finally, strain-specific haplotypes are conserved across large regions of the genome, such that strainspecific SNPs are strongly clustered (Khan et al., 2005a). This leads to a situation where most of the informative markers on some chromosomes (i.e. VI, VIII, XI, XII) are unique to the type I lineage. An extreme example of this is chromosome XI, where the genetic linkage map is based almost exclusively on progeny from the I × III cross. Hence, this chromosome is effectively invisible in the II \times III cross. This situation also leads to linkage disequilibrium between chromosomes VI and VIII, which are strongly linked despite physical evidence that they are separate (Khan et al., 2005a). Additional crosses to include I × II progeny will be needed to resolve these problems, and this may ultimately lead to a revised genetic lineage map.

14.5.2 Assembly of the *T. gondii* genome

When the whole genome sequence of *T. gondii* was undertaken, it was done as a shotgun approach without benefit of a physical map. The positions of the genetic markers were then used to assemble the large scaffolds from the genome into wholechromosome super-assemblies (Khan *et al.*, 2005a). Scaffolds are assemblies that consist of contigs (overlapping sequence reads) joined in some cases by short gaps that are bridged by end sequences from specific clones. The chromosome maps for *T. gondii* are comprised of all the larger scaffolds (greater than 100 kb), which collectively account for >95 percent of the estimate 65-Mb genome (Figure 14.3). The remaining scaffolds are mostly small contigs of >10 kb that cannot be



FIGURE 14.2 Genetic linkage maps for *T. gondii*. There are 14 recognized linkage groups for *T. gondii*. Vertical maps are provided for each chromosome, calibrated in centiMorgans (listed at the left and totaled at the bottom of each chromosome). Individual markers are listed at the right of each chromosome. Further details can be found at http://ToxoMap.wustl.edu. Reproduced from Khan *et al.* (2005a), with permission.



FIGURE 14.3 Physical maps of the *T. gondii* chromosomes. Placement of the genetic linkage markers shown in Figure 14.2 onto the whole genome scaffolds (http://ToxoDB.org) was used to assemble the genome into 14 chromosomes. Chromosome markers are indicated to the far left, and total sizes in bp are shown in the second column. Shaded bars correspond to the criteria used for mapping, that were based on genetic linkage analysis, physically overlapping contigs, and BAC-end clones. Scaffold numbers are given within or above each box representing a single scaffold. Further details can be found at http://ToxoMap.wustl.edu. Reproduced from Khan *et al.* (2005a), with permission.

easily aligned with the rest of the genome. Scaffolds that have been physically mapped fall into several different groups, depending on the degree of concordance between genetic and physical mapping data (Figure 14.3). For all major scaffolds, the genetic linkage map and physical assembly agrees quite closely. Separate scaffolds were assigned to particular chromosomes and ordered based on the assortment of genetic markers that they contain. In the case of smaller scaffolds, often genetic linage data could only be used to assign the relative position within the chromosome, but not the orientation. This occurs when the ends of the scaffold lie at the same genetic node, and it is a direct consequence of the local crossover frequency. Some of these scaffolds were correctly oriented based on the end sequences of BAC clones mapped to the genome by BLAT analysis. Finally, some scaffolds were linked solely by end sequences from BAC clones. A database integrating both the genetic linkage maps and physical maps was developed based on CMap, a genetic mapping tool provided by the Genetic Model Organisms Database (GMOD: http://www.gmod.org). The CMap for *T. gondii* can be accessed at: http://ToxoMap.wustl.edu. Comparison of the physical and genetic maps can be used to examine localized differences in crossover rate and to identify contigs/scaffolds and their associated markers.

14.5.3 Mapping drug resistance

Drug resistance in parasitic protozoa has been analyzed by genetic linkage mapping in Eimeria tenella (Chapman, 1984; Shirley and Harvey, 2000), P. chabaudi (Carlton et al., 1998), and P. falciparum (Wellems et al., 1991). The most successful of these examples is the mapping and positional cloning of the chloroquine-resistance gene in P. falciparum (Fidock et al., 2000). Drug-resistance markers in T. gondii, originally generated for convenient screening of recombinant progeny, have also been mapped to specific loci by genetic linkage. The association between resistance in the progeny and specific genetic markers has been evaluated for several of the drug-resistance markers, including FUDR, SNF, and AraA (Khan et al., 2005a). Resistance to each of these compounds is primarily determined by a single genetic locus. Their association with specific genetic markers was evaluated using quantitative trait locus (QTL) mapping (Lander and Botstein, 1989; Lander and Kruglyak, 1995) to analyze both single-locus effects and secondary loci that influence resistance. QTL analysis showed a single strong association for each of these compounds with no detectable contribution from other loci (Figure 14.4).

The molecular targets for two of these compounds, FUDR and AraA, were previously known, and they serve as controls for the precision of mapping by linkage analysis. The target for FUDR is the uracil phosphoribosyl transferase (*UPRT*) gene, located in the center of chromosome XI. Mapping FUDR resistance showed perfect correspondence to this locus (Figure 14.4). The target for AraA is adenosine kinase, which is located at the end of chromosome XII between markers AK165 and AK163 (Figure 14.4). Although the target for

SNF in *T. gondii* is presently unknown, the resistance phenotype mapped to a region on chromosome XII associated with a cluster of markers that includes AK123. SNF is known to inhibit methylation reactions (Martin and McMillan, 2002), although preliminary examination of this region of the genome does not identify an obvious target that fits this prediction (http://ToxoDB.org).

The relatively large genomic intervals defined by these mapping studies illustrates one of the primary limitations of the linkage mapping in T. gondii: the low rate of recombination limits resolution. The map unit (centiMorgan) in T. gondii is approximately 100 kb (Khan et al., 2005a), and a large number of progeny would have to be evaluated to map a trait to a region this small. Typically such a region contains 10-20 genes, many of which have complex splicing of multiple exons. Consequently, positional cloning will likely require screening progeny for recombination across specific regions, and corresponding higher density maps in regions where candidate loci have been identified. Once a candidate locus has been identified, the newly completed genome sequence (http://ToxoDB.org) makes it feasible to readily align markers to specific regions and identify candidate genes, thus allowing application of reverse genetic approaches.

14.5.4 Mapping quantitative traits

Many phenotypic traits, such as growth and virulence, are not governed by a single locus but rather by the combination of genes at different loci. Additionally, other regions of the genome can modulate traits that are strongly influenced by a single locus. Studies by Lander and Botstein (Lander et al., 1987; Lander and Botstein, 1989; Lander and Kruglyak, 1995) provided the foundation for genetic analysis of complex traits. In particular, these studies demonstrated that interval mapping based on genetic markers can reliably map quantitative loci (QTLs). The power of QTL mapping strategies for analyzing complex phenotypes has led to great interest in application of this technology in parasites (Su and Wootton, 2004). QTL analysis not only estimates the contributions



FIGURE 14.4 Genome-wide scans for mapping drug resistance in *T. gondii*. QTL scans were performed for the drug resistance markers SNF, FUDR, and AraA. Plots indicate the log likelihood ratios for association between genetic makers (listed sequentially along the bottom axis) and drug resistance phenotypes. The total number of markers here (175) corresponds to those that are informative for the I \times III cross. Chromosome boundaries are shown by the vertical lines. Resistance to SNF is mapped to chromosome IX, FUDR is mapped to chromosome XI, and AraA is mapped to chromosome XII. The lower horizontal dotted line corresponds to a likelihood of 1.7, while the upper line corresponds to a likelihood of 3. Reproduced from Khan *et al.* (2005a), with permission.

of different loci; it also provides statistical methods to account for the potential for false-positive associations that occurs with large datasets (Lynch and Walsh, 1998). In most biological experiments, statistical tests based on pair-wised comparisons generally have a cutoff of p < 0.05. However, when large numbers of pair-wise comparisons are conducted in a single analysis, as is the case with linkage studies, this false-positive rate is unacceptably high. Typically this is accounted for by a likelihood ratio that relates the probability that a given association is real versus the probability that it might occur by chance. The outcomes of these calculations are referred to as the likelihood ratio statistic or log odds ratio (i.e. LOD score) (Lynch and Walsh, 1998). Typically, LOD scores of > 3 are considered significant, but very strong support requires LOD scores of > 5-6. However, even low LOD scores can provide meaningful clues about loci that may contribute to a trait, and this might justify analysis of further progeny in order to confirm or refute the preliminary association.

14.5.5 Genetic approaches for defining virulence genes

One of the main advantages of classical genetics is that it provides forward genetic analyses of complex phenotypes. In other words, genetic linkage mapping has the potential to identify yet unknown genes that mediate important, naturally occurring biological phenotypes. The strength of this approach is that it requires no a priori assumptions about the underlying biology of the trait, so long as it differs between natural isolates and can be reliably measured. Forward genetics can answer the important question: which gene(s) mediates this phenotype? Reverse genetic approaches, on the other hand, are best suited for asking more defined questions, such as: does a specific gene mediate a particular phenotype? While reverse genetics can in theory be applied to any gene (essential genes may require a regulated system for disruption), it cannot prioritize genes for study or systematically identify unknown genes that mediate a phenotype. These two alternative strategies give access to very different sets of genes thus, when faced with the question: what genes are important for virulence? Reverse genetic approaches have defined several genes in T. gondii that are important for infection - including MIC1-MIC3 (Cérede et al., 2005), GRA2 (Mercier et al., 1998), M2AP (Huynh et al., 2003), SAG3 (Dzierszinski et al., 2000), MyoA (Meissner et al., 2001), AMA1 (Mital et al., 2005), and CPSII (Fox and Bzik, 2002). However, these genes do not explain natural differences in acute virulence between strains of T. gondii; nor can reverse genetics be used to find such genes efficiently.

Strains of *T. gondii* differ substantially in their ability to cause disease in animal models. More specifically, strains of the type I genotype are acutely virulent in the mouse model. Type I strains are uniformly lethal in mice and, even at low inocula, infected animals do not survive challenge with tachyzoites given by i.p. inoculation (translates into an effective LD_{100} of a single organism) (Sibley and Boothroyd, 1992a; Howe *et al.*, 1996). In contrast, types II and III are relatively non-virulent and typically have LD_{50} s of >10³ (Sibley and

Boothroyd, 1992a; Howe *et al.*, 1996). Human infections are most often caused by type II strains (Howe and Sibley, 1995; Howe *et al.*, 1997; Ajzenberg *et al.*, 2002a, 2002b), yet several studies suggest that type I strains, or strains harboring alleles typically found in type I strains, are also more pathogenic in humans (Fuentes *et al.*, 2001; Grigg *et al.*, 2001b; Khan *et al.*, 2005b). The mouse model offers the greatest potential to uncover biological differences in acute virulence between natural isolates of *T. gondii.*

Quantitative trait mapping has been used to evaluate the acute virulence of T. gondii in the mouse model (Su et al., 2002). To explore the genetic basis of acute virulence, a highly virulent type I strain called GT-1 (Dubey, 1980) was crossed with a non-virulent type III strain CTG (Pfefferkorn et al., 1977). GT-1 has a virulent phenotype typical of type I strains, while CTG has an LD_{50} of ~ 10^3 in outbred mice (Su et al., 2002). To identify unique clones, progeny were genotyped using polymorphic RFLP markers distributed across the genome. Separately, each clone was tested to establish the virulence phenotype in mice following inoculation with 10¹, 10² or 10³ tachyzoites. Virulence was defined based on cumulative mortality: virulent clones do not give rise to chronic infection (all infected animals succumb), while clones with intermediate levels of virulence were categorized as high (70-95 percent mortality, but with chronically infected survivors), intermediate (30-70 percent mortality) and low (< 30 percent mortality) virulence.

Phenotypic analysis of recombinant progeny revealed a range of phenotypes from fully virulent to non-virulent, and including intermediate levels not seen in either parental strain (Su *et al.*, 2002). These results suggest that virulence is largely heritable rather than epigenetic, and that it is likely multigenic. When acute mortality is considered as single gene model, it maps to a single QTL in the central region of chromosome VIIa (Su *et al.*, 2002; Khan *et al.*, 2005a). Additionally, when cumulative mortality is modeled based on the assumption that it may be due to several genes, the major contribution is still located on chromosome VIIa. The QTL on chromosome VIIa is flanked by markers M95-cS10-A6 with minor contributions from other regions of the genome (Su *et al.*, 2002; Khan *et al.*, 2005a). This finding implies that a single genetic locus is largely responsible for the virulence phenotype of toxoplasmosis in mice. Earlier studies have highlighted the potential for other regions of the genome to also contribute to this phenotype, notably chromosome IV (Su *et al.*, 2002); however, at present the statistical support for these regions is low, and they may not be borne out by further analysis. Nonetheless, it is logical to assume that other genes will influence the trait of acute virulence.

The natural differences seen in virulence between the three lineages is likely due to the different assortment of alleles they acquired following the relatively few genetic crosses since their common origin (Grigg and Suzuki, 2003; Su et al., 2003a). Two models could explain the much higher level of virulence in the type I lineage. First, this lineage may have inherited a pre-existing virulence gene from one of the two parental strains shared by the three clonal lineages. Alternatively, neither parental strain may have expressed this trait, and only through the reassortment/recombination of alleles did virulence fully manifest in the progeny. There is precedence for this latter model, as recombination between progeny of a II × III cross led to enhanced virulence of one of the progeny (albeit not to the extreme level exhibited by type I strains) (Grigg et al., 2001a). Further analysis of this virulence QTL should enable identification of the gene responsible for enhanced mortality. Reverse genetic approaches can then be used to test the role of specific genes by swapping of alleles between lineages, or by gene deletion analysis (Roos et al., 1994). Additionally, a broader survey of natural isolates may reveal clones with an ancestral genotype that might help to resolve the origin of the extreme virulence manifest by the type I lineage.

14.6 FUTURE CHALLENGES

14.6.1 Overcoming major limitations

Classical genetic analysis in *T. gondii* has been extremely useful for defining basic parameters of

recombination, mapping drug-resistance loci, assembling the genome, and probing the molecular basis of pathogenesis. Despite this progress, classical genetics remains a relatively difficult and expensive process in *T. gondii* compared to model organisms. Improvements that would accelerate progress include:

- 1. Development of *in vitro* methods for generation of gametocyte formation. Such techniques have been reported for *Eimeria* (Hofmann and Raether, 1990) and *Plasmodium* (Al-Olayan *et al.*, 2002). Successful completion of the life cycle *in vitro* would remove the single biggest obstacle to performing genetic crosses in *T. gondii*.
- 2. Conducting increased numbers of genetic crosses, especially between the type I and II lineages.
- Analysis of self-crossing vs. out-breeding in experimental crosses not driven by drug selection.
- 4. Development of parallel analysis of a lager number of markers by microarray hybridization to detect SNPs. This would allow more rapid genotyping and almost more precise pinpointing of crossovers.
- 5. Conducting genetic crosses with more exotic lineages that may exhibit additional or different phenotypes not found in the clonal lineages.

14.6.2 Additional phenotypes

In additional to the above-mentioned phenotypes, there are several interesting biological questions that could be probed by classical genetic mapping. Differences in the migration rate of lineages of *T. gondii* have been linked to virulence (Barragan and Sibley, 2002); however, genetic associations for this trait have not been reported. Differences in growth rates have been described for strains of *T. gondii* (Radke *et al.*, 2001), although it is not known to what extent these contribute to pathogenesis. Differences in the induction of cytokines (Robben *et al.*, 2004) and migratory responses of dendritic cells to chemokines (Diana *et al.*, 2004) have been reported to be strain-dependent in the mouse model. The development of intestinal

necrosis (Liesenfeld, 2002) and CNS pathology (Suzuki *et al.*, 1989) in the mouse model are also influenced in part by the genotype of the parasite. Genetic linkage studies may enlighten us about genes that contribute to these unique aspects of parasite biology, and better define their roles in pathogenesis.

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15

Genetic Manipulation of *Toxoplasma gondii*

B. Striepen and D. Soldati

- 15.1 Introduction
- 15.2 The mechanics of making transgenic parasites
- 15.3 Using transgenic parasites to study the function of parasite genes
- 15.4 Perspectives

15.5 The *Toxoplasma* Maniatis: a selection of detailed protocols for parasite culture, genetic manipulation, and phenotypic characterization *Acknowledgements References*

15.1 INTRODUCTION

The first genetic manipulations applied to Toxoplasma were performed by using chemical mutagenesis. These studies were pioneered in the 1970s by Elmer Pfefferkorn (Pfefferkorn and Pfefferkorn, 1976; Pfefferkorn, 1988), who perfected protocols to reproducibly cultivate tachyzoites in a tissue culture system and to mutagenize, select, and finally clone parasites by limiting dilution. Based on these protocols, a series of chemically induced mutants were used to map out the parasites' nucleotide biosynthetic pathways. These studies were critical for the establishment of protocols for genetic crosses in the cat (Pfefferkorn and Pfefferkorn, 1980). Crosses can be used to map a given phenotype to a single or multiple genome loci. This classical forward genetic approach has been instrumental to map virulence factors and to

analyze *Toxoplasma* population structure and evolution. (See Chapters 3 and 14 for further discussion of these topics.)

The reverse genetics approach, which introduces foreign DNA into parasites, was achieved using electroporation. Initially the transient transfection of plasmid DNA containing reporter genes flanked by T. gondii 5' and 3' flanking sequences allowed the expression of reporter genes used for the characterization of the elements controlling transcription. This methodology was rapidly utilized to identify and validate several selectable marker genes, which then opened an avenue for stable transformation and the development of an invaluable panoply of tools associated with DNA transfection. A wide range of positive and negative selectable markers has been tailored for homologous recombination leading to allelic replacement and gene knockouts. In addition, non-homologous



FIGURE 15.1 Sources of information and manipulation strategies. Schematic drawing of an intracellular parasite with the subcellular structures and organelles and the list of the tools currently available for functional analysis. Figure modified from Soldati, D. and Meissner, M. (2004). *Toxoplasma gondii* a model organism for the Apicomplexans. In: *Malaria Parasites: Genomes and Molecular Biology*. Norwich: Horizon Press, pp. 135–167.

random integration vectors have been designed to express transgenes and as a strategy for random insertional mutagenesis.

The recent completion of the Toxoplasma genome sequencing project (www. Toxodb.org) and the availability of other apicomplexan genomes for comparison are delivering an unprecedented amount of exciting information. In this new area of post-genomics, the accessibility of T. gondii to multiple genetic manipulations approaches and to high-throughput studies makes it a very attractive and powerful system to improve our understanding of the basic biology of apicomplexan parasites. Figure 15.1 summarizes the available sources of information and experimental approaches. There is no limitation to the identification of relevant genes, and little or no barrier to experimentally unraveling their biological function on a relatively large scale.

The purpose of this chapter is to recapitulate and describe the strategies associated with DNA transfection, including the most recent acquisitions, and to provide a list of the most useful protocols, reagents, and strains available to researchers.

15.2 THE MECHANICS OF MAKING TRANSGENIC PARASITES

15.2.1 Transient transfection

Successful manipulation of the *Toxoplasma* genome is critically dependent on the efficiency of DNA transfection. Electroporation was and still remains the method of choice to introduce DNA into tachyzoites. Importantly, the combination of this method with media mimicking the cytosolic

ion composition of the cells (cytomix) confers the best survival rate (van den Hoff *et al.*, 1992). The protocol, initially established using a BTX electroporator, led to an efficiency of transient expression that oscillated between 30 and 50 percent (Soldati and Boothroyd, 1993). The optimal settings chosen on the BTX Electroporator were fixed for the RH strain (type I, virulent strain) and were slightly modified for the cyst-forming strains (ME49 and Prugniaud; type II strains). It has been frequently observed that the cyst-forming strains are less amenable to genetic manipulation, probably due to several factors.

To monitor transfection efficiency, chloramphenicol acetyl transferase (CAT) and β -galactosidase were originally used as reporter genes, and subsequently β -lactamase, alkaline phosphatase, and fire-fly luciferase (LUC). These enzymes are classically used as reporters because their activities can be monitored with great sensitivity and in a quantitative fashion. Additionally, these enzymes are absent in eukaryotic cells, leading to virtually no background activity.

Interestingly, β-lactamase and alkaline phosphatase exhibit no activity within the parasite, probably due to the presence of inhibitors, and can be exploited to study the secretory pathway and quantify parasite secretion (Karsten et al., 1998; Chaturvedi et al., 1999). LacZ activity can be measured using a colorimetric assay that transforms yellow chlorophenol red-β-D-galactopyranoside (CPRG) substrate into a red product using an absorbance spectrophotometer at 570 nm (Seeber and Boothroyd, 1996). This colorimetric readout assay can be monitored in live parasites using culture medium without phenol red and in multiwell plates allowing (at a high throughput level) the screening of the efficacy of a drug against the parasite (McFadden et al., 1997).

Faithful expression of a reporter gene requires adequate 5' and 3' flanking sequences that are derived from *T. gondii* genes. The flanking sequences must contain the control elements necessary to drive an optimal level of transcription. The monocistronic nature of transcription in *T. gondii* facilitated the identification of promoter elements that are usually in close proximity to the transcription start site. Numerous vectors suitable for transfection are currently available, and, as they exhibit different range of promoter strength and stage specificities, they can be chosen appropriately according to the purpose of the experiment.

It has been frequently observed that the strength, and probably also the timing of expression with respect to the cell cycle, critically influence the outcome of an experiment, especially when studying the subcellular localization of a given gene product. For example, the overexpression of microneme proteins often results in accumulation in the early compartment of the secretory pathway, or leakiness into the parasitophorous vacuole (Soldati *et al.*, 2001). The use of the native promoter is highly recommended if transgenic gene expression results in toxicity or mistargeting (Gubbels *et al.*, 2006b).

A constitutive level of expression can be obtained by using vectors derived from TUB1 (α -tubulin), DHFR (dihydrofolate reductase), ROP1 (rhoptry protein 1), MIC2 (microneme protein 2), several GRA (dense-granule proteins) and HXGPRT (hypoxanthine-guanine phosphoribosyl transferase) genes. The strength of these and other promoters has not been very systematically compared, but the GRAs and MIC2 promoters are the strongest, TUB1 and ROP1 promoters are intermediate, while DHFR-TS is a weak promoter.

Stage-specific expression can be achieved using the 5-flanking sequences of stage-specific genes, and so far no stage-specific regulatory elements have been mapped in the 3'UTR sequences. Tachyzoite-specific expression is conferred by vectors derived from SAG1 (surface antigen 1), ENO1 (enolase 1), and LDH1 (lactate dehydrogenase 1) genes. In contrast, vectors constructed from BAG1 (bradyzoite antigen 1), ENO2 (enolase 2) or SAG4 genes confer expression in the bradyzoite stage exclusively. Detailed promoter analyses and identification of cis-acting elements have only been undertaken for a limited number of genes (Soldati and Boothroyd, 1995; Mercier et al., 1996; Bohne et al., 1997; Yang and Parmley, 1997; Matrajt et al., 2004; Kibe et al., 2005). Chapter 16 provides a discussion of regulation of gene expression.

In addition to the promoter elements, sequence features carried on the mRNAs also contribute to the success of transfection. Sequence information derived from the 5' and 3' untranslated regions likely affects gene expression, but this level of regulation has not been rigorously investigated to date. The 3' UTR is an important element as transcription drops to less than 10 percent when such element is not included. In *Plasmodium*, partial deletion of 3' UTR regions has been exploited to modulate the level expression of essential genes, offering a way to analyze their function (Thathy *et al.*, 2002).

At the start codon, a consensus sequence termed the 'Kozak sequence' is recognized by the ribosome as a favorable sequence to initiate translation. A compilation of abundant expressed genes in T. gondii was used to establish a consensus translational initiation sequence gNCAAaATGg, which is similar but not identical to the Kozak sequence found in higher eukaryotes (Seeber, 1997). Several genes including GFP were initially very difficult to express using their native sequence, but the lack of expression was solved by the generation of fusions at the N-terminus (Striepen et al., 1998). These observations suggested significant influence of the N-terminal а amino-acid sequences in recombinant protein expression. A systematic analysis aiming at the evaluation of the importance of the amino acid following the initiation methionine confirmed the existence of an N-end rule in T. gondii (Matrajt et al., 2002a). Amino acids such as Ala, Glu, and Asp confer high level of expression of the transgene.

15.2.2 Stable transformation and positive and negative selectable markers

Most of the selectable marker genes commonly used for eukaryotic cells are not suitable for selection of stable transformants in *T. gondii* (*T. gondii* is an obligate intracellular parasite). Only drugs selectively affecting the parasite while keeping the host cells intact could be considered. In spite of this restriction, various selection protocols have been developed and are listed in Table 15.1.

Chloramphenicol shows a potent but delayed parasiticidal effect, allowing the use of *E. coli* chloramphenicol acetyl transferase (CAT) not only as reporter enzyme but also as a tight selectable marker gene (Kim *et al.*, 1993). Parasites must complete up to three cycles of host-cell lysis (up to 7 days) before an effect of the drug is evident. At this point parasite are cloned in 96 well plates for about 5 days, starting in the presence of drug selection.

Another selection strategy based on the resistance to a drug can be achieved by exploiting the protective effect of the *ble* gene product of Streptoalloteichus or Tn5 against the DNA-breaking activity of phleomycin (Messina et al., 1995; Soldati et al., 1995). Parasites expressing ble become resistant to the drug; however, this selection needs to be applied on extracellular parasites to be effective. Phleomycin selection has been used successfully for the random insertion of transgenes (Soldati et al., 1995), and to disrupt genes by homologous recombination (Mercier et al., 1998). As an alternative to drug resistance, stable selection can be achieved by complementation of the naturally occurring tryptophan auxotrophy of Toxoplasma by addition of indole to the culture medium (Sibley et al., 1994) following the introduction of the bacterial tryptophan synthase (trpB) gene.

Two genes coding for non-essential nucleotide salvage pathway enzymes have been exploited as negative selectable markers. Loss of uracil phosphoribosyl transferase (*UPRT*) activity confers resistance to the prodrug 5'-fluo-2'-deoxyuridine (FUDR) (Donald and Roos, 1995), and, in the absence of hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) activity, 6-thioxanthine (6-Tx) cannot be converted into an inhibitor of GMP synthase (Donald *et al.*, 1996). In HXGPRT-deficient mutants this gene can also be used for positive selection strategies, since mycophenolic acid efficiently kills parasites lacking the enzyme. THE MECHANICS OF MAKING TRANSGENIC PARASITES

Selectable marker genes	Recipient strain	Drug or selection procedure	Concentration range
CAT, E. coli	Wild type	Chloramphenicol; drug treatment for 7 days before cloning	20 µM CM
DHFR-TS, T. gondii	Wild type	Pyrimethamine; treatment for 2 days before cloning	1 μM PYR
Ble, <i>Streptoalloteichus</i> , or Tn5	Wild type	Phleomycin: two cycles of treatment for 5–10 hours on extracellular parasites	5 μg/ml PHELO
HXGPRT, T. gondii	RHhxgprt-, ME49hxgprt-, PRUhxgprt-	Positive selection: Mycophenolic acid + xanthine: treatment for 3 days before cloning	25 μg/ml MPA, 50 μg/ml XAN
		Negative selection: 6-Thioxanthine	80 μg/ml 6-TX
UPRT, T. gondii	RH uprt-	Negative selection: 5′-fluo-2′-deoxyuridine	5 μM FUDR
GFP/YFP, Aequorea victoria	Wild type	FACS	
Essential genes, T. gondii	TATi-1 conditional KO	Anhydrotetracycline	Max. 1 µM ATc
Cre recombinase, Enterobacteria phage P1	Transgenes flanked by loxP sites (recycling of markers)	Transient transfection with Cre expressing plasmid; cloning immediately after electroporation	No selection
TK, Herpes simplex	Wild type	Ganciclovir, 24 hours treatment	10 µM GCV
CD, E. coli	Wild type	5-fluorocytosine	40 µM FLUC

TABLE 15.1 Selection strategies, gene markers, and conditions

The frequency of stable transformation fluctuates significantly depending on the type of selectable marker used. The conformation of the transfection plasmid (circular versus linearized by restriction) can also affect transfection efficiency. A much higher frequency of stable transformation is achievable using pyrimethamine resistance vectors derived from the parasite's bifunctional dihydrofolate reductase-thymidylate synthase, DHFR-TS. An artificially mutated *dhfr-ts* gene from *T. gondii* was used to design an expression vector pDHFR*–TSc3 (No. 2854) that confers pyrimethamine resistance (Donald and Roos, 1993). The DHFR-TS based selection is unique, and shows an exceptional frequency of chromosomal integration of up to 5 percent (Donald and Roos, 1993). The flanking sequences of the DHFR-TS genes are responsible for this unusual property, which can be partially conferred on other selectable marker genes such as the HXGPRT if this latter is controlled by the DHFR-TS flanking sequences.

Furthermore, restriction enzyme-mediated integration (REMI) can be used to further enhance the frequency of transformation up to 400-fold (Black *et al.*, 1995) and enables co-transfection of several unselected constructs together with a single selectable marker. Any of the selectable markers genes listed above can, if needed, be efficiently recycled by the action of the site-specific Cre recombinase. The adaptation of the *cre loxP* system from bacteriophage P1 to *T. gondii* enables the specific *in vivo* excision of any introduced sequence which was flanked by *loxP* sequences (Brecht *et al.*, 1999).

15.2.3 Homologous recombination and random integration

Unlike the situation in many protozoans, where integration into chromosomes occurs exclusively by homologous recombination and requires only a short segment of homology, homologous recombination is not favored in T. gondii. Vectors lacking long stretches of contiguous genomic DNA typically integrate into chromosomal DNA at random. The high frequency of transformation and random integration throughout the small genome size of haploid T. gondi tachyzoites was developed as an efficient strategy to mutagenize the entire genome of T. gondii within one single electroporation cuvette (Roos et al., 1997). Such genomic scale tagging allows identification of any gene whose inactivation is not lethal to tachyzoites and for which a suitable functional selection or screen is available.

Homologous recombination leading to gene replacement (Figure 15.2) is instrumental to studying gene function, and can be accomplished in *T. gondii* provided that sufficient contiguous homologous DNA (several kilobases) is used to target the locus (Donald and Roos, 1994). Nevertheless, the efficiency of homologous recombination remains very low, and a counter-selection strategy has been developed to raise the yield. A gene-targeting system based on HX*GPRT* as a positive/negative selectable marker enables the generation of genetic knockouts or allelic replacement by 'hit and run' mutagenesis for loci where no direct selection is available. A positive selection produces first a duplication of the target gene at the endogenous locus by single-site homologous recombination. Subsequently, a negative selection resolves the pseudo diploid to produce either wild type (revertant) or allelic replacement (gene knockout).

Alternatively, a second negatively selectable marker can be inserted into a knockout construct outside of the homologous flanking regions in order to eliminate all the transformants originating from random integration or single homologous recombination. The herpes simplex virus thymidilate kinase (TK) and the bacterial cytosine deaminase (CD), which confer sensitivity to ganciclovir and to 5-fluorocytosine respectively (Fox et al., 1999, 2001; Radke and White, 1999) have been successfully used as negative selectable markers in T. gondii. The fusion of these genes with CAT or DHFR-TS elegantly creates additional positive/ negative selectable markers. Finally, counterselection by FACS using fluorescent protein markers has also been applied successfully (Mazumdar et al., 2006).

Positive/negative selection can be employed for knockout experiments or used to devise selection schemes for mutants and promoter traps. The HXGPRT gene has been exploited to identify genes that are expressed in a stage-specific fashion (Knoll and Boothroyd, 1998). Parasites expressing HXGPRT under the control of a bradyzoite-specific promoter were mutagenized by random insertion of a plasmid and subjected to *in vitro* tachyzoiteto-bradyzoite conversion under 6-thioxanthine selection to isolate mutants deficient in differentiation (Matrajt *et al.*, 2002b)

15.3 USING TRANSGENIC PARASITES TO STUDY THE FUNCTION OF PARASITE GENES

15.3.1 Tagging subcellular compartments

Visualizing different subcellular compartments is an essential tool for cell biological analysis.



FIGURE 15.2 Exploiting non-homologous insertion and homologous recombination to manipulate the *T. gondii* genome.

- (A) Schematic representation of insertional genomic tagging using a DHFR-TS plasmid (based on Roos *et al.*, 1997). Plasmid DNA is indicated at top, genomic insertions below. For insertional mutagenesis, expression of the DHFR-TS pyrimethamine resistance gene is driven by its own promoter; the insertion therefore is not necessarily within the open reading frame but might also act through inactivating a regulatory region (e.g. promoter). In the case of promoter trapping, DHFR-TS does not carry its own promoter, and expression of the resistance gene depends on insertion close to an active promoter, or in-frame fusion into an expressed gene. Tandem insertions can complicate the identification of the tagged locus by plasmid rescue (using restriction enzyme X) and/or inverse PCR (using restriction enzyme X or Y). However, simultaneously applying restriction enzyme Z cuts the tandem into two fragments incompatible with plasmid rescue or inverse PCR (Roos *et al.*, 1997; Sullivan *et al.*, 1999).
- (B) Schematic representation of gene knock-out through double homologous recombination. The homologous regions destined for homologous recombination are represented by white boxes. Restriction enzymes A and B are used to generate fully homologous ends. In this case YFP is used as a negative selectable marker to enrich for homologous recombination (YFP is lost and parasites are FACS-negative).
- (C) Schematic representation of allelic replacement through single homologous recombination. In this strategy a circular plasmid inserts and tags the locus with a YFP fusion (which can be omitted, or replaced by a shortened ORF to create a functional knock-out). The gene-locus 3' of the plasmid backbone is functionally inactivated by the lack of a promoter.

This figure is taken from Gubbels, M.J., Mazumdar, J., van Dooren, G. and Striepen, B. (2006b). *The Biology* of Toxoplasma gondii: *Manipulating the* Toxoplasma *Genome*. Norwich: Horizon Press.

Specific antibodies raised against subcellular fractions or individual proteins are widely used for this purpose at the light- and electron-microscopic level. This approach, however, requires the production of antigen, either by purification from the parasite or by recombinant expression and subsequent immunization, which is time-consuming and not always technically feasible. Through transfection experiments, proteins can be tagged by gene fusion using a generic epitope (for which antibodies are already available) or using an autofluorescent protein. Autofluorescent proteins can be detected in vivo with minimal manipulation, providing a unique tool to follow biological processes over time (see Figure 15.3, and Gubbels and Striepen, 2004, for an in-depth review).

Numerous versions of green fluorescent protein (GFP) and related autofluorescent proteins have been successfully expressed in T. gondii (Striepen et al., 1998; Kim et al., 2001), and a range of colors is available now for the simultaneous use of multiple markers. Cyan (CFP) and yellow fluorescent protein (YFP) are a suitable pair for double-labeling experiments, and have been used in in vivo microscopic studies of Toxoplasma organelle biogenesis (Striepen et al., 2000; Joiner and Roos, 2002; Pelletier et al., 2002). A tandem repeat of the YFP gene yields exceptionally bright fluorescent transgenics which are now widely used to track parasites in tissue culture and in infected animals (Gubbels et al., 2003, 2004, 2005; Egan et al., 2005). Red fluorescent proteins (RFP) further extend the options. DsRed produces brightly fluorescent parasites (Striepen et al., 2001); however, the requirement for tetramerization of this marker can be problematic if the tagged protein is part of a complex or structure. Monomeric variants of RFP (e.g. mRFP; Campbell et al., 2002) can help overcome these problems, but suffer from considerably weaker fluorescence. The new 'cherry' and 'tomato' variants (Shaner et al., 2004) provide a reasonable compromise, and a tandem tomato marker produces exceptionally bright fluorescence when expressed in T. gondii (Giel vanDooren and BS, unpublished).

A large number of organelle-specific fluorescent protein markers is now available (see Figure 15.3 for examples). However, not all proteins can be studied in this way, as the bulky GFP tag can affect targeting, maturation, or function of its fusion partner. In such a case, epitope tags can provide an alternative approach. These tags can be inserted internally or placed at the N- and C-terminus. Due to their short length, epitope tags cause limited steric hindrance. Epitope tags require fixation and staining with a specific antibody before visualization. While not suitable for live-cell imaging, they can be used for subcellular and ultrastructural localization and immunoprecipitation experiments, or to monitor protein processing during targeting or maturation. A number of epitope tags have been used successfully in Toxoplasma - for example, cMyc (Delbac et al., 2001), HA (Karsten et al., 1997), FLAG (Sullivan et al., 2005), and Ty-1 (Herm-Gotz et al., 2002).

Parasites expressing fluorescent proteins can also be analyzed and sorted by flow cytometry. Cell sorting is amenable for positive and negative selectable markers. To obtain clonal parasite lines stably expressing fluorescent protein, two rounds of fluorescence-activated cell sorting (FACS) and expansion of sorted parasites in culture (Gubbels *et al.*, 2003) are routinely used. Multiple fluorescent proteins can be used and sorted simultaneously; however, an instrument with multiple lasers might be required (see protocol section). Fluorescent protein expression can also be detected using a plate reader. This provides a convenient growth assay for drug screening and genetic selections (Gubbels *et al.*, 2003).

15.3.2 Genetic analysis of essential genes

In order to study the function of essential genes in a haploid organism, tools need to be developed to ectopically and selectively control gene expression while avoiding pleiotropic effects. One widely used approach is based on the *E. coli* tetracyclinerepressor system, which controls gene expression at the transcriptional level. The original tetracyclinerepressor system interferes with transcription, and has been optimized and coupled to T7 polymerase to tightly regulate gene expression in *Trypanosoma*



FIGURE 15.3 For legend see next page.

FIGURE 15.3 Tagging subcellular compartments with fluorescent protein markers in T. gondii. This figure provides examples of single and dual fluorescent protein labeling *T. gondii*; all images were obtained by live cell microscopy.

(A) Dense granules and parasitophorous vacuole, P30-GFP (Striepen et al., 1998); (B) centrocones (outermost dots) and posterior IMC rings of mother (innermost) daughter cells (lines), MORN1-YFP (Gubbels et al., 2006a); (C) nuclei, PCNA-GFP (Radke et al., 2001); (D) plasma membrane, P30-GFP-GPI (Striepen, unpublished); (E) micronemes, MIC3-GFP (Striepen et al., 2001); (F) cytoplasm, YFP-YFP (Gubbels et al., 2003); (G) inner membrane complex, IMC3-YFP (Gubbels et al., 2004); (H) microtubules, YFP-TUB (Hu et al., 2002); (I) mitochondria, HSP60-RFP (van Dooren, unpublished); (J) dividing tachyzoites IMC3-YFP and H2b-mRFP (Hu et al., 2004); (K) nuclear division and cytokinesis, H2b-mRFP and MORN1-YFP (Gubbels et al., 2006a); (L) apicoplast division, FNR-RFP and MORN1-YFP (Striepen et al., 2000); (M) Golgi division, GRASP-RFP and MORN1-YFP; (N) apicoplast, ACP-GFP (Waller et al., 1998); (O) rhoptries, ROP1-GFP (Striepen et al., 1998); (P) endoplasmatic reticulum, P30-GFP-HDEL. This figure is reproduced in color in the color plate section.

brucei (Wirtz et al., 1999). The tet-repressor system has also been developed for other protozoan parasites, including T. gondii (Meissner et al., 2001). Gene fusion of the tet-repressor, as recently reported (van Poppel et al., 2006), has led to higher transgene expression and tighter regulation.

Although suitable for the expression of toxic genes and dominant negative mutants, this system proved not to be appropriate for the generation of conditional knockouts in T. gondii. Indeed, the necessity to keep the parasites in the presence of drug (anhydrotetracycline, ATc) for a prolonged period in order to maintain the expression of an essential gene led to generation of revertants that lost regulation.

To improve the system, a genetic screen based on random insertion was designed to identify a functional transcriptional activating domain in T. gondii and to establish a tetracycline transactivatorbased inducible system (Meissner et al., 2002). This screen led to the isolation of two artificial transactivators that were not functional in HeLa cells, illustrating the differences between the transcription machinery in the parasite and its higher eukaryotic hosts. Interestingly, these transactivators corresponding to short stretches of rather hydrophobic amino acids were also active in P. falciparum and allowed the establishment of an inducible system for the malaria parasite (Meissner and Soldati, 2005). This system is suitable for the conditional disruption of essential genes with no apparent reversion effect, and operates on the parasites in the animal model. In addition to TgMyoA, the tet system has been implemented to functionally analyze TgAMA-1 (Mital et al., 2005) and to determine the importance of several other genes, including TgMIC2 (Huynh and Carruthers, 2006), TgACP (Mazumdar et al., 2006) and profilin (Platnner and Soldati, unpublished).

So far, the tet-inducible system has been relatively laborious, requiring two steps of selection. The first step is the construction of a stable line expressing an inducible copy of the gene of interest. The second step is the actual knockout of the target gene (see protocol section for details). It is also conceivable to generate inducible knockouts by direct targeting of the inducible construct into the gene of interest by knock-in, replacing the endogenous promoter with the tet-inducible promoter. This could be achieved either by singlehomologous recombination or, preferably, by double-homologous recombination to avoid potential reversion of the phenotype by excision of the plasmid and restoration of the wild-type locus. Once an essential gene has been conditionally disrupted by the tet system, it is possible to use ATc as a positive selectable drug to complement the knockout mutants with either a wild-type or modified form of the gene of interest.

Approaches that specifically lower the level of mRNA and consequently the level of the corresponding protein are powerful genetic tools

available in many organisms. Various technical solutions relying on antisense RNA/oligonucleotides, ribozymes, or dsRNA interference (RNAi), have been developed. Such knockdown of genes is usually easier and faster to accomplish than in the generation of conditional knockouts using a tet-regulated system. In *T. brucei*, a combination of efficient RNAi and tight tet-regulated transcription is routinely applied in large-scale functional genomic screens (Ullu *et al.*, 2004).

In contrast, the efficiency of RNAi in apicomplexans is still a matter of debate. Previous studies have reported the successful use of antisense/ ribozyme in *T. gondii* (Nakaar *et al.*, 1999, 2000). More recent studies suggest that RNAi can operate in *T. gondii* (Al-Anouti and Ananvoranich, 2002; Al-Anouti *et al.*, 2003). Unfortunately, these approaches are currently not sufficiently robust to be used in broad screens.

15.3.3 Insertional mutagenesis and promoter trapping as tools of functional genetic analysis

Random high-frequency integration of a genetic element into the parasite genome can be used to disrupt loci and produce pools of insertional mutants. The integrated sequence can subsequently be exploited to identify the targeted gene with modest effort (Figure 15.2). The exceptionally high frequency of non-homologous recombination of transgenes in T. gondii allows the use of simple plasmid constructs, similar to the way transposons are used in other organisms (Donald et al., 1996). Several non-essential genes have been identified using random insertion of a DHFR-TS or HXGPRT element (Donald and Roos, 1995; Chiang et al., 1999; Sullivan et al., 1999; Arrizabalaga et al., 2004). The genomic locus tagged by the insertion can be identified by plasmid rescue or inverse PCR strategies (Roos et al., 1997).

The insertional strategy is not limited to gene disruption; it can also be used to trap promoters and genes. Bradyzoite-specific genes (Bohne and Roos, 1997; Knoll and Boothroyd, 1998) as well as genes controlling differentiation (Matrajt *et al.*, 2002b; Vanchinathan *et al.*, 2005) have been identified using differential HXGPRT selection under

culture conditions that favor differentiation to bradyzoites followed by counter-selection under 'tachyzoite' conditions. The trapping of native *T. gondii* transcription factors might also be achievable. For this a recipient strain harboring a YFP-YFP marker under the control of a tet-regulated promoter would be randomly inserted. The tagging plasmid would harbor a tet-repressor gene lacking a stop codon and 3' UTR sequences. Translational fusion of this marker with a transcription factor should result in transactivation and hence green fluorescence.

The fact that tachyzoites are haploid precludes the identification of essential genes by insertional mutagenesis. Nevertheless, it is possible to generate a library of parasite mutants for essential genes by coupling random insertion to the tet-inducible system (Gubbels and Striepen, unpublished).

Signature-tagged mutagenesis is another strategy that has been used to identify essential genes by insertional tagging. In this case, screening is performed in a different life-cycle stage or under different growth conditions to permit the identification of 'differentially essential' genes. This approach has recently been adapted for Toxoplasma (Knoll et al., 2001). Wild-type parasite clones are first tagged with unique oligonucleotide insertions (the signature-tag). These clones are then mutagenized (chemical or insertional) followed by another cloning step. Pools of mutants, which are distinguishable by their tag, are subsequently exposed to a selective condition - for example, infection into an animal. Tagging of genes that are essential in this condition will result in loss of the mutant. 'Missing' mutants are then identified by comparing the tags present in pools before and after selection. Several candidate genes important for parasite persistence in the mouse have been identified using this approach (Knoll, personal communication).

15.3.4 Forward genetic analysis using chemical mutagenesis and complementation cloning

Genetic analysis of pathways essential for growth in culture requires conditional mutants.

Temperature sensitivity (ts) due to chemicallyinduced point mutations can be exploited to obtain strains that are viable at the permissive temperature and display a mutant phenotype at the restrictive temperature. For Toxoplasma, heatsensitive (Pfefferkorn and Pfefferkorn, 1976, Radke et al., 2000; Gubbels and Striepen, unpublished) and cold-sensitive (Uyetake et al., 2001) mutants have been isolated. ENU (N-ethyl-N-nitrosourea) induces random point mutations, and has been the mutagen of choice in most T. gondii studies. Chemical mutagenesis has been successfully used in T. gondii to produce mutants with defects in stage differentiation (Singh et al., 2002), invasion and egress (Black et al., 2000; Uyetake et al., 2001), and cell division and cell-cycle progression (Radke et al., 2000; White et al., 2005).

While generating chemical mutants is straightforward, identifying the mutated gene responsible for the phenotype is not. The two avenues most commonly used to accomplish this goal are physical mapping through crosses, and phenotypic complementation by transfection with a wild-type DNA library. While crosses are feasible in T. gondii, their limited throughput makes them less practical as a general tool for mutant analysis (also, the RH strain used as the molecular biology work horse for T. gondii is unable to complete the sexual life cycle). The second approach to identify the gene affected in a given mutant is phenotypic complementation using a library of wild-type DNA. This strategy faces two technical challenges: full representation of the genome (or transcriptome) in the complementation library, and efficient recovery of the complementing sequence. Black and colleagues identified a genetic element that maintains stable episomes in T. gondii (Black and Boothroyd, 1998), allowing convenient rescue by Hirt lysis and transformation of bacteria. A library harboring an episomal maintenance sequence on the backbone successfully complemented the HXGPRT locus in the knockout mutant under mycophenolic acid selection. Analysis of the recovered plasmids, however, suggested that they might undergo extensive recombination,

potentially decreasing their stability and usefulness (Black and Boothroyd, 1998).

The second effort to generate a complementation system was built on high-frequency integration of library plasmids (Striepen et al., 2002). Mutants are transfected with a plasmid library and subjected to selection. Subsequently, complementing DNA sequences (carried as stable chromosomal insets) are rescued into plasmid using an in vitro recombination protocol (Invitrogen Gateway system; Hartley et al., 2000). Rescued library inserts can be shuttled back into a parasite expression plasmid through a second recombination step to confirm their complementation capacity. A cDNA library built on this model successfully complemented the Toxoplasma HXGPRT locus at high efficiency (Striepen et al., 2002), and was used to identify a phenotypic suppressor of the T. gondii ts cell-cycle mutant C9-11 (Radke et al., 2000; White et al., 2005). An analogous library carrying Cryptosporidium parvum genomic DNA was used for heterologous complementation, resulting in the identification of a Cryptosporidium gene encoding the purine salvage enzyme IMPDH (Striepen et al., 2002; Umejiego et al., 2004).

Several ts mutants could not be complemented using the cDNA libraries described above (Gubbels, White and Striepen, unpublished). Genes encoding large mRNAs and/or transcribed at low levels are typically underrepresented in cDNA libraries. To overcome these problems, a large insert (40-50 kb) genomic cosmid library build on a DHFR-TS containing super-cos vector was constructed. This library provides sufficient coverage and transformation efficiency to complement the lack of HXGPRT in every transfection reaction attempted. In addition, these researchers recently complemented a mutant with a ts cell division defect. Three overlapping cosmids were identified from independent transfections which all harbored the gene for a kinase known to be involved in cell-cycle regulation (Gubbels and Striepen, unpublished; cosmid complementation was observed in four additional ts mutants, M. White, personal communication).

15.4 PERSPECTIVES

T. gondii has proven itself as an excellent experimental model, and reverse genetic approaches were key to building a detailed molecular picture of apicomplexan biology. The reverse genetic toolbox has seen constant extension and refinement; however, several technological challenges remain. One of these challenges is the now completed genome sequence. The genome is obviously a tremendous resource, but the number of 'candidate' genes produced by computational screens easily overwhelms the throughput of many of the functional genetic assays currently available. Knockouts and conditional knockouts are powerful genetic experiments, but are currently very time-consuming and not successful in all cases.

Protocols that would increase the frequency of homologous over non-homologous recombination could be very helpful to streamline the process. A better mechanistic understanding of apicomplexan DNA recombination and the enzymes involved in the process is needed (Dendouga et al., 2002). Based on such knowledge and following the example of several fungal systems, the recombination pathway could be remodeled by the disruption of non-homologous recombination pathways (Navak et al., 2006) or, conversely, the overexpression of elements of the homologous recombination pathway (Shaked et al., 2005) could improve knockout frequency. RNA-mediated knockdown presents an attractive alternative to gene targeting. While promising, RNAi in T. gondii currently is not sufficiently robust for large-scale screens (Al-Anouti and Ananvoranich, 2002; Al-Anouti et al., 2003; Sheng et al., 2004). Improvements could be derived from optimized protocols to choose and deliver interfering RNAs. It might also be feasible to screen genetically for strains that show higher responsiveness to dsRNA.

Forward genetic approaches have seen considerable progress as well. These approaches could hold the key to mechanistic analysis of phenomena for which the genome does not immediately present an obvious list of candidate genes and proteins. While the tools to complement mutants have improved and may now be at a level to permit robust analysis, the ways to generate and select such mutants still lag behind. Robust screens that reduce a complex cell-biological phenomenon to a phenotype that can be easily scored in thousands of mutants with limited effort are needed. The success of visual screens using automated microscopic detection (Carey *et al.*, 2004) points to one avenue to reach this goal. The past decade has seen tremendous progress, driven by the ability to transfect and genetically manipulate the parasites. The next decade will require a set of tools with sufficient throughput to take full advantage of the genome sequence.

15.5 THE TOXOPLASMA MANIATIS: A SELECTION OF DETAILED PROTOCOLS FOR PARASITE CULTURE, GENETIC MANIPULATION, AND PHENOTYPIC CHARACTERIZATION

15.5.1 Propagation of *Toxoplasma* tachyzoites in tissue culture

T. gondii is promiscuous in its choice of host cell, and will infect almost any mammalian cell commonly used in tissue-culture work. In general, large, spread-out cells like fibroblasts or Vero cells are most suitable. Infection of these cells results in distinctive rosettes, which makes it easy to monitor parasite development by microscopy. Many laboratories use transformed cell lines such as Vero or 3T3, which produce high parasite yields. Immortal lines grow fast, are easy to culture, and can be obtained from many sources.

Primary cell lines such as human foreskin fibroblasts (HFF) are also widely used. Their strong contact inhibition and slow growth makes them the cell of choice for plaque assays, bradyzoite-induction experiments, genetic selections or any experiment in which cultures have to be maintained for longer periods of time. They also provide excellent microscopy for cell-biological analysis. The disadvantage of primary lines is that they have to be managed more carefully, as they will die at higher passage number due to senescence. A sufficient amount of early passage cells has to be cryopreserved to reinitiate the culture at that point. HTERT cells (BD Biosciences) have emerged as a compromise; these cells are immortal, but retain many characteristics of primary fibroblasts. The authors have found these cells to be equivalent to HFF cells in almost all applications. The protocols below are based on HFF cells, but can be used for HTERT cells as well (note the difference in glutamine concentration). Many companies supply reagents for tissue culture; the suppliers mentioned in the following are the ones used by the authors, but products from other sources might work just as well.

15.5.1.1 Maintenance of HFF cells

T25 flask tissue cultures typically yield $4-7 \times 10^7$ parasites (yields are typically lower for the type II and III cyst-forming strains). The protocols below are based on this scale. If more material is needed, larger flasks (e.g. T175), roller bottles, and cell factories have been used successfully with appropriately scaled protocols.

- Warm media and trypsin solution in a 37°C water bath.
- Aspirate medium from a confluent culture and add 2.5 ml of trypsin solution to the flask (0.25% trypsin and 0.2 g/l EDTA in HBSS (Hyclone); store this solution in smaller 5-ml aliquots at -20°C for convenience). Carefully 'wash' monolayer by tilting flask several times, then aspirate most of the solution and leave enough to just cover the cells (~ 0.5 ml). Incubate at 37°C for 2 minutes. Inspect cells for rounding and detachment using an inverted microscope equipped with phase or interference contrast optics. If cells are still attached after 2 minutes, tap flask with flat hand and/or prolong incubation. HFF cells are relatively fragile, so take care not to overtrypsinize.

• Immediately take up detached cells in a defined volume of Dulbecco's Modified Eagle's Medium (DMEM (Hyclone); if large batches are used the medium can be prepared from powder, otherwise use ready-made medium) supplemented with 10% newborn calf serum (NBCS, Hyclone, cosmic calf serum), penicillin and streptomycin (1:200 of a 10 000-unit/ml of antibiotic stock, Hyclone) and glutamine (1:100 of a 200 mM stock in water; note: for HTERT cells do not add glutamine to avoid overgrowing of cultures) and split 1:8 into new flasks. If fungal contaminations are a frequent problem, use 1:100 Fungizone (250 µg/ml amphothericin B, Invitrogen). Move to incubators gassed to 5% CO₂ at 37°C. Allow gas exchange by loosening caps. Confluent cultures can be kept for several weeks prior to T. gondii infection.

15.5.1.2 Maintenance of tachyzoites

- Aspirate medium from a confluent HFF culture.
- Add 10 ml of infection medium (DMEM supplemented with 1% fetal calf serum (FCS, Invitrogen; for experiments which require tight control over the small molecule composition, use dialyzed fetal calf serum), penicillin, and streptomycin (1 : 200 of a 10 000-unit/ml of antibiotic stock, Hyclone).
- Infect a new flask with culture supernatant of a freshly lysed culture. As a rule of thumb, passing 0.5-1.0 ml into a T25 culture will result in complete lysis within 2-3 days for RH derived strains. A high inoculum is preferable if parasites are to be used, e.g. in a transfection experiment, as the majority of the tachyzoites will egress synchronously, resulting in high overall parasite viability. To maintain strains, pass a smaller number of parasites (e.g. 100 µl of a lysed culture). Transfection efficiency and invasion efficiency are greatly enhanced by using freshly lysed parasites. Host cells should not be over-infected. Ideally, every host cell should be infected with one parasite.

15.5.1.3 Cryopreservation of host cells and parasites

In general, the aim is to freeze slowly and to thaw quickly. Wear a lab coat, face protection, and appropriately insulated gloves when handling liquid nitrogen. For best results, have all tubes and reagents prepared and labeled, chill them on ice, and work quickly (if you have to freeze many vials at a time, divide them into smaller batches).

- Label 2-ml cryo vials (fitted with silicone O-ring, Nalgene) using a pen dispensing ink that resists liquid nitrogen, and chill on ice.
- Prepare an isopropanol/water-containing freezing container (VWR; using this simple and inexpensive device will result in about 1°C/min cooling in a -80°C freezer; alternatively, use a thin-walled foam container to slow cooling).
- Use 'freshly' confluent (T175) HFF cultures for freezing. Trypsinize cells as described above, and recover detached cells in DMEM 10% newborn calf serum into a 15-ml sterile centrifuge tube. Pellet cells in a table-top centrifuge at 900 g for 10 minutes at 4°C using a swing bucket rotor.
- Discard the supernatant and resuspend cells in 1.8 ml chilled DMEM (no serum). Add 1.8 ml freezing medium (25% tissue culture grade DMSO and 20% FBS in DMEM) and mix quickly. Immediately dispense 0.5-ml aliquots into chilled freezing tubes, tightly cap tubes, and move into chilled (ice) freezing container and place into a -80°C freezer.
- Thaw one vial the next day to insure that your stocks are viable, and move the remaining vials into a liquid-nitrogen storage container. Solid bookkeeping which keeps track of rack, box, and vial position is essential, as it is not easy to 'search' for vials in liquid nitrogen stocks.
- Parasites are preserved as extracellular tachyzoites. Pellet a freshly lysed culture (1500 g, 20 minutes, 4°C) and then proceed as described for host cells above. Plan to freeze 2×10^8 per vial, which means that you will produce three vials from a single T25 culture using 0.8 ml of DMEM and 0.8 ml of freezing medium. Test for

viability by thawing before you discontinue the culture of the given line.

- Parasites can also be cryopreserved in host cells at the rosette stage in DMEM with 50% FBS/10% DMSO.
- To thaw HFF cells, prepare a flask with medium warmed to 37°C. Remove one vial at a time from liquid nitrogen, using tongs, and immediately immerse in a beaker filled with water warmed to 37°C, gently shaking the vial. Once the medium has thawed, transfer cells to the flask and incubate as described for standard culture. Replace medium after 12 hours.
- To thaw parasites, use above procedure and inoculate a confluent T25 culture.

15.5.1.4 Mycoplasma detection and removal

Mycoplasma contamination is a frequent plague of tissue cultures. Heavy infection can affect the growth of host cells; mycoplasma DNA can produce unwanted background in genetic experiments; and bacterial contamination is a severe problem for immunological experiments because mycoplasma-derived molecules potently stimulate a variety of immune cells and functions. A simple test for contamination can be performed by DNA staining:

- Culture cells (and/or parasites) for two passages in the absence of antibiotics (which will lead to massive amplification of the bacteria), then transfer to six well plates with coverslips.
- Stain coverslip cultures for bacterial DNA using DAPI and the standard IFA protocol provided below (more sensitive staining can be obtaining by acid/alcohol fixation and Hoechst staining; see Chen, 1977 for a detailed protocol).
- In contaminated cultures, numerous small dots of DNA staining (about the size of the typical apicoplast genome staining) can be observed throughout the cytoplasm of the host cell.
- More sensitive PCR (ATCC, Stratagene) or luciferase-based (MycoAlert from Cambrex) assays are also available.

- If recent contamination is suspected, discard the cultures, thaw a fresh vial from the liquid nitrogen, and retest. Protocols to screen strains obtained from other laboratories should be routine.
- If you have to 'rescue' your particular strain, treat with *Mycoplasma* Removal Agent according to the manufacturer's guidelines (an inhibitor of bacterial gyrase, e.g. MP Pharmaceuticals) for three passages and then retest (this antibiotic is reasonably tolerated by *T. gondii* at the suggested concentration). Other commercial agents kill *T. gondii* and should be screened prior to use as *Mycoplasma* elimination agents.
- Alternatively, passage of the strain through a mouse and re-isolation into tissue culture will remove mycoplasmas.

15.5.1.5 Passaging Toxoplasma tachyzoites in mice

- Tachyzoites of any strain can be maintained by passage in the peritoneal cavities of mice; 10⁴ (type I strain, i.e. RH) or 10⁵ (type II or III strain, i.e. ME49 or Prugniaud) are injected intraperitoneally into the mouse.
- Replicating *T. gondii* can be harvested from the peritoneal cavity 3 days later (for type I strains) and 5 days later (type II or III strain) by peritoneal lavage with 4 ml of sterile saline or PBS.
- This material can be used to serially passage the strain in the peritoneal cavities of mice or to infect tissue culture cells. Murine inflammatory cells (macrophages and neutrophils) will also be seen in this lavage material.
- Passage through mice can be useful to remove microorganisms which have contaminated *T. gondii* tissue culture, provided that they cannot replicate in murine peritoneum. Anecdotal data indicate that periodic murine passage of a *T. gondii* strain passaged continuously in tissue culture helps to maintain the vigor and biologic characteristics of the strain.

15.5.1.6 Passaging Toxoplasma bradyzoite cysts in mice

- Persistent cyst-forming strains such as 76K, ME49 (PLK), and Prugniaud can be passed by infection of mice with cysts collected from the brains of infected animals, or by infection with tissue-culture-derived organisms (for these strains, tissue cultures produce a mixture of tachyzoites and bradyzoites).
- Cysts or tissue-culture-derived organisms can be administered orally, or injected subcutaneously or intraperitoneally. Oral infection with cysts has anecdotally been reported to produce the highest number of brain cysts. In general, 10–100 tissue cysts are usually sufficient for oral infection.
- To prepare tissue cysts for infection, 3 ml of sterile 0.9% NaCl or PBS is added to one or two infected murine brain(s) containing tissue cysts, and this tissue is disrupted by passage through a syringe with an 18-gauge needle.
- The number of tissue cysts is determined by examining a 25–50 µl aliquot of this material under a tissue-culture microscope. Maximal reported yields of tissue cysts from the brain occur 4–8 weeks following infection; however, tissue cysts will persist for the life of the infected animals. Anecdotally, repeated serial passage of a tissue cyst-forming strain every 4 weeks in mice will increase the number of brain cysts formed by a strain.
- Cysts can be purified from the brains of infected mice by centrifugation using the method of Cornelissen *et al.* (1981). Mouse brains are homogenized using a ratio of one of brain to 1.5 ml of 0.9% NaCl at seven to ten strokes in a Potter tube (using the loose-fitting pestle).
- An isopycnic Percoll gradient is formed by centrifugation of 30 ml of 45% (v/v) Percoll in PBS pH 7.2 for 20 minutes at 27 $138 \times g$; 5 ml of the brain suspension is layered on this preformed gradient and the material is centrifuged for 15 minutes at $1250 \times g$.

- After centrifugation, the bottom 4 ml containing red blood cells is discarded and the next 20 ml is collected. This 20-ml fraction (specific gravity 1.056) contains the tissue cysts. This material is diluted with 20 ml of PBS and centrifuged for 10 minutes at 1000 \times *g*, to concentrate the cysts as a pellet free from Percoll. Recovery of cysts has been estimated at 80 percent with this technique.
- It is also possible directly to mix 5 ml of brain suspension with 30 ml of 45% (v/v) Percoll in PBS pH 7.2, followed by centrifugation for 20 minutes at 27 $138 \times g$. Cysts are found in this method in the same 20-ml fraction as when a preformed gradient is used. Recovery of cysts with this alternative method has been estimated to be 40 percent.

15.5.2 Transfection and stable transformation protocols

15.5.2.1 Transient transfection

- Cytomix (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄ pH 7.6, 25 mM HEPES pH 7.6, 2 mM EGTA, 5 mM MgCl₂) can be prepared in larger batches, filter sterilized and stored in aliquots at -20°C or 4°C (van den Hoff *et al.*, 1992).
- Weigh 12 mg ATP and 15.2 mg glutathione, add to 10 ml of cytomix and sterilize by passing through a 0.22-µm filter.
- Sterilize DNA by ethanol precipitation. Adjust 50 µg of plasmid DNA (typically in ~10µl and purified using a commercial plasmid purification kit such as from Qiagen) to 100µl with TE (pH 8.0). Add 11µl 3M NaOAc, and 250µl ethanol. Precipitate DNA for 5 minutes at -20°C and spin at full speed in a microcentrifuge.
- Wash the pellet with 1 ml cold 70% ethanol by gently inverting the tube, and spin for 2 minutes in a microcentrifuge.
- Move tubes into the laminar flow hood and discard the ethanol (keep an eye on the pellet).
- Let ethanol evaporate for 5–10 minutes (be careful not to 'over-dry', as it can be hard to

redissolve DNA). Resuspend DNA in $100\,\mu$ l cytomix.

- Filter parasites from a freshly lysed T175 flask into a 50-ml polypropylene tube and count t in a hemocytometer (dilute sample 1:10 in PBS for counting). Pellet parasites at 1500 g, 20 minutes, 4°C, and resuspend in complete cytomix to 3.3×10^7 parasites per ml (if required, the parasite concentration can be increased up to eight times).
- Mix 100 μ l plasmid DNA and 300 μ l parasites in a 2 mM gap electroporation cuvette (Genetronix) and electroporate parasites with a single 1.5-kV pulse, a resistance setting of 25 Ω , and a capacitor setting of 25 μ F, using a BTX ECM 630. If using a BioRad electroporator, set it to 1.5 kV, 25 μ F, and square wave; if employing an Amaxa system, use the T-cell solution instead of cytomix and set the electroporation conditions to program U33.
- Transfer parasites immediately into a confluent T25 HFF culture (for selection and biochemical experiments) or onto coverslips for microscopy (see below).
- Expression of the transgene can be detected beginning 8 hours after transfection (depending on the transgene and the sensitivity of the assay employed), peaking around 36 hours after electroporation. To measure transient transfection efficiency, electroporate with a robust and easy-to-score visual marker (e.g. plasmid tubYFP-YFPsagCAT; Gubbels *et al.*, 2003). Inoculate the coverslips and count total number of vacuoles and number of fluorescent vacuoles for several fields. All three electroporators yield transient efficiencies of 30–50 percent 24 hours after electroporation.

15.5.2.2 Selection of stable transformants

• CAT: selection for chloramphenicol acetyl transferase (CAT) can start immediately after electroporation in the presence of 20 μ M chloramphenicol (34-mg/ml stock in ethanol). Since the effect of the drug is delayed, it is important to passage the parasites every 2 days

by inoculating at least 10^6 parasites to keep the pool of parasites as heterogeneous as possible. The minimal amount of plasmid required to generate stable transformants depends on the vector used, but $10-50 \ \mu g$ of linearized plasmid will usually yield stable transformants.

- DHFR-TS: electroporate parasites with 50 µg of a plasmid encoding the drug-resistant dihydrofolate reductase-thymidylate synthase allele (Donald and Roos, 1993), such as plasmid pDHFR*-TScABP (Sullivan *et al.*, 1999). After electroporation, culture in the presence of 1µM pyrimethamine (1µl of a 10mM stock in ethanol). This plasmid results in the highest frequency of transformation (1–5 percent). Be careful handling transgenic strains, as pyrimethamine is used in the treatment of human toxoplasmosis.
- HXGPRT: this selection requires a hypoxanthine-xanthine-guanine phosphoribosyltransferase null mutant (such mutants are available now for multiple strains; see, for example, Donald *et al.*, 1996 for RH). Twenty-four hours after transfection add 25 µg/ml mycophenolic acid (25 mg/ml stock in ethanol) and 50µg/ml xanthine (50 mg/ml stock in 0.1 N KOH). MPA/xanthine should kill parasites within 2–3 days.
- BLE: for phleomycin selection, electroporate parasites with an expression vector encoding the resistance marker BLE (Messina et al., 1995), transfer to HFF cells until complete lysis of the host culture occurs (12-24 hours later). The lysed culture is forced three times through a 25-ga needle to assure that all the parasites are extracellular (see safety section for concerns about needle-passing before using this protocol). The suspension of parasites is adjusted to 5mg/ml of phleomycin (stock solution: 20 mg/ml in water and stored at -20°C) and incubated at 37°C for 10 hours. Parasites are transferred for recovery to HFF cultures in media containing 5 µg/ml of phleomycin. After a new cycle of lysis the extracellular parasites are treated again in the presence of the drug for 10 hours, and cloned thereafter by limiting dilution in 96-well microtiter plates containing

HFF cells in the presence of $5\mu g/ml$ of phleomycin.

15.5.2.3 *Restriction enzyme mediated integration (REMI)*

Transformation efficiency can be enhanced by adding 50–100 U of BamHI, NotI, or SacII to the cuvette immediately prior to electroporation (these three enzymes have worked in the past; choose one that does not cut an essential part of your plasmid(s)). Note that REMI often results in multi-copy integration of plasmid(s) (Black *et al.*, 1995; Gubbels *et al.*, 2004).

15.5.2.4 Cloning of transgenic lines by limiting dilution in 96-well plates

- Seed tissue-culture-treated 96-well plates with HFF cells and grow to confluency. Remove medium and add $100\,\mu$ l DMEM 1% FCS to each well.
- Harvest freshly lysed parasites by filtration and centrifugation as described above.
- Count, using a hematocytometer, and dilute to 250 parasites per ml.
- Add 100µl (25 tachyzoites) to each well in the first and seventh vertical columns.
- Using a multichannel pippetor perform a serial dilution from left to right, transferring 100µl at each step (mix each well by pippetting up and down three times). Discard medium after reaching column 6, and start over at row 7.
- Incubate for 7 days without disturbing the culture.
- Inspect each row from left to right, using an inverted microscope, and identify wells that contain a single plaque. Mark those wells. Expand clonal lines by passage into a T25 flask.

15.5.3 Measuring parasite survival and growth

15.5.3.1 Plaque assay

Plaque assays are a reliable way to measure the number of viable and infectious parasites in a

sample, and are well suited to measuring stable transfection efficiency. The following protocol will measure stable transformation using a DHFR-TS resistance plasmid.

- Electroporate tachyzoites as described above using 50 µg of pDHFR*-TScABP (Sullivan *et al.*, 1999). After electroporation, dilute 50 µl of the contents of the cuvette into 950 µl cytomix or medium.
- Infect T25 HFF cultures in drug-free medium with 3μ l and 6μ l diluted parasite suspension, and two cultures with 6μ l and 60μ l to be cultured in the presence of 1μ M pyrimethamine.
- Incubate for 7 days without disturbing the flasks (the optimal time may depend on the strain used; 2–3-mm plaques are best for scoring; a few extra flasks can be added in a larger experiment to be 'developed' individually to test when the right plaque size is achieved). The period of selection takes longer with type II and III strains.
- To stain the monolayer, aspirate the medium, rinse with PBS, fix for 5 minutes with ethanol, and stain for 5 minutes with a crystal violet solution (dissolve 12.5 g crystal violet in 125 ml ethanol and mix with 500ml 1% ammonium oxalate in water).
- Remove crystal violet solution and rinse with PBS.
- Air dry, and count the number of plaques.

This assay can also be used to quantify parasite growth by measuring plaque area. To do this, scan stained flasks with a standard flat-bed scanner at 600 dpi and use image analysis for measurements. The area of plaques can be reasonably approximated using an ellipse. Measure the longest and shortest diameters of each plaque, and use $\pi ab/4$ to calculate the area.

15.5.3.2 Fluorescence assay

This assay will produce dynamic growth curves over the time of the experiment (usually a week).

- Seed tissue-culture-treated black 384- or 96-well plates with special optical bottoms (Beckton Dickinson) with HFF cells. For larger-scale assays, an automatic liquid dispenser (e.g. Genetix Q-Fill) will increase throughput and reproducibility.
- Once plates are confluent, replace the medium with DMEM (without phenol red, Hyclone), 1% FCS, and antibiotics, as described above.
- Infect each well with 2000 (384-well) or 5000 (96-well) tachyzoites (e.g. the YFP-YFP strain; Gubbels *et al.*, 2003). Plan to have quadruple wells for each experimental condition (e.g. drug concentration) and include negative (no parasites) and positive controls on each plate. Fill all wells with the medium, but do not use the outermost wells because they evaporate faster, which affects parasite growth.
- Measure fluorescence daily for each well, for 5–8 days, using a sensitive plate reader (BMG Fluostar, or Molecular Devices SpectraMax M2, bottom excitation and emission 510/12 and 540/12 nm respectively).
- Plot the results (average of four wells and standard deviation) as percent positive in relation to the untreated positive control in each plate.

15.5.3.3 β -Galactosidase (LacZ) assay

This is an endpoint growth assay that can be used in multi-well formats (McFadden *et al.*, 1997); a yellow substrate will be turned into a red product.

- Seed HFF cells into standard tissue-culture-treated 384-well plates, as described above.
- Change the medium of confluent cultures to DMEM 1% FCS without phenol red (50 ml/well), and infect with 2000 β -galactosidase-expressing tachyzoites (wash parasites in PBS before infection to eliminate phenol red).
- At the desired read-out day (usually 5 days after infection optimal staining has to be established empirically for each strain and condition), add 4.5 μ l chlorophenol red- β -galactopyranoside (CPRG, Boehringer Mannheim, 4.5 mM stock in medium without phenol red).
• Develop color to desired intensity (if left too long, all the wells will turn red; use your negative and positive controls as a guide), and read absorbance at 570 nm. Plot endpoints as percent positivity, as described above.

15.5.3.4 Uracil incorporation assay

In contrast to mammalian cells, *T. gondii* can directly salvage uracil through UPRT. This can be exploited to measure parasite growth as a function of [³H]-uracil incorporation into parasite TCA precipitable nucleic acids (Pfefferkorn and Guyre, 1984; Roos *et al.*, 1994). The advantage of this assay is that it can be used in all strains and does not require a transgene. Recently a 96-well real-time format has been developed for this assay; this is described in detail in Nare *et al.* (2002).

- Infect 24 well cultures with parasites and incubate under test conditions (e.g. in the presence of a drug).
- Add 5 μCi of [5,6-³H]-uracil (30–60 Ci/mmol) to each well and incubate for 2 hours at 37°C.
- Chill cultures and add an equal volume of icecold 0.6 N trichloroacetic acid to the medium of each well, then incubate on ice for at least 1 hour.
- Remove TCA and rinse plates under running water overnight (make sure to use a sink designated for radioactivity work).
- Dry plates, add 500 µl of 0.1 N NaOH to each well, incubate for 1 hour, and measure radioactivity in half of the sample by liquid scintillation counting. Depending on the scintillation cocktail used, neutralization of the base can help to avoid background.

15.5.4 Live-cell and indirect immunofluorescence microscopy

• Sterilize round 23-mm glass cover slips in 70% ethanol (or autoclave) and transfer to a six-well plate. Seed cover slips with host cells, and culture to confluency. Infect wells with tachyzoites 24–36 hours before microscopic examination.

- To observe parasites expressing fluorescent protein transgenes, remove the coverslip from the dish with sterile forceps, wipe off the medium from the bottom side, and gently invert onto a microscope glass slide. If longer observation is required (for example, for timelapse microscopy), use spacer circles (e.g. Secure Seal, Invitrogen) to generate a small reservoir of medium. Alternatively, use dishes that have a coverslip bottom (e.g. ΔT3 dishes, Bioptechs).
- To use antibodies to stain cells, remove medium and fix cells in 2 ml 3% paraformaldehyde in PBS for 10–20 minutes.
- Remove fixative, and permeabilize cells in 2 ml 0.25% Triton X100 (in PBS) for 10 minutes.
- Block in 2 ml 1% w/v BSA in PBS/0.25% Triton X100 for 30 minutes.
- React with primary antibody (diluted 1:100 to 1: 5000 in PBS/BSA/0.25% Triton X100, depending on titer) for 1 hour. This can be done with minimal reagent by inverting the coverslip onto 100-µl drops on parafilm in a moist chamber.
- Place back into six-well dish (cell side up) and wash three times with 3 ml PBS (5 minutes each).
- React with secondary antibody diluted in BSA/PBS for 1 hour.
- Wash four times in 3 ml PBS (5 minutes each). To counterstain DNA, add 2 μl of a 2 mg/ml DAPI stock solution to the first wash.
- Apply a drop of mounting medium to a microscope slide.
- Briefly wash coverslip in dH₂O (to prevent crystal formation after drying) and invert into mounting medium (cells down).

Some epitopes are sensitive to aldehyde fixation. In that case, use 2 ml of methanol for 20 minutes as a fixative (methanol will also permeabilize the cells, and no Triton treatment is required). This protocol also works better to stain proteins secreted into the parasitophorous vacuole (these are often washed out by Triton permeabilization). A more elaborate protocol for secreted protein, which preserves subcellular

structures better than methanol, can be found in Lecordier *et al.* (1999).

15.5.5 Cytometry of parasites and infected cells

Toxoplasma tachyzoites can be efficiently sorted using a fluorescence-activated cell sorter (FACS) after labeling with specific antibodies to the surface of the parasite (Kim and Boothroyd, 1995; Radke *et al.*, 2004), or based on the expression of autofluorescent protein (Striepen *et al.*, 1998; Gubbels and Striepen, 2004; Gubbels *et al.*, 2004). Parasites expressing fluorescent proteins can also be sorted within their host cells (Gubbels and Striepen, 2004; Gubbels *et al.*, 2005).

- For sorting autofluorescent parasites, harvest a freshly lysed culture and filter parasites through a 3-µm polycarbonate filter. Count parasites, and take up in sterile PBS at 10⁷/ml.
- Use a high-speed sorter equipped with a 488-nm argon laser and the following filter and mirrors. GFP or YFP: DM, 555 SP; F: 530/40 BP; DRFP or Tomato DM: 555 SP; F: 570/40 BP. Note that for sorting the flow the stream is broken into droplets, which carries the potential to produce aerosolized parasites. Extra safety can be provided by an evacuated and HEPA-filtered enclosure of the sorting chamber. Discuss biosafety aspects with the FACS facility director and operator.
- For enrichment, sort into tubes preloaded with 0.5 ml of PBS or medium, and transfer to a confluent T25 HFF culture. For cloning, sort directly into seeded multi-well plates. Using a MOFLO sorter, the authors found three events per well to result in the maximum number of single clones per plate.
- To sort infected cells, inoculate parasites into a confluent HFF culture 1–24 hours prior to sorting.
- Aspirate medium and wash twice with sterile PBS.
- Trypsinize cells as described above, and recover in 10 ml DMEM 1% FCS.

 Filter through a 75-µm cell strainer (Becton Dickinson), spin down and resuspend in 0.5 ml PBS, and sort as described above.

Detail on antibody staining for FACS of tachyzoites is provided in Radke *et al.* (2004).

15.5.6 Disruption of non-essential genes

T. gondii is haploid, and non-essential genes can be disrupted by homologous recombination using single or double crossover. As discussed in detail, the main challenge is to overcome the background of non-homologous plasmid insertion. Above we have described and cited several approaches; here we describe a CAT/YFP positive/negative selection for homologous recombination by double crossover in detail (Mazumdar *et al.*, 2006).

- Construct a targeting plasmid that flanks a sagCATsag selectable marker cassette with 1.5–3-kb homologous sequence from the target gene (typically the 5' and 3' genomic sequences flanking the actual coding sequence). Introduce a YFP expression cassette 3' adjacent to the 3' homologous flanking region. Be sure that your targeting plasmid contains a unique restriction site that will allow you to linearize the construct without cutting into markers or flanking regions (e.g. in the multi-cloning site of the plasmid backbone).
- Test for YFP expression in a transient transfection experiment (~30 percent of the vacuole should show cytoplasmic fluorescence).
- Transfect with 10, 25, and 50 µg of linearized plasmid, and select for stable transformation in the presence of 20 µM chloramphenicol.
- Subject the drug-resistant population (typically after three or four passages) to FACS (use the non-transfected parent strain and a YFP-expressing strain as positive and negative controls). Gate events to be sorted to 'viable' tachyzoites by forward and side scatter, and clone non-fluorescent parasites by sorting into confluent 96-well plate cultures.

- Leave plates undisturbed, check for single plaques after 7 days, and mark clones.
- Suspend parasites by pipetting up and down, and transfer 100µl of each well into a well of a six-well plate. Replenish medium in the 96-well plate, and keep in the incubator.
- Six-well cultures will lyse within 3–4 days. Resuspend lysed parasites by pipetting, and harvest by centrifugation.
- Wash parasites with PBS and pellet again.
- Resuspend parasites in 500µl TE, add 1µl RNAse (10 mg/ml), 10µl 10% SDS, and 20µl proteinase K (10 mg/ml).
- Incubate at 55°C for at least 1 hour (can be left overnight).
- Extract twice with 500 µl phenol : chloroform : isoamylalcohol (25 : 24 : 1, molecular biology grade), and once with chloroform; always keep the water phase.
- Add 1/10 volume of 3M NaOAc and 2.5 volumes of ethanol and precipitate DNA for 20 minutes at –20°C.
- Spin for 10 minutes at full speed in a microcentrifuge, wash pellet with 70% ethanol, spin again, briefly airdry, and resuspend DNA in 50 µl TE.
- Use 5 μl as the template in a PCR reaction with primers that will produce different-sized products for the native and the KO locus (make sure that your primers do not pick up the ectopic mini-gene copy, see below).
- Confirm putative allelic replacements by Southern blot using appropriate probes.

15.5.7 Disruption of essential genes

As detailed above, this approach has three steps:

- 1. Introducing an ectopic tet-regulatable copy of the target gene
- 2. Targeting the native locus by homologous recombination
- 3. Knockdown of the expression of the ectopic copy using ATc treatment.

The choice of selectable markers may differ from experiment to experiment (the tet-transactivator line [Meissner *et al.*, 2002] is resistant to mycophenolic acid); this example will use CAT, YFP, and DHFR-TS.

- Construct a plasmid for ectopic expression of the target gene, for example by replacement of the ACP coding sequence in plasmid ptet07sag4-ACPmyc/DHFR-TS (Meissner *et al.*, 2002; Mazumdar *et al.*, 2006). If you omit the stop codon, this should result in an N-terminal translational fusion to a c-myc epitope tag.
- Transfect into the TAti transactivator line (Meissner *et al.*, 2002), select stable transformants in the presence of 1 μ M pyrimethamine, and clone by limiting dilution.
- Test clones for transgene expression by IFA and Western blot using an anti-cmyc antibody (mAb 9E10, Roche).
- Choose clones that express the transgene at a similar level to the native gene. Depending on the size of the target gene, addition of the tag may result in a noticeable mobility shift on SDS PAGE. In this case, an antibody against the target protein can be used to compare both proteins side by side.
- It is critical to identify a tightly regulated clone before proceeding to the KO experiment. Careful characterization of clones will pay off with a clean, interpretable phenotype. Test for regulation by culturing parasites in the presence or absence of 1 µg/ml of ATc (0.2 mg/ml stock in ethanol) followed by IFA and Western blot. Note that stable proteins might have to be diluted out by growth. Do the first screen after 5 days of treatment, and then titer the minimal treatment time for complete suppression using your tightest clone.
- Target the native locus as described above (using CAT/YFP positive/negative selection), establish allelic replacement, and analyze regulation of the ectopic copy in confirmed KO clones by IFA and Western blot.

15.5.8 Insertional mutagenesis and tag rescue

• Electroporate tachyzoites, using 50µg of linearized (e.g. restricted with NotI) plasmid

pDHFR*-TScABP. Select for stable transformants in 1 μ M pyrimethamine, and apply the desired phenotypic screen. Clone mutants by limiting dilution, expand into T25 cultures and isolate genomic DNA as described above.

- Set up parallel 20-µl restriction digests using several restriction enzymes which cut once in your plasmid (e.g. EcoRI, HindIII, XhoI, XbaI for pDHFR*-TScABP; see Sullivan *et al.* (1999) for maps and a detailed discussion of enzyme choice). Use 2 µg genomic DNA for each digest, and incubate overnight at 37°C.
- Purify DNA from the digest using a Qiagen spin column, following the manufacturer's protocol, and elute in 30 µl elution buffer.
- Mix 5 μ l eluate with 2 μ l 10x NEB ligase buffer, 13 μ l H₂O, and 1 μ l T4 DNA ligase, and incubate overnight at 16°C.
- Add 1μl glycogen, 2μl 3M NaAc, pH 5.2, and 50μl ethanol, and precipitate DNA for 30 minutes at -20°C.
- Wash pellet with 1µl 70% ethanol, airdry briefly, and resuspend pellet in 10µl H₂O.
- Electroporate 1µl into 25µl library efficient electrocompetent bacteria (we found DH12S to result in best recovery).
- Transfer into sterile microcentrifuge tube, add 200 µl LB medium, and incubate for 1 hour at 37°C while shaking.
- Plate entire transformation onto an LB agar plate containing suitable antibiotic (in this case, ampicillin).

Tags can also be rescued by inverse PCR – see Sullivan *et al.* (1999) for primer design and a detailed protocol.

15.5.9 Chemical mutagenesis

- ENU is highly toxic and carcinogenic. Use utmost care with all the materials that have come into contact with this chemical – label tubes and flasks to warn members of your laboratory, and dispose of contaminated solutions appropriately.
- The mutagenic potency can vary from batch to batch, and has to be titrated by plaque assay. Prepare a stock solution (100 mg/ml in DMSO)

and store multiple aliquots at -20° C. Perform triplicate plaque assays using 0, 25, 50, and 75 µl of mutagen. Optimal mutagenesis results in 70 percent parasite killing compared to untreated controls (the protocol below assumes 50 µl as the optimal dose).

- Infect two confluent T25 HFF cultures with 1.2 ml of a freshly lysed culture 24 hours prior to the experiment.
- Replace medium with 10 ml DMEM 0.1% FBS medium.
- Incubate at 37°C for 30 minutes.
- Add 50 µl ENU to flask A and 50 µl sterile tissueculture-grade DMSO to flask B.
- Treat for 4 hours at 37°C.
- Wash cultures three times with 10 ml cold sterile PBS, and discard into a dedicated waste container.
- Add 10 ml PBS, scrape cells with a cell scraper, liberate parasite by two passages through a 25-ga needle (see safety section), and filter through a 3-µm polycarbonate filter.
- Transfer to a 50-ml tube, add 40 ml PBS, and spin at $1500 \times g$ at 4°C for 20 minutes.
- Resuspend in 5 ml PBS and count the parasites. Proceed to cloning by limiting dilution. It is advisable to control the mutagenesis efficiency of each experiment by plaque assay.

15.5.10 Complementation cloning using the ToxoSuperCos library

- Prepare 50 large and 10 small LB-agar Petri dishes (10 µg/ml kanamycin)
- To titer the ToxoSuperCos library, prepare five 1.5-ml Eppendorf tubes with 135µl LB (no antibiotics), one with 1ml LB, and one empty tube.
- Remove library from the -80°C freezer and keep on ice (work quickly to avoid thawing and immediately refreeze library).
- Scrape a small amount of library (~20 $\mu l)$ into the empty tube.
- Add 1 µl of thawed scraped bacteria to 1 ml LB (1:10³ dilution).
- Keep the remainder of the thawed library at 4°C (stable for 1–2 days).

- Prepare a dilution series (10^4-10^8) , plate $100 \mu l$ of each dilution on pre-warmed small LB-Kan plates, grow overnight at $37^{\circ}C$, and count colonies to calculate the number of colony-forming units (cfu) per ml.
- To amplify the library DNA, pre-warm large LB-Kan plates at 37°C. Prepare 10 ml of LB containing 50 000 cfu/ml, and plate 200 μ l per plate.
- Grow overnight at 37°C (incubate longer if colonies are too small).
- To harvest, add 2 ml of LB to the plate and scrape colonies using a cell scrape, transfer into a 250-ml centrifugation bottle (on ice), and wash with 1 ml of LB. Repeat for each plate and pool.
- Pellet bacteria in a table-top centrifuge, remove liquid, and weigh the pellets (bacteria can be stored at -20° C at this step).
- Purify cosmids using a commercial kit (e.g. Qiagen large construct kit), according to the manufacturer's instructrons; resuspend DNA pellet in 150 µl TE per column, and store cosmid DNA at 4°C in the dark.
- To complement *T. gondii* mutants, perform five independent transfections as described above (8 × 10⁷ parasites and 25 μg cosmid DNA per cuvette). Include at least one mock transfection to control for reversion.
- Transfer independently into T175 HFF cultures, incubate overnight at permissive conditions, then apply selective pressure. The ToxoSuperCos backbone contains a DHFR-TS cassette. In addition to the selective pressure, you can also apply pyrimethamine selection. This can be helpful to ensure integration of the entire cosmid, facilitating rescue of a tag later on.
- For ts mutants, plaques can be identified 10–14 days after transfection.
- Clone by limiting dilution, prepare genomic DNA, and rescue a sequence tag exploiting the Kan marker on the ToxoSuperCos backbone, as described for insertional mutagenesis (use BgIII, HindIII and XhoI).
- BLAST rescued sequences against ToxoDB. You should obtain hits to the same genomic region from independent complementations.

Check if your candidate region is represented among the sequenced and arrayed cosmids displayed on ToxoDB, acquire these cosmids, and test for complementation.

15.5.11 Safety concerns working with *T. gondii*

Several aspects of the parasite's biology make work with *T. gondii* relatively safe. In immunocompetent persons, the infection produces usually no or only modest symptoms. Depending on the region of the world, 20–70 percent of the population is already infected and resistant to reinfection. Lastly, the tachyzoite stage, which is most widely used in experimental work, is not highly infective by aerosol or ingestion. However, *T. gondii* is a human pathogen with the ability to cause severe disease, and should be handled with appropriate care (serious lab accidents have occurred in the past).

The following section summarizes a few ground rules, although it does not represent a comprehensive laboratory safety manual.

- Laboratory workers who belong to a specific risk group (active or potential severe immunosuppression, pregnancy) should not work with live parasites.
- Safety procedures should frequently be reviewed with all members of the laboratory.
- Parasites must be handled in designated biosafety cabinets. Label accordingly all the work areas, flasks, tubes, and waste containers that might harbor infectious material.
- Wear a lab coat, gloves, and goggles. Goggles are especially important for workers who do not wear glasses; an eyesplash could potentially deliver a high inoculum of parasites.
- The main route of infection with tachyzoites is direct inoculation by injury or through eyesplash. Be extremely careful in all situations that involve sharps. Note that coverslips, microscope slides, and plastic or glass tubes can break and produce sharp edges. Should you break something, sterilize using 70% ethanol before attempting cleanup. Needle sticks are the most

common source of laboratory infections. The safest approach to minimize such situations is to avoid them. Consider whether the use of sharps is really essential to the experiment. If you really have to needle-pass infected cells to liberate parasites, leave the plastic sheath on the needle and cut off its tip, using sturdy scissors, several millimeters before the tip of the actual needle. This can help to protect you from accidental sticks, and provides extra safety at no additional cost or effort.

- Be especially careful working with strains that encode resistance to drugs commonly used for treatment of humans, including pyrimethamine, sulfadiazine, clindamycin, and azithromycin.
- Sterilize all materials that have been in contact with live parasites (autoclave all plastic tissue-culture material, bleach all liquids accumulating in, for example, vacuum bottles, and frequently sterilize surfaces by spraying and wiping down with 70% ethanol).
- Have a plan for a potential accident. While the goal is to prevent accidents, they might happen nonetheless. Establish local as well as national contacts to infectious disease specialists who could provide advice for diagnosis and treatment. (Reference laboratories include the Palo Alto Research Foundation (http://www. pamf.org/serology) and the Laboratory of Parasitology and FAO/WHO International Centre for Research and Reference on Toxoplasmosis, Statens Seruminstitut, 2300 Copenhagen S, Denmark).
- Ensure good communication about lab safety, and always disclose any contamination, accident or inoculation. Inform the head of your laboratory about any accident, even if it appears to be a minor incident.

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16

Gene Regulation

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16.1 Introduction

- 16.2 The transcriptome of *Toxoplasma*
- 16.3 Transcriptional control in Toxoplasma
- 16.4 Chromatin remodeling in Toxoplasma

16.5 Evidence of post-transcriptional mechanisms in *Toxoplasma*16.6 Conclusions Acknowledgements References

16.1 INTRODUCTION

Toxoplasma gondii is distinct from nearly all other members of the large coccidian family (phylum Apicomplexa) owing to the exceptional range of animals (virtually all warm-blooded animals) that serve as host for its intermediate life cycle. Like other coccidians, Toxoplasma completes the definitive life cycle in a single animal host (Dubey et al., 1970); however, the ability of oocysts (shed from the feline host) as well as tissue cysts produced in intermediate hosts to infect either host type (Dubey and Beattie, 1988) has enabled Toxoplasma to increase its host range (Su et al., 2003). Sexual stages in the feline host lead to the development of oocysts that are shed into the environment (Long, 1982), where contamination of soil or water has led to epidemics of human

toxoplasmosis (Stray-Pedersen and Lorentzen-Styr, 1980; Konishi and Takahashi, 1987; Bowie *et al.*, 1997; Choi *et al.*, 1997; Isaac-Renton *et al.*, 1998). Moreover, oocysts are the primary source of *Toxoplasma* infections of livestock destined for slaughter and human consumption (Andrews *et al.*, 1997; Mateus-Pinilla *et al.*, 1999).

Given the importance of *Toxoplasma* infections to human populations, understanding developmental mechanisms initiated by sporozoites or bradyzoites leading to tissue cyst formation will be central to ultimately controlling transmission and chronic disease. Studies of *Toxoplasma* primary infections in animals and of sporozoite- and bradyzoite-infected cultures *in vitro* (Dubey and Frenkel, 1976; Dubey, 1998; Jerome *et al.*, 1998; Radke *et al.*, 2003) indicate that development initiated by either the sporozoite or bradyzoite stage is similar, and likely the consequence of a unified genetic program (Radke et al., 2003). Thus, defining the changes in gene expression that accompany this development pathway will be important to understand the underlying mechanisms responsible for toxoplasmosis caused by either route of infection. The following four sections review the current understanding of the Toxoplasma transcriptome (section 16.2) as it changes during the parasite intermediate life cycle. These studies demonstrate that mRNA pools are dynamic, and indicate that transcriptional control is a primary means to regulate developmental transitions in this parasite. It is in this context that we also discuss the evidence that Toxoplasma possesses a similar repertoire of mechanisms to regulate transcription as observed in other well-studied eukarvotes, from yeast to multicellular animals (sections 16.3 and 16.4). Section 16.5 briefly considers observations that indicate that posttranscriptional regulation may also be active in this parasite.

16.2 THE TRANSCRIPTOME OF TOXOPLASMA

16.2.1 Current genome resources

Early efforts to accelerate gene discovery in Toxoplasma led to the sequence for >120 000 ESTs from RH- and ME49-strain tachyzoites as well as ME49-strain bradyzoites and VEG strain oocysts (Ajioka et al., 1998; Manger et al., 1998; Li et al., 2003; L. Li et al., 2004). The ApiDots site (http://www.cbil.upenn.edu/apidots/) provides access to these data and those from other apicomplexan parasites of clinical and veterinary interest, including Eimeria tenella, Neospora caninum, Plasmodium falciparum, and Sarcocystis neurona. EST sequences have been assembled to generate consensus sequences, and then annotated by similarity to known nucleic acid and protein sequences (Li et al., 2003). This dataset has facilitated the identification and characterization of individual genes and helped to define intermediate stages of the parasite life cycle (L. Li et al., 2004). At the time of writing, a Toxoplasma SAGE project and a 10X-whole genome project for the type II-Me49B7 strain were completed. BAC clone end sequencing (http://www.sanger.ac.uk/Projects/ T_gondii) and 10X shotgun genomic sequencing projects (http://www.tigr.org/tdb/e2k1/tga1/) have converged, and the ToxoDB (http://ToxoDB.org) repository containing these data provides a genome resource for Toxoplasma (Kissinger et al., 2003). This database offers a growing array of data-mining tools for the analysis of the draft sequence. Further genome sequence to provide 5X coverage of a type I and a type III strain is currently underway, and whole genome microarrays based on the type II-Me49B7 reference strains are also in development. Features of the genome project are described and in summary form in the current chapter. In addition, we have summarized the major findings of the SAGE project (Radke et al., 2005).

16.2.2 Microarrays and the parasite transcriptome

Due to intense scientific interest, and as a beneficiary of an early genome project (http://plasmodb.org/ PlasmoDB.shtml), Plasmodium whole-genome microarrays were the first to provide a global view of mRNA expression in this parasite family (Le Roch et al., 2004; Bozdech and Ginsburg, 2005). From detailed analyses of mRNA expression during Plasmodium development, some key concepts about gene expression have emerged (reviewed in Llinas and DeRisi, 2004). More than 80 percent of the transcripts monitored in microarray experiments were regulated, with most having a peak expression within a single timeframe in the sexual stages or intraerythrocytic cycle. Stagespecific mRNA expression accounts for a large fraction of *Plasmodium* transcripts, and there was little shared expression between stages: nearly 200 genes were expressed in the gametocyte, 41 were sporozoite-specific, and 20 percent of all transcripts were specific for the intraerythrocytic cycle (merozoites). Proteomic studies appear to confirm these results, as over half of the 948 proteins detected in a recent proteomic analysis were uniquely expressed by a single developmental

stage (Hall et al., 2005). Plasmodium genes that display patterns of mRNA co-regulation (based on kinetics and mRNA level) encompass large groups of proteins (tens to hundreds) that are involved in development, parasite motility, and invasion, as well as metabolic pathways (Le Roch et al., 2004; Bozdech and Ginsburg, 2005). The proper timing of mRNA accumulation applies not only to genes associated with parasite cell cycles that might be expected to have similar kinetics (Bozdech and Ginsburg, 2005), but also to genes encoding protein components of subcellular structures, as the narrow window when these structures form during the division cycle may also require coordinated gene expression (Triglia et al., 2000). The large changes in transcript levels in Plasmodium suggest that mRNA expression is governed by 'just-in time' mechanisms, and the relatively low proportion of constitutive mRNAs in these parasites may reflect this concept (Llinas and DeRisi, 2004). Taken together, these results demonstrate that transcription is a major mechanism controlling gene expression in these parasites, and this view is supported by a recent comparison of the changes in the Plasmodium transcriptome and proteome that indicates alterations in mRNA levels have a higher correlation to protein changes in this parasite than is observed in yeast or higher eukaryotes (Le Roch et al., 2004).

The EST projects for apicomplexan protozoa were undertaken with the expressed goal of gene discovery, although crude estimates of the changes in mRNA expression have also enabled developmentally regulated genes to be identified, and this information has since been used to construct a limited Toxoplasma cDNA microarray (Cleary et al., 2002; Matrajt et al., 2002; Singh et al., 2002). These first arrays focused on tachyzoite-bradyzoite transitions in cell-culture models of bradyzoite differentiation (Cleary et al., 2002), and explored gene expression in mutants that were unable to differentiate (Matrajt et al., 2002; Singh et al., 2002). Although variation in hybridization replicates diminishes some interpretations from these experiments (Ellis et al., 2004), these studies confirm that changes in mRNA levels correlate with the expression of known bradyzoite

protein antigens and support a role for transcriptional mechanisms in determining developmental stage characteristics in Toxoplasma. They also provide evidence for co-regulation of transcription in this parasite (Singh et al., 2002). In the transcripts of four mutants generated by chemical mutagenesis, and selected against the ability to differentiate, a common set of mRNAs was affected (unable to be induced), while other affected mRNA groups appeared to cluster with two or three mutants, suggesting that hierarchical gene expression may direct bradyzoite development (Singh et al., 2002). The significance of these results will await genome-based arrays; however, the SAGE project described below provides additional support for co-regulated transcription and also demonstrates that some of the general concepts emerging from the Plasmodium studies apply to gene expression in Toxoplasma.

16.2.3 Serial analysis gene expression (SAGE) in *Toxoplasma*

In order to define parasite gene expression as it relates to Toxoplasma development in the intermediate host, SAGE libraries (Velculescu et al., 1995) were constructed from VEG strain populations spontaneously arising from sporozoite infections of human fibroblasts (Jerome et al., 1998), and from laboratory-adapted strains representing the major genotypic variants of Toxoplasma (type I-RH, type II-Me49B7, and type III-VEGmsj). SAGE libraries were constructed using mRNA sources derived from key transitions in the developmental program from sporozoite to bradyzoite, as described in earlier studies (Jerome et al., 1998). A total of 290 000 tags were generated by this project, and individuals may access the full dataset at TgSAGEDB (http://vmbmod10.msu.montana.edu /vmb/white-lab/newsage.htm) or view SAGE data in ToxoDB-GBrowse (http://toxodb.org/gbrowse/).

16.2.3.1 SAGE global statistics and the chromosome landscape

The ~300 000 tags generated by the SAGE project represent 8880 unique 14 mer sequences that

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match Toxoplasma genome sequence with limited redundancy, suggesting that nearly 9000 unique mRNA transcripts are expressed in Toxoplasma during the intermediate life cycle. An estimated 6300 genes (based on CAP3 assembly of the 2-kbp genomic sequences 5'-upstream of each tag) are represented by these tags, with the redundancy explained by differential polyA+ choice and potential splice variants. In addition, the SAGE project has identified mRNAs containing single nucleotide polymorphisms, as well as non-coding RNAs representing potential antisense transcripts. The average content of SAGE tags by chromosome was remarkably consistent with a SAGE tag for every 6000 bp across all chromosomes. Thus, roughly half the Toxoplasma genome is occupied by gene-transcription units with the relative spacing of genes ~2000 bp (using an average ~4000 bp per gene derived from whole genome annotation; Mackey and Roos, unpublished observations). Restrictions of distance are a common feature of promoters in unicellular eukaryotes with compact genomes (Stamatoyannopoulos, 2004), and the handful of Toxoplasma promoters studied are consistent with this structural organization - for example, all ciselements mapped to date fall within a few hundred nucleotides of the protein open reading frame (ORF) (see section 16.3 for a detailed discussion) (Soldati and Boothroyd, 1995; Mercier et al., 1996; Bohne et al., 1997; Roos et al., 1997; Nakaar et al., 1998; Ma et al., 2004; Matrajt et al., 2004; Kibe et al., 2005).

Nearly 49 percent of SAGE tags from VEG developmental stages show a >2.5-fold change in frequency in at least one library (66 percent >2 fold), indicating that transcriptional regulation is extensive in this parasite. The mRNA abundance classes as defined by three SAGE tag frequencies (Velculescu et al., 1995) - high >100, moderate 11-99, and low 2-10 - are partitioned into 70 percent low abundance tags, 25 percent moderate tags, and 5 percent high abundance tags. In comparison to yeast (Velculescu et al., 1997), highand moderate-abundance pools are smaller in this parasite (half the size of yeast), indicating there is less constitutive gene expression in Toxoplasma (Figure 16.1). Unlike the high expression of metabolic or structural genes in animal cells, nearly one third of the Toxoplasma-abundant mRNA group are Apicomplexa-specific genes that have simple genomic structures containing few, if any introns (based on frequency, one in five SAGE tags corresponds to highly regulated Apicomplexa-specific transcripts from dense granules, SAGs, MICs, ROPs; Figure 16.1). Therefore, many transcripts encoding proteins of the basal metabolic machinery and subcellular structures appear to be transcribed in Toxoplasma only when needed during parasite growth and development. This is consistent with the 'just-in time' concept put forth from studies of Plasmodium (Llinas and DeRisi, 2004). Overall, development-specific genes (sporozoite, tachyzoite, bradyzoite), genes encoding proteins from biochemical pathways, and genes representing mRNA abundance classes are dispersed between all Toxoplasma chromosomes. Gene clusters rarely



FIGURE 16.1 The high abundance mRNA class in Toxoplasma is enriched for Apicomplexa-specific genes. Normalized SAGE tags were parsed into three classes based on frequency. Tags found at >100 were considered highly abundant, while tags with frequencies between 99 and 11 or <10 were moderately or low abundant, respectively. Based on these frequency classes, ~270 unique transcripts encompass 66 percent of all tags in the total SAGE project. One in five SAGE tags encodes an Apicomplexa-specific gene involved in parasite transmission and invasion - e.g. proteins in micronemes, rhoptries, dense granules, and surface antigens (56 582 were from Api-tags with > 100 average frequency, 1999 were from 99-11 frequency, and 426 tags were from <10 frequency). The number of unique moderate tags totaled 1541, and 6286 unique tags had a low abundance frequency.

occur, and in those cases mRNA expression patterns do not appear to be strongly influenced by physical proximity. For example, genes encoding enolase 1 and 2 are less than 1500 bp apart on chromosome VIII, yet are expressed exclusively in bradyzoite or tachyzoite stages, respectively (Lyons et al., 2002). These observations indicate that local changes in chromatin structure or the recruitment of RNA polymerase to promoters has little influence on nearby genes. Altogether, the emerging view of gene organization and global mRNA expression provided by the SAGE project indicates that transcriptional mechanisms play a key role in regulating the developmental program of Toxoplasma; and the general features of parasite mRNA transcription (e.g. less constitutive) make it more likely that important changes in gene expression can be identified through functional genomic strategies.

16.2.3.2 SAGE analysis of primary VEG strain parasites from sporozoite to tachyzoite to bradyzoite in comparison with laboratory strains

Global comparisons of SAGE tags from VEG primary libraries (sporozoite, Day 4, 6, 7, 15 postsporozoite infection and pH-shift mRNA sources used to construct primary SAGE libraries; Jerome et al., 1998) demonstrate unique changes in mRNA levels defining each of the developmental stages (Pearson correlations r = 0.0229-0.32), with the exception of sporozoite and parasites emerging from sporozoite infections at Day 4 (r = 0.728). SAGE tags uniquely associated with each developmental library encompass 23 percent of the total tags sequenced, and ranged from 1.5-5.3 percent of the tags in each library. Globally, our results parallel similar studies in Plasmodium that indicate mechanisms coordinating developmental mRNA expression in these parasites are largely stage-specific. Furthermore, the discovery of large groups of stage-specific genes in Toxoplasma adds support to the concept that developmental gene expression in this parasite likely follows a hierarchical order (Singh et al., 2002). Thus, our SAGE studies (and earlier microarray experiments; Singh

et al., 2002) provide evidence that mRNA expression in *Toxoplasma* falls into distinct co-regulated classes and that groups of mRNAs, which are likely regulated by development-specific trans-acting factors, control phenotypic transitions associated with the *Toxoplasma* intermediate life cycle.

Analysis of gene expression across parasite development has revealed expected patterns of mRNA expression as well as surprising mRNA profiles that may provide new biological insights into the genetic program expressed by Toxoplasma, and help to explain variations in developmental capacity that occur between strains. We have reported that parasites emerging from primary sporozoite inoculations (VEG strain) undergo ~20 rapid divisions before synchronously slowing their growth rate and initiating bradyzoite gene expression approximately 1 week post-sporozoite infection (Jerome et al., 1998). Thus, it was not unexpected that we observed a downregulation in the frequency of SAGE tags corresponding to growth-related genes (e.g. TgPCNA1, DHFR-TS, and adenosine transporter) and some energy-related genes (e.g. fructose-1,6-phosphate, gyceraldehyde-3-phosphate, 3-phosphglycerate) in the Day-7 library (and all subsequent post-growth shift libraries) congruent with the dramatically slower doubling time of these parasites. Further agreement with earlier measurements of BAG1 in these populations (2 percent BAG1 at Day 7 post-sporozoite; Jerome et al., 1998) was reflected in the absence of the known bradyzoite genes in this library. Parasites from Day-15 post-sporozoite infection are a mixture of tachyzoite and bradyzoite forms (50 percent BAG1; Jerome et al., 1998), and thus SAGE tags corresponding to bradyzoite markers SAG4.2 and ENO1 were evident in this library along with expression of tachyzoitespecific genes like SAG1. Exposing VEG primary parasites to pH stress shifts the mRNA pool further towards the bradyzoite stage (Radke et al., 2003), as all of the published bradyzoite markers were detected (Lyons et al., 2002), and had higher frequencies, in the pH-shifted SAGE library, than were observed in the Day-15 library. Equally important, and predicted, was the near complete downregulation of tachyzoite-specific genes

(e.g. SAG1, LDH1, ENO1) in the pH-shifted populations. In each of the developmental transitions sampled in this study there was an opportunity to discover novel parasite gene expression, and this remains an area of current investigation. For example, a novel NTPase that is related by sequence to NTPI and II (Bermudes et al., 1994) was identified whose expression was detected in the Day-15 parasites (but not at all in Day-7 parasites) and was among the highest expressed genes in the pH-shifted library (see section 16.3 below; Radke and White, unpublished results). Comprehensive lists of genes that summarize development-specific expression at each transition can be found at TgSAGEDB (http://vmbmod10.msu.montana.edu/ vmb/white-lab/newsage.htm).

Previous studies of sporozoite infections have demonstrated that differentiation was an active and rapid process both in vitro and in vivo (Jerome et al., 1998). Although relatively few markers were monitored in these experiments, the results led to speculation that differentiation from the sporozoite to the tachyzoite stage was essentially complete in the first 1-2 days following a sporozoite infection. Whole-cell measurements by SAGE tells a different story, as mRNA pools of parasites emerging even 4 days post-sporozoite infection retain a significant fraction of sporozoite-specific mRNAs such that the correlation between these pools is high (sporozoite vs. Day 4, r = 0.728). This observation was unexpected and suggests that, in trying to understand Toxoplasma infections initiated by oocysts, sporozoite-specific biology may have significant influence during the early stages of development in the intermediate host.

Global comparisons of SAGE datasets from types I, II, and III laboratory strains within the context of VEG development provided other unexpected results that illuminate important biological differences between strains. There was a striking correlation between upregulated SAGE tags derived from VEG Day-6 post-sporozoite populations and those in the type I-RH strain that were not observed in comparison to the other primary VEG populations or strain types. This unique relationship in gene expression may reflect a shared biology: Day-6 VEG populations, like RH parasites, lack any evidence of sporozoite or bradyzoite mRNA expression, and grow with a similarly fast doubling time (Jerome et al., 1998; Radke et al., 2001). Importantly, SAGE libraries constructed from type II-Me49B7 and type III-VEGmsj parasites did not have elevated Day-6 SAGE tags, but unlike RH/Day-6 datasets, SAGE tags corresponding to bradyzoite genes were found in the libraries from these strains. This difference likely relates to the greater capacity of VEGmsj and Me49B7 parasites to enter the bradyzoite development pathway where RH is developmentally incompetent (Dubey et al., 1999). It is unknown whether this differential pattern of gene expression also applies to other virulent type I strains whose inefficiency at forming tissue cysts is also well established (Sibley and Boothroyd, 1992; Dubey, 1997, 1980), but the absence of bradyzoite gene priming in virulent strains that is evident in the microarray analysis of avirulent strains has recently been reported (Saeij et al., 2005). Thus, the mRNA patterns detected in laboratory strains appear to mirror characteristics from the natural development pathway with their comparative position with respect to the growth-shifted populations that occur at Day 7 post-sporozoite infection (Jerome et al., 1998). Strains whose gene expression places them to the left (and earlier) of this developmental boundary, like RH, are further removed from bradyzoite differentiation, and may be more virulent. By contrast, gene expression patterns consistent with populations to the right (i.e. evidence of basal bradyzoite gene expression) indicate a greater capacity for bradyzoite development, which is associated with avirulence (Saeij et al., 2005). Sorting out the gene expression mechanisms that control these developmental transitions and understanding how alterations in these controls result in the variation in strain developmental capacity is an important area for future studies.

16.3 TRANSCRIPTIONAL CONTROL IN TOXOPLASMA

16.3.1 RNA synthesis in *Toxoplasma*

The basal transcriptional complex that controls the expression of protein-encoding genes (class II) in most eukaryotes is RNA polymerase II and its associated general transcription factors (GTFs). Comparisons of these transcription factors, as well as the similarities in the three nuclear polymerases (Ranish and Hahn, 1996), have shown that these mechanisms are largely conserved throughout evolution, from the Archea to mammals. In wellstudied unicellular and multicellular eukaryotes, transcription involves a series of co-regulatory complexes that work in concert to control the synthesis of RNA from a particular genomic region. Activating transcription factors (ATFs) bind to cis-acting promoter element(s) and recruit chromatin remodeling enzymes which relax the chromatin around the *cis*-element-containing region, as well as recruiting the multi-subunit Mediator complex that contacts the RNA polymerase II pre-initiation complex (PIC) directly (Blazek et al., 2005). The accessibility of the cis-element to ATF binding is dependent upon the interaction with these remodeling enzymes, but can also be influenced by other factors, such as the chromatin state at the regulatory sequence and the phase of the cell cycle (Fry and Peterson, 2002). In turn, the relaxed chromatin state allows for the formation of the PIC at the core promoter elements through activities contained within the Mediator that facilitate recruitment of RNA polymerase II and the GTFs. It is notable that current models change the view of ATFs to suggest that activation of RNA polymerase II by these factors occurs indirectly through their recruitment of ATP-dependent chromatin remodeling complexes (Featherstone, 2002; Y.J. Li et al., 2004; Blazek et al., 2005). The cooperative model described here is a general one, although variations to this basic model are now well recognized in the studies of individual genes (for an overview, see Fry and Peterson, 2002).

Within some protozoa there are examples of differences in the regulation of protein encoding genes. For example, while RNA polymerase II-mediated transcription of class II genes in *Trypanosomes* has been documented, the transcription of VSG and the procyclic acidic repetitive protein (PARP) genes have been shown to be dependent on RNA polymerase I (Belli, 2000). This is unusual for a eukaryote, as is the polycistronic

nature of Trypanosome transcripts and the posttranscriptional regulation of gene expression (Belli, 2000). The analysis of protein encoding genes in the Apicomplexa indicates that conventional RNA polymerases with similarity to other crown eukaryotes are present (recently reviewed in Meissner and Soldati, 2005). Homologs for all known required eukaryotic RNA polymerases have been found in the Toxoplasma genome: RNA polymerase I (transcribes ribosomal RNA), RNA polymerase II (transcribes protein-encoding transcripts), and RNA polymerase III (transcribes small RNA) (Li et al., 1989, 1991; Fox et al., 1993; Meissner and Soldati, 2005). Moreover, this classification is supported by studies in Plasmodium showing that synthesis of 18S rRNA is α-amanitin insensitive, while both sense and antisense transcription of class II genes are inhibited by this polymerase IIspecific toxin (Militello et al., 2005). These results indicate that Plasmodium and Toxoplasma possess the conserved eukaryotic machinery whereby RNA polymerase II transcribes proteinencoding genes.

The core elements of class II eukaryotic promoters include TATA box, Initiator (Inr), and downstream promoter elements (DPE) that are recognized and bound by several GTFs: TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Ranish and Hahn, 1996; Featherstone, 2002; Blazek et al., 2005; Ruvalcaba-Salazar et al., 2005). The core of the GTF family includes the TATA binding protein (TBP), TFIID and RNA polymerase II. Studies of the Plasmodium TBP protein has shown that this portion of the TFIID complex indeed binds to a specific TATA box element in promoter regions (Ruvalcaba-Salazar et al., 2005), although the inability to find other core elements has led to speculation that the conventional bipartite mechanism of cis- and basal transcription is either absent or more primitive in these parasites (Coulson et al., 2004; Callebaut et al., 2005; Meissner and Soldati, 2005). More recently, a combination of two-dimensional hydrophobic cluster analysis with profile-based search methods using PSI-BLAST (Callebaut et al., 2005) has extended earlier investigations (Coulson et al., 2004; Meissner and Soldati, 2005), such that ~60 percent of the known eukaryotic GTFs in the

Plasmodium genome, including important components of the TFIID complex, have now been identified. Similar 'in silico' surveys for components of the eukaryotic transcriptional machinery demonstrate the presence of these factors in the Toxoplasma genome. Homologs for various subunits for the GTFs (TFIID, TFIIE, TFIIF, and TFIIH) and a subunit of Mediator (mdt6) (Meissner and Soldati, 2005; Behnke and White, unpublished) have been found, and comparison with the newly found Plasmodium GTFs (Callebaut et al., 2005) reveals that genes with significant similarities also exist in Toxoplasma: TFIID-Taf1 to TGG 993790; TFIID-Taf2 TGG 993790; TFIIE-α-subunit TGG_994351-TgTwinScan_5763; TFIIE-β-subunit TGG_994289-TgSwinScan_3074; TFIIF-β-subunit TGG_994699-TgTwinScan_3381; TFIIH-TFB5 TGG 994580. It is also notable that components of the CCR4-Not complex have been identified in Toxoplasma SAGE expression data, including homologous sequences for CCR4 subunit 7, Not1, and Caf-1 (Behnke and White, unpublished). This complex has been conserved from yeast to human, and serves as a regulatory component for responses to nutrient-level changes and stress through interaction with the PIC complex (Collart, 2003). Altogether, these investigations demonstrate that while GTFs are less conserved in the Apicomplexa, much of the basal transcriptional machinery and chromatin remodeling factors (see section 16.4) required for cooperative control of gene transcription in eukaryotes are present in these pathogens.

16.3.2 Autonomous control of gene expression in *Toxoplasma*

Functional features of gene expression in Apicomplexa protozoa can be gleaned from the variety of efforts of many labs to develop expression vectors and transfection models in *Plasmodium* and *Toxoplasma* (Goonewardene *et al.*, 1993; Soldati and Boothroyd, 1993; Roos *et al.*, 1994; Wu *et al.*, 1995; Crabb and Cowman, 1996; Dechering *et al.*, 1997). These experiments confirmed the basic principle of monocistrony previously established by run-on transcription

(Lanzer et al., 1992) and, through trial and error, demonstrated that genomic regions flanking parasite ORFs act autonomously to confer a characteristic strength of expression and, in some situations, developmental specificity on endogenous and foreign protein coding regions (see, for example, Soldati and Boothroyd, 1993; Bohne and Roos, 1997). The inaptness of mammalian promoter elements to drive gene expression in Plasmodium, which was equally unsuccessful when the experiment was reversed, was also a principle established by these efforts that gave an early hint to the uniqueness of Apicomplexa transcription mechanisms (Horrocks et al., 1998). This idea has since been borne out by the challenges to identify conserved transcription factors in Apicomplexa genome sequence, as discussed above (Coulson et al., 2004; Callebaut et al., 2005; Meissner and Soldati, 2005). Initial attempts to express proteins in Toxoplasma demonstrated the presence of regulatory elements in genomic sequence upstream of the exons encoding SAG1, ROP1, and TUB1 (Soldati and Boothroyd, 1993). The promoter fragments in these studies encompassed several hundred to a few thousand nucleotides of 5'-flanking genomic sequence, and displayed differential strength (TUB1 > ROP1 > SAG1). Promoter autonomy has since been exploited to construct expression vectors containing genomic sequences able to induce highlevel mRNA expression so as to elevate encoded protein levels in Toxoplasma and in the related parasite Neospora caninum (e.g. Figure 16.2, GRA1 promoter; Howe et al., 1997). Identical strategies have been employed to achieve developmental expression of reporter genes (Bohne et al., 1997). The specific location of regulatory elements within the promoter sequences used in these vectors is not often known, nor are sites of transcriptional initiation typically mapped, leaving open the possibility that expression levels inherent to these promoter fragments are in part influenced by post-transcriptional mechanisms contributed by 5'-UTR sequences (Seeber and Boothroyd, 1996). The influences of the 3'-UTR regions used in these constructs are also not fully vetted; however, there is some experimental evidence that 3'-UTR sequences do not play a significant role in controlling developmental expression outcomes (Bohne and Roos, 1997; Kibe *et al.*, 2005; White, unpublished).

We have recently developed a vector system utilizing recombinational cloning (Hartley *et al.*, 2000) that is based on the principle of *Toxoplasma* promoter autonomy. The basic features of this model consist of a variable-length promoter fragment that is produced by PCR from genomic sequence with small recombination sites (att sites) incorporated at each end to allow for modular, recombinational cloning. The fragment is then cloned 5'- to a single exon encoding firefly or renilla luciferase (dual luciferase model is well described in Promega manual 058) by recombination (the 3'-attB2 site is translated). The advantages of this model lie in its improved flexibility and potential to rapidly clone multiple promoter regions. Using the dual luciferase model, we have reaffirmed the influence of intergenic regions in regulating gene expression in Toxoplasma during bradyzoite differentiation. Fourteen 5'-intergenic flanking regions from previously known bradyzoiteand tachyzoite-specific genes have been examined to date. The promoter regions comprised 1-1.5 kbp of 5'-flanking region fused to the firefly luciferase via recombination cloning, and expression has been evaluated in a transient transfection format employing atubulin-renilla luciferase (atub-RENLUC) co-transfection controls to normalize differences in electroporation efficiency. Native a-tubulin was chosen because mRNA levels are relatively unchanged during tachyzoite differentiation into the bradyzoite (Dzierszinski et al., 2001). Figure 16.2 shows the stress-induction of five



FIGURE 16.2 Dual luciferase assay for several *Toxoplasma* promoters. Promoter fragments were cloned into the pDEST-FLYLUC-dhfr-3' vector and transfected in combination with α tub-RENLUC (40/20 µg) into VEGmsj parasites by electroporation. Parasites were plated into replicate T-25 cm² flasks (2 million/flask), incubated overnight, and then induced under stress conditions. Infected monolayers were scraped at 48 h, cells pelleted, and lysates prepared directly without parasite purification. Sequential measurement (four replicates/construct) of the firefly and renilla luciferase in each extract was determined using published protocols (manual 058, Promega Co.). Induced values ranged from 5000 to 100 000 light units. Fold increase over α tub-RENLUC controls: black bars = tachyzoite media conditions; gray bars = 48-h stress-induction.

bradyzoite promoter constructs: SAG4.2 (p18), LDH2, BAG1, 65-kDa cyst wall protein (CW), and Brady-NTPase. Note that the BSR4 construct was constitutively expressed in these assays, consistent with the SAGE and EST frequencies obtained for this gene, and in agreement with constitutive reassignment of this gene (Laura Knoll, personal communication). A number of other promoters tested had low to high levels of expression in tachyzoites, including GRA1 (shown here), as well as constructs containing 5'-flanking regions for SAG1, LDH1, ENO2, DHFR, α-tubulin, ROP4, and fructose-1,6-bisphosphate aldolase (not shown). The GRA1 promoter construct was consistently expressed at higher levels than all other promoters, and 100-fold higher than the lowest expressed DHFR construct in tachyzoite transient assays (Behnke and White, unpublished). Thus, the tachyzoite and bradyzoite promoter regions examined using the dual luciferase model have, thus far, displayed the characteristic pattern of accumulated expression associated with the Toxoplasma native protein and/or mRNA (developmental specificity of many of these markers is reviewed in Lyons et al., 2002).

16.3.3 Gene-specific *cis*-elements

Classical promoter mapping strategies utilizing conventional protein reporters, including chloramphenicol acetyltransferase (CAT), β-galactosidase, green fluorescent protein or firefly/renilla luciferase, have now been employed to map regulatory sequences in several promoters in *Plasmodium* and Toxoplasma (reviewed below and in Horrocks et al., 1998; de Koning-Ward et al., 2000; Meissner and Soldati, 2005). Regulatory *cis*-elements that influence basal as well as regulated transcription in Plasmodium lie nearby or at a limited distance upstream of the transcriptional start (Crabb and Cowman, 1996; Dechering et al., 1999; Porter, 2001; Osta et al., 2002), although promoter ciselements have also been found within the intron sequences (Calderwood et al., 2003) of var genes and in the coding region immediately downstream of the start of translation in pgs28 (Chow and Wirth, 2003). In Toxoplasma, deletion studies to identify promoter cis-elements have been reported for various GRA genes, DHFR-TS, tachyzoitespecific SAG1 and enolase 2, and for a number of bradyzoite genes including hsp30/BAG1, hsp70, LDH2, and enolase 1. Together, these results confirm that promoter elements are primarily located upstream from the translational start site (Soldati and Boothroyd, 1995; Mercier et al., 1996; Bohne et al., 1997; Roos et al., 1997; Nakaar et al., 1998; Ma et al., 2004; Matrajt et al., 2004; Kibe et al., 2005). Promoter elements were observed to be active in either DNA strand, but may have a limited working distance from the transcriptional start or lose their influence when located downstream of the coding region (Soldati and Boothroyd, 1995). The level of detail within these studies varies, and minimal sequence elements were determined in only a few studies (below and in Mercier et al., 1996; Matrajt et al., 2004); moreover, no published study has fully resolved the question of functional sufficiency for any putative cis-element. Nonetheless, it is evident that a 27-bp repeat sequence (6X repeat) in the SAG1 promoter is required for function and a sequence element (A/TGAGACG) found in the GRA promoters was demonstrated to be required for basal expression within the context of a 53-bp minimal promoter (Mercier et al., 1996). It is interesting that GAGACG is present in the central core of the SAG1 27-bp repeats, and this element is also found in regions implicated to contain regulatory cis-elements by deletion analysis of the NTPI/II and DHFR-TS promoters (Nakaar et al., 1998; Matrajt et al., 2004). A GAGACG motif or closely related sequence has emerged from the application of pattern algorithms trained on Toxoplasma whole genome sequence flanking known and predicted proteins (Mullapudi and Kissinger, unpublished), suggesting that GAGACG might be the Toxoplasma equivalent to RNA polymerase II basal elements (TATA or CAAT box) of higher eukaryotic promoters that are absent from Apicomplexa promoters. This intriguing question needs to be experimentally tested, as this element is not universally present in genome sequence flanking predicted or known first exons or even other SAG-related promoter regions. Furthermore, the presence of et al., 2004). As noted in the above section, developmentspecific changes in mRNA levels are a dominant feature of the Apicomplexa transcriptome. Nearly one-fourth of the transcripts detected in the recent Toxoplasma SAGE project were observed to be uniquely expressed during parasite development; similar observations have emerged from functional genomics studies of the Plasmodium intra-erythrocytic cycle (Le Roch et al., 2004; Bozdech and Ginsburg, 2005). Developmental expression of a heterologous protein reporter in Toxoplasma parasites was first achieved using plasmid constructs containing promoter sequences from the hsp30/BAG1 gene. These studies employed alkaline stress to stimulate expression of the CAT reporter (Bohne et al., 1997), and an alkaline-responsive cis-element was mapped by sequential deletion analysis to a 40-bp region located immediately upstream of the major transcription start site in the BAG1 promoter (284-324 bp upstream of the translational start). A GC-palindromic sequence was noted to lie within this region, although its importance was not tested. Promoters controlling bradyzoitespecific genes, hsp70 and LDH2, have now been mapped using alkaline-stress induction at a similar resolution to BAG1 (Yang and Parmley, 1997; Ma et al., 2004); while these studies support the role of promoter elements in regulating stressresponse in Toxoplasma, their resolution is too low to allow for the identification of common ciselements. The reciprocal regulation of enolases 1 (bradyzoite-specific) and 2 (tachyzoite-specific) is of particular interest, given their close proximity in the genome (in an ordered tandem array of enolase-2-enolase-1). Repression of enolase-1 expression in tachzyoites appears to require a distal region > 600 bp from the enolase-1 ATG, and these elements are distinct from inductive elements which were mapped closer to the start of transcription (Kibe et al., 2005). Similarly, two regions in the enolase-2 promoter may play a role in repression of this gene under bradyzoite conditions, although this analysis is difficult to interpret

because downregulation of enolase-2 constructs was not fully achieved in these experiments. As with other studies, the lack of functional validation of putative enolase-1 elements by internal deletion or mutagenesis limits the ability to draw a consensus view of the critical sequences that regulate bradyzoite-specific transcription.

Employing the dual luciferase model described above, we have recently mapped bradyzoitespecific cis-elements within a newly identified Toxoplasma gene encoding a novel NTPase (Brady-NTPase; chromosome X, 42.m03416). A series of sequential and internal deletions followed by 6-bp substitution mutagenesis has identified a 15-bp cis-element that is responsible for induction of the Brady-NTPase promoter under a variety of drug and stress conditions that co-induce native bradyzoite gene expression. This element lies within the first 500 bp of the Brady-NTPase promoter, and 90 percent of the induction is lost when the element is mutated in the context of the full-length promoter fragment (1495 bp). Mutation of this element does not lead to increased expression in the tachyzoite stage, indicating that it is a true inductive element. Fine mapping of the Brady-NTPase element is now underway in order to define the precise nucleotide boundaries. By inspection of regions implicated in bradyzoite induction of the LDH2 and SAG4.2 promoters, we have found overlap with the Brady-NTPase element, and while these comparisons are intriguing, validation of the relationship between these promoters must await further functional analyses.

16.3.4 Gene-specific transcription factors

Functional studies of *Plasmodium* and *Toxoplasma* promoters support the view that Apicomplexa promoters possess a similar bipartite organization of basal and *cis*-elements, and suggest that transcriptional initiation in these parasites follows principles similar to other eukaryotes (Horrocks *et al.*, 1998). This view is supported by the presence of at least 60 percent of the GTFs associated with eukaryotic RNA polymerase II in

Apicomplexa genomes (Coulson *et al.*, 2004; Callebaut *et al.*, 2005), including important components of the TFIID complex that recruit RNA polymerase II to the site of transcriptional initiation.

Studies have demonstrated that cell typespecific components are found associated with the core transcriptional complex in multicellular organisms. Additions to the basal machinery may have evolved to regulate the increased complexity of gene expression needed for these cells to specialize (Hochheimer and Tjian, 2003). Comparably, less is known about the factors that act in trans to regulate gene-specific transcription in apicomplexan parasites. Nevertheless, it is clear that this deficiency will likely be short-lived, as there is now evidence that proteins containing nucleotide binding domains are present in Apicomplexa genomes and their activity has been detected in parasite extracts. We have documented SAGE tag evidence for the expression of a number of proteins containing zinc-finger domains in Toxoplasma, and these proteins can be found in the gene ontology classification of SAGE data on the TgSAGEDB website (Behnke and White, unpublished). Indications of specific DNA binding activity in Apicomplexa parasite extracts are drawn from reports of distinct nuclear factors that bind functional cis-elements that are conserved within var genes (Voss et al., 2003) and observations of protein factors in gamete nuclear extracts that have specific binding activity to a gametespecific cis-element from pfs25 (Dechering et al., 1999). In Toxoplasma, nuclear extracts obtained from parasites exposed to alkaline stress show increased DNA binding activity to the enolase-1 stress-responsive cis-elements (Kibe et al., 2005) and gel mobility shift experiments reveal DNA binding activity that is specific for stress-responsive elements found in the hsp70 promoter (Ma et al., 2004). Until recently, functional validation of a gene-specific DNA binding factor had not been reported in any Apicomplexa model. However, using a dsRNA knockdown approach, the expression of DNA-binding protein PfMyb1 was linked to a transcriptional network required for parasite growth, and chromatin immunoprecipitation detected PfMyb1 bound to promoter sequences of several members of this regulatory network (Gissot *et al.*, 2005).

16.4 CHROMATIN REMODELING IN TOXOPLASMA

Current models of eukaryotic transcriptional activation implicate a significantly greater number of cofactors than was appreciated more than a decade ago. The simple binding of gene-specific ATFs (activating transcription factors) to local sequences in nucleosomal DNA (Naar et al., 2001) is now recognized to be insufficient to recruit the RNA polymerase II-PIC. ATFs recruit chromatin remodelers to facilitate the assembly of the PIC on core promoter sequences (recently reviewed in Spector, 2003; Ehrenhofer-Murray, 2004; Y.J. Li et al., 2004). These findings bring chromatin dynamics to the forefront of gene expression research, and the discovery that histone proteins can be chemically modified in ways that enhance or inactivate transcription, along with ATPases capable of repositioning nucleosomes, has prompted intensive investigation into how these mechanisms act cooperatively to regulate gene expression. Although the order and assembly of transcriptional factors has not been demonstrated in any apicomplexan parasite, including Toxoplasma, the initial forays into understanding transcriptional regulation reveal that these parasites possess essential features of the basal transcription machinery (discussed above) as well as a significant collection of chromatin remodeling machinery. Research into chromatin remodeling mechanisms for the purpose of new drug target discovery is an important area of investigation, as is illustrated by the HDAC inhibitor, apicidin, which has broad spectrum activity against a variety of apicomplexan parasites, including human pathogens Toxoplasma and Plasmodium, and veterinary pathogens from the Eimeria genera (Darkin-Rattray et al., 1996).

The fundamental building block of chromatin is the histone protein. Four canonical types of histones exist (H2A, H2B, H3, and H4) that form an octamer complexed with DNA (nucleosome). Histone tails, particularly those of H3 and H4, are subject to a diverse array of covalent modifications that have different consequences on gene transcription (Peterson and Laniel, 2004). Like many other eukaryotes, Toxoplasma H3 and H4 are exceptionally well conserved; and each residue in the N-terminal tail reported to be susceptible to chemical modification is present (Sullivan et al., 2003, 2006). The existence of histone variants, which may be substituted to modulate DNA-driven processes, is also conserved in Toxoplasma. For example, Toxoplasma contains a homolog of variant H3.3 in addition to the canonical H3, the former being associated with genes undergoing transcription in other species (Sullivan et al., 2003). Two variants exist for both H2A and H2B in Toxoplasma (Sullivan et al., 2006). Similarly to yeast, Toxoplasma does not appear to possess H1, the extra-nucleosomal 'linker' histone involved in solenoid formation during chromatin condensation.

The histone N-terminal tails are also rich in positively charged amino acids and interact tightly with negatively charged DNA, facilitating condensation. The assembly of genomic DNA into histone nucleosomes and then into higher-order chromatin structures is associated with transcriptional repression, and 'silenced' chromatin is thought to be the default mechanism guiding the formation of chromatin following DNA replication (Ehrenhofer-Murray, 2004). Thus, active steps must be taken to alter the normal state of chromatin in order to achieve stable transcriptional activation. A myriad of chemical modifications to histones are now known and are proposed to operate in combinatorial fashion, constituting a 'histone code' that reflects corresponding changes in the local activation (and inactivation) of specific genes (Strahl and Allis, 2000). Consistent with this hypothesis is the discovery of protein motifs capable of binding specific histone modifications - for example, bromodomains interact with acetylated lysines, chromodomains can bind methylated lysines, and macro domains recognize ADP-ribose moieties (Dhalluin et al., 1999; Bannister et al., 2001; Karras et al., 2005). In the following sections,

we present a summary of histone modifications and discuss what is known about their occurrence in *Toxoplasma*.

16.4.1 Histone-modifying enzymes in *Toxoplasma*

In the past decade, numerous enzymatic complexes capable of restructuring the chromatin environment have been documented. Chromatin remodelers fall into one of two distinct classes; those capable of covalently modifying the histone tails, and those that utilize ATP to reposition nucleosomes (SWI2/SNF2 family ATPases). Previous reports catalog members of both classes of chromatin remodelers present in Apicomplexa: a histone acetyltransferase (HAT) and deacetyltransferases (HDACs), as well as a SWI2/SNF2 ATPase homolog in Plasmodium (Ji and Arnot, 1997; Joshi et al., 1999; Fan et al., 2004; Freitas-Junior et al., 2005) and a HAT and SWI2/SNF2 ATPase homolog in Toxoplasma (Hettmann and Soldati, 1999; Sullivan and Smith, 2000; Sullivan et al., 2003).

16.4.1.1 Histone acetylation In other eukaryotes, acetylation of lysine residues in the N-terminal histone tails is linked to gene activation; conversely, the removal of acetyl groups is associated with transcriptional repression. A wide variety of HATs and HDACs have been characterized among eukaryotes that control the acetylation status of nucleosomal histones, and hence play an important role in the regulation of gene expression (Sterner and Berger, 2000; Thiagalingam et al., 2003). The Toxoplasma genome harbors at least seven HATs, four of which have been experimentally confirmed (details below). Homologs for cytoplasmic Hat1, TAF1 (TAF1250), and Elp3 (TgTwinScan_0366, 1728, and 1958, respectively) are based solely on sequence similarity at present.

Two MYST family HAT proteins (MOZ, Ybf2/Sas3, Sas2, Tip60) exist in *Toxoplasma*, each possessing a chromodomain and the atypical C2HC zinc finger domain upstream of the HAT domain (Smith *et al.*, 2005). The predicted proteins, named TgMYST-A (AY578183) and -B (DQ104220),

have features consistent with the 'MYST + CHD' subclass, homologous to yeast Esa1, human Tip60, and MOF (Utley and Cote, 2003). Previous studies demonstrate that this type of HAT has a preference for acetylating lysines in H4, and the observation that recombinant TgMYST-A also prefers H4 as substrate in assays using free-core histones (Smith et al., 2005) functionally validates this classification. Given this similarity, it was not surprising that the TgMYST-A genomic locus could not be disrupted by homologous recombination, as the Esa1 homolog in yeast is also an essential gene (Smith et al., 1998). TgMYST-A is not amendable to stable overexpression unless the recombinant protein is mutated to nullify its HAT activity, suggesting that a delicate balance of TgMYST-A-mediated acetylation exists in Toxoplasma. A peptide antibody to TgMYST-A reveals that the protein is much more abundant in tachyzoites relative to bradyzoites, providing the first demonstration of a stage-specific HAT in protozoal parasites (Smith et al., 2005).

Two HAT proteins of the GCN5 class also exist in Toxoplasma. These proteins are designated TgGCN5-A (AAF29981) and TgGCN5-B (AAW72884), and, to the best of our knowledge, two TgGCN5s in a single cell have not been documented for any other invertebrate, although mammals have two GCN5 HAT genes, referred to as GCN5 and PCAF (p300/CBP Associating Factor). The mRNA encoding TgGCN5-A is more abundant in tachyzoites than that of TgGCN5-B. Whether the TgGCN5 genes have redundant or independent functions is not known; however, deletion of mouse GCN5 results is embryonic lethal, while the loss of PCAF has no discernable phenotype (Xu et al., 2000; Yamauchi et al., 2000). Dual TgGCN5s are reminiscent of the duplicate genes encoding proliferating cell nuclear antigens that also occur in this parasite (Guerini et al., 2000, 2005). All GCN5 family members studied show a strong preference to acetylate H3, particularly lysine 14 {K14}, and recombinant TgGCN5-A was found to have an exquisite selectivity to acetylate H3 at lysine 18 {K18}, while TgGCN5-B was more prototypical and capable of targeting H3 {K9}, {K14}, and {K18} in vitro (Saksouk et al., 2005; Bhatti et al., 2006). Another difference between the TgGCN5 HAT proteins is their ability to bind with the ADA2 co-activator, for which two homologs have been identified in Toxoplasma (TgADA2-A, DQ112184; TgADA2-B, DQ112185). By yeast two-hybrid assay, TgGCN5-B has been shown to interact with either TgADA2 homolog, while TgGCN5-A can only associate with TgADA2-B (Bhatti et al., 2006). Apicomplexan GCN5 HATs contain an unusual N-terminal extension upstream of the well-conserved HAT and bromodomains. Curiously, the length and amino-acid composition varies greatly among the Apicomplexa, and even among the pair of GCN5s in Toxoplasma. Most GCN5s from early eukaryotes do not have appreciable sequence upstream of the HAT domain. In contrast, mammalian GCN5 and PCAF have N-terminal extensions, but they are very similar to each other. The function of the N-terminal extension may be to mediate protein-protein interactions (e.g. the binding of CBP) and/or substrate recognition, as GCN5 lacking the N-terminal extension can only acetylate free histones and not nucleosomal histones (Xu et al., 1998). The N-terminal extensions of TgGCN5-A and -B are required for nuclear localization, but are dispensible for enzyme activity on free histones (Bhatti and Sullivan, 2005; Bhatti et al., 2006). Further, a six amino-acid motif in the N-terminal extension of TgGCN5-A has been mapped as a necessary and sufficient nuclear localization signal that interacts with the nuclear chaperone importin alpha (Bhatti and Sullivan, 2005).

Previous work has also noted that HDAC proteins exist in *Plasmodium* (Joshi *et al.*, 1999; Freitas-Junior *et al.*, 2005), and analysis of *Toxoplasma* genomic sequence indicates that there are six potential HDAC genes with one experimentally characterized to date (TgHDAC3). Recombinant TgHDAC3 exhibits histone deacetylase activity that is inhibited by butyrate, aroyl-pyrrole-hydroxy-amides, and trichostatin A, and a native TgHDAC3containing complex has been recently purified (TgCRC for CoRepressor Complex) (Saksouk *et al.*, 2005). The TgCRC contains several protein components that are homologous to subunits found in the human N-CoR and SMRT complexes, as well as two large parasite-specific proteins of unknown function (Saksouk *et al.*, 2005).

16.4.1.2 Histone methylation

The addition of methyl groups occurs on lysine and arginine residues in H3 and H4, and can lead to gene activation or silencing (Zhang and Reinberg, 2001). Toxoplasma possesses five protein arginine methyltransferase (PRMT) homologs, designated TgPRMT1-5 (TgTwinScan_1589, 0364, 2891, 0419, 1298). Recombinant TgPRMT1 (AY820756) is capable of methylating H4 $\{R3\},$ while TgPRMT4 (referred to as TgCARM1 - co-activator associated arginine methyltransferase, AY820755) methylates H3 {R17} (Saksouk et al., 2005), which parallels the substrate specificity of their human homologs. Human CARM1 has been associated with SWI2/SNF2 ATPases, including the Snf2related CBP activator protein, or SRCAP (Monroy et al., 2003; Xu et al., 2004). An SRCAP is present in the list of Toxoplasma SWI2/SNF2 homologs discovered by surveys of the Toxoplasma genome (see following section and Sullivan et al., 2003). Recombinant TgCARM1 incubated with parasite extract enriched for ATP-dependent nucleosome disruption activity indicated that TgCARM1 is likely to interact with a Toxoplasma SWI2/SNF2 member (Saksouk et al., 2005).

Lysine methyltransferases share a common feature known as the SET domain (Dillon *et al.*, 2005). Searching for this domain in the predicted proteins contained in the *Toxoplasma* genome reveals at least fourteen potential candidates in this parasite. With regard to the removal of methyl groups from lysines, *Toxoplasma* appears to encode a homolog of lysine-specific demethyltransferase (LSD1/BHC110, TgTwinScan_1683) (Shi *et al.*, 2004). Enzymes that remove methyl groups from arginines are believed to exist in eukaryotes, but have not been identified to date.

16.4.1.3 Other histone covalent modifications

Histones are also susceptible to phosphorylation, ADP-ribosylation, ubiquitylation, and sumoylation

(Peterson and Laniel, 2004). There is no evidence at this time to support that these modifications exist in Apicomplexa; however, the Toxoplasma genome contains homologs that may perform these activities. Snf1, for example, phosphorylates H3 {S10} to enhance transcription (Lo et al., 2001), and Toxoplasma encodes two possible proteins with strong similarity to the Snf1 sequence (TgTwinScan_5974 and 0259). Toxoplasma also appears to possess proteins containing PARP and PARG domains, required for the addition or removal (respectively) of ADP-ribose subunits. There is also no shortage of ubiquitin-conjugating enzymes in this organism, including Ubc9, which is implicated in gene repression via the sumoylation of H4 (Shiio and Eisenman, 2003). Histone residues known to be targeted are also conserved in Toxoplasma, including H2A {S1}, H3 {S10}, and H4 {S1}, which are candidates for phosphorylation, and H2A {K120} and H2B {K116}, which are sites for ubiquitylation.

In summary, previous reports coupled with bioinformatic analyses of the completed genome demonstrate that *Toxoplasma* is capable of mediating all known histone modifications. This observation further underscores the antiquity of this phenomenon in the evolution of the eukaryotic cell, and implies that, like other eukaryotes, epigenetic mechanisms are important components of the transcriptional machinery in *Toxoplasma*.

16.4.2 SWI2/SNF2 ATPases in *Toxoplasma*

The second broad class of chromatin remodeling complexes in eukaryotes is comprised of the SWI2/SNF2 DNA-dependent ATPases that have roles in transcriptional repression as well as activation (Mohrmann and Verrijzer, 2005). While the mechanism of action of these factors is incompletely understood, it is believed the energy of ATP hydrolysis is used to reposition or relocate the nucleosome (Johnson *et al.*, 2005). All members of the SWI2/SNF2 family contain a distinctive ATPase domain consisting of an N-terminal DEXDc portion and a C-terminal HELICc portion. Further classification based on sequence homology and additional structural features leads to four separate types: Snf2 members (contain a bromodomain), ISWI (contain a SANT domain), Mi-2 (contain a chromodomain), and Ino80/SRCAP/p400 (contain a lengthy insert between the DEXDc and HELICc domains). Previous reports have described SWI2/SNF2 factors in Apicomplexa, including an ISWI homolog in *Plasmodium* and an SRCAP homolog in *Toxoplasma* (TgSRCAP, AAL29689), *Cryptosporidium*, and *Plasmodium* (Ji and Arnot, 1997; Sullivan *et al.*, 2003).

Only TgSRCAP has been studied in any great detail. Like human SRCAP, TgSRCAP can function to enhance CREB-mediated transcription in the presence of the HAT CBP in vitro (Sullivan et al., 2003). However, a protein with similarity to CBP or CREB has not been found; therefore its role in Toxoplasma remains to be elucidated. To facilitate a better understanding of what TgSRCAP may do, a yeast two-hybrid screen was conducted using the lengthy 'spacer' region separating the DEXDc and HELICc domains as 'bait' (Nallani and Sullivan, 2005). The corresponding region in human SRCAP binds CBP (Johnston et al., 1999). Most of the strongest interacting proteins isolated and confirmed by in vitro co-immunoprecipitation are novel parasite-specific proteins having no homologs in other eukaryotes. A few of these are from genes predicted to encode domains suggestive of a role in DNA processes - including transcription. Of particular interest is the first protein described in Toxoplasma to contain Kelch repeats and a BTB/POZ domain (Nallani and Sullivan, 2005). POZ domains from several zinc finger proteins have been shown to mediate transcriptional repression and to interact with components of histone deacetylase co-repressor complexes. The gene has subsequently been cloned (DQ174778) and termed TgLZTR, since it is most similar to human Leucine-Zipper-like Transcriptional Regulator - a gene deleted in people with DiGeorge syndrome (Kurahashi et al., 1995). Future studies should elucidate the role of TgLZTR and whether it associates with TgSRCAP in vivo.

In addition to TgSRCAP, the *Toxoplasma* genome contains at least 15 possible SWI2/SNF2 homologs.

None of the predicted proteins has a bromodomain, but two (TgTwinScan_2264 and 1843) have high sequence similarity to Snf2 subclass members. One additional protein contains a predicted SANT domain (TgTwinScan_2326), making it a likely ortholog of ISWI, and another contains a chromodomain (TgTwinScan_6651), making it a probable Mi-2 ortholog. Other predicted SWI2/SNF2 family ATPases in *Toxoplasma* include one that has strong ISWI homology (TgTwinScan_7555) but has an AT hook domain instead of a SANT domain, and several harbor RING (TgTwinScan_2150 and 5444) or PHD zinc fingers (TgTwinScan_2820), which are potential protein–protein interaction domains.

16.4.3 Evidence for chromatin signatures of gene regulation in *Toxoplasma*

Significant progress has been made in the past couple of years to begin mapping epigenetic marks that are associated with gene activation and repression in *Toxoplasma*. A key advance in facilitating this research was the optimization of a chromatin immunoprecipitation (ChIPs) protocol for these parasites. ChIPs can be employed to detect specific histone modifications *in vivo* at selected promoters, and can also be used to identify chromatin remodeling factors bound to promoters of interest. A summary of the evidence for epigenetic modifications and associated factors that may mediate them in *Toxoplasma* is shown in Figure 16.3.

As mentioned above, the N-terminal tails of TgH3 and TgH4 are indistinguishable from most other eukaryotes, so many of the commercially available antibodies raised to specific covalent histone modifications are cross-reactive to *Toxoplasma* histones. Using ChIPs with antibodies to either acetylated H3 or H4, it was demonstrated that in *Toxoplasma* relative acetylation versus deacetylation can be correlated with specific gene activation or repression, respectively (Saksouk *et al.*, 2005). This was shown in the context of parasite differentiation, demonstrating that in parasites cultured as tachyzoites, H3 and H4 were acetylated at tachyzoite-specific promoters like

SAG1 and SAG2A, while no acetylation is detected at bradyzoite-specific promoters such as LDH2 and BAG1 (Saksouk et al., 2005). Conversely, in a parasite population induced to enter the bradyzoite pathway, acetylation at tachyzoite promoters was diminished, while acetylated histones were associated with bradyzoite promoters. As expected, intergenic regions upstream of constitutively expressed genes were found in the acetylated state in either population in vitro. Confirmation of the differential state of histone acetylation that is associated with specific remodeling enzymes was also observed in parasite transgenic lines expressing epitope-tagged TgGCN5-A and TgHDAC3 proteins. HAT protein TgGCN5-A was present at tachyzoite promoters in tachyzoites,

but absent at bradyzoite promoters, whereas TgHDAC3 was associated with promoters that are downregulated in the tachyzoite stage (Saksouk *et al.*, 2005).

Studies have also shown that the TgCARM1mediated methylation of H3 {R17} is another signature of gene expression in *Toxoplasma*, with the presence of this protein at active genes in either the tachyzoite or bradyzoite stage (Saksouk *et al.*, 2005). While recombinant TgPRMT1 was shown to methylate H4 {R3}, this histone modification has not yet been confirmed *in vivo*. Finally, ChIPs has shown that H3 {K4} can be di- or tri-methylated in *Toxoplasma*. More specifically, tri-methylated H3 {K4} is enriched at tachyzoite promoters during the tachyzoite stage, and becomes enriched at



FIGURE 16.3 Epigenetics and chromatin remodelers in *Toxoplasma*. Cartoon displays histone modifications that have been observed on the N-terminal (Nt) tails of *Toxoplasma* histones H3 and H4, either *in vitro* or *in vivo*. Four HATs associated with gene activation are shown, along with any reported associating factors (depicted as feathers). The preferred substrate is depicted with a bold arrow. Enzymatic specificity of TgMYST-B has yet to be resolved. The TgHDAC3-containing complex, TgCRC, is associated with deacetylated promoters of transcriptionally inactive genes. Two histone methyltransferases are also shown, along with their substrate target. Di- and tri-methylation of TgH3 {K4} has been reported, but the enzyme responsible has yet to be determined. Also shown is TgSRCAP, the SWI2/SNF2 ATPase homolog, which may associate with the *Toxoplasma* homolog of Leucine-Zipper-like Transcriptional Regulator (LZTR). It is unknown at present whether TgSRCAP associates with the other chromatin remodelers shown, but SWI2/SNF2 family members are found in complexes containing CARM1 or HDACs in other species. Ac = acetylation; Me = methylation.

bradyzoite promoters following differentiation, thus representing another potential mark of gene activation in this parasite (Saksouk *et al.*, 2005).

16.4.4 Final observations and future directions

There is no doubt that continued research will demonstrate a critical role for chromatin remodeling in various aspects of parasite physiology. A role for epigenetic mediators, specifically the HDAC PfSir2, has been established in antigenic switching a process that occurs in Plasmodium and facilitates its ability to evade host immunity (Duraisingh et al., 2005; Freitas-Junior et al., 2005). There is no evidence of antigenic switching in Toxoplasma, but other aspects of its life cycle that are critical to pathogenesis, such as tachyzoite-bradyzoite interconversion, involve changes in the expressed genome that correlate with specific alterations in histone acetylation levels (Saksouk et al., 2005). Interference with parasite histone methylation may also disrupt control of differentiation. While the reasons remain unclear, pre-treating tachyzoites with a CARM1 inhibitor leads to a higher frequency of bradyzoite development upon infecting host cells in vitro (Saksouk et al., 2005).

It is now clear that the main features of Toxoplasma chromatin that have been linked to gene expression in other eukaryotes are well conserved in this parasite. It might be expected that the enzymes orchestrating these histone modifications would also be conserved, and to a degree this is true: catalytic domains on Toxoplasma histone-modifying homologs contain no overt differences. However, the unique domains contained in these proteins require further investigation in order to understand their contribution to function. A number of HAT and HDAC inhibitors are known, and their potential effect against Toxoplasma is worth examining in greater detail (Thiagalingam et al., 2003; Schafer and Jung, 2005). However, selective toxicity may be an issue, as the catalytic domains are generally well conserved between host and parasite. A better opportunity for specifically interfering with the parasite chromatin machinery may be targeting of the unique regions of these chromatin factors, or the parasite-specific proteins associated with them.

16.5 EVIDENCE OF POST-TRANSCRIPTIONAL MECHANISMS IN *TOXOPLASMA*

In animal cells and yeast, studies comparing mRNA transcript levels with those of corresponding protein clearly demonstrate that, while regulation of gene expression at the post-transcriptional and post-translational levels are evident, transcriptional control remains the primary mechanism dictating protein levels. For example, in a study where mRNAs were first isolated from both resting and mitogenically activated fibroblasts, using ribosome loading to distinguish global changes in the translational state of mRNAs (Zong et al., 1999), less than 1 percent (of 1200) were found regulated after transcription. Similarly, in the yeast Saccharomyces cerevisiae, a comparison of 1400 proteins isolated by two-dimensional gel electrophoresis and their corresponding mRNA showed strong correlation between protein abundance and mRNA levels; and typical post-translational control imposed by mRNA stability and protein turnover were found to be insignificant (Futcher et al., 1999). Studies in Plasmodium demonstrate that regulation of transcription appears more closely related to yeast and animal cells. The mRNA transcripts and the level of the cognate protein(s) have been measured for nine different stages of the parasite life cycle (Washburn et al., 2001; Florens et al., 2002; Graumann et al., 2004; Le Roch et al., 2004), and a recent analysis based on a subset of these data has determined that changes in mRNA usually match protein levels with discrepancies between mRNA and protein levels often explained by a time shift between the first detection of the transcript and the cognate proteins (Le Roch et al., 2004).

While the above results demonstrate the distinct predisposition to regulate protein levels by mRNA transcription in both multi- and uni-cellular eukaryotes, post-transcriptional mechanisms to regulate the level of specific proteins among the protozoa have been described (Shapira et al., 2001; Garcia-Salcedo et al., 2002; Chow and Wirth, 2003; Larreta et al., 2004; Rochette et al., 2005). Transcription in Leishmania is polycistronic, and thus post-transcriptional transplicing mechanisms are required to achieve mature mRNA (Shapira et al., 2001; Campbell et al., 2003); and in Leishmania and Trypanosoma species, mRNA levels are dictated mostly by these post-transcriptional events or the inherent stability of the mRNA itself (Haile et al., 2003; Cevallos et al., 2005; Purdy et al., 2005a, 2005b; Webb et al., 2005a, 2005b). RNA-binding proteins with demonstrated roles in the regulation of translation and RNA stability have been found in Plasmodium (Cui et al., 2002), and transcription-associated proteins with known roles in modulating mRNA decay and translation were also found in the genome (Coulson et al., 2004). As such, indications of post-transcriptional control have been described for protozoal genes with defined roles in differentiation (Vervenne et al., 1994; Dechering et al., 1997; Sullivan et al., 2004), mitochondrial RNA processing (Rehkopf et al., 2000), and surface antigens (Lanzer et al., 1993; Levitt et al., 1993; Spano et al., 2002). In Toxoplasma, unbalanced ratios of mRNA and protein have been observed for SAG-related Toxoplasma surface proteins, designated SAG5A, SAG5B, and SAG5C (Spano et al., 2002), and for mRNAs encoding the proliferating cell nuclear antigens, TgPCNA1 and TgPCNA2 (Guerini et al., 2000). TgPCNA1 mRNA was found to be seven-fold higher than that of TgPCNA2, yet TgPCNA1 and 2 on Western blots were expressed at nearly equal levels in all strains examined (Guerini et al., 2000). These discrepancies in the levels of mRNA and protein indicate that post-transcriptional events occur in Apicomplexa parasites, although the mechanisms of translational control in these parasites are not understood. In Toxoplasma, the phosphorylation of eukaryotic initiation factor-2, eIF2, which is a known prerequisite to stress remediation in eukaryotic cells, has been shown to occur during stress induction of parasite development (Harding et al., 2000; Clemens, 2001; Narasimhan et al., 2004; Sullivan et al., 2004; Zhan et al., 2004). A novel Toxoplasma eIF2 protein kinase,

designated TgIF2K-A (Toxoplasma gondii initiation factor-2 kinase), contains sequence conserved among members of the eIF2 kinase family, and is able to phosphorylate recombinant TgIF2a in vitro (Sullivan et al., 2004). Overexpression of TgIF2K-A in a yeast strain minus endogenous eIF2 kinase (designated GCN2; Narasimhan et al., 2004) results in unregulated phosphorylation of eIF2 and a subsequent reduction in translation initiation that leads, ultimately, to slowed cell growth. The phosphorylation of TgIF2 α in tachyzoites was demonstrated in response to heat shock or alkaline stress (Sullivan et al., 2004) - both conditions known to induce parasite differentiation in vitro. Whether this mechanism affects mRNA translation generally or serves to regulate specific aspects of developmental gene expression in Toxoplasma remains to be determined.

The few examples for regulation at the level of mRNA utilization suggests that, if not a global strategy in Apicomplexa parasites, these mechanisms are likely to be important for specific genes. The completion of the *Toxoplasma* genome sequence and development of whole-genome microarrays, combined with advances in proteomics, will allow for more global characterization of mRNA and protein levels, and will lead to the identification of those mRNAs subject to post-transcriptional regulatory activity.

16.6 CONCLUSIONS

We now have sufficient knowledge of global mRNA expression in *Plasmodium* and *Toxoplasma* to conclude that transcriptional mechanisms play a major role in regulating the developmental program of these parasites. The observations that co-regulated genes are dispersed across parasite chromosomes, along with the presence of much of the conventional eukaryotic transcriptional machinery in the Apicomplexa genomes including chromatin remodelers, is consistent with growing evidence that promoter structures in these parasites contain *cis*-elements that are regulated by transacting factors. The details of these mechanisms remain to be worked out, and we anticipate that this will be forthcoming in the next few years. Apicomplexa protozoa are evolutionarily distinct from other eukaryotes, and the unique enrichment of parasite-specific genes in their mRNA pools suggests that transcriptional regulatory mechanisms in these parasites will have unique characteristics. Transcription factor conservation appears to be a function of evolutionary distance (Coulson and Ouzounis, 2003), indicating that the structural constraints required to preserve the basic core mechanisms are flexible with respect to protein sequence. Thus, future dissection of transcriptional mechanisms in Toxoplasma and other Apicomplexa will require the use of biochemical approaches that have been well developed in other eukaryotic models.

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17

The Secretory Protein Repertoire and Expanded Gene Families of *Toxoplasma gondii* and Other Apicomplexa

T.J. Templeton

17.1 Introduction

- 17.2 The EC protein repertoire of
- Toxoplasma gondii
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17.1 INTRODUCTION

During the evolutionary process of specialization that led to parasites of the genus *Toxoplasma*, the most rapidly evolving events involved the acquisition or streamlining of metabolic pathways and, the subject of this chapter, the invention of new proteins that function at the parasite membrane surface. For the parasitic protozoans, great breadth and capacitance is required within the catalog of extracellular (EC) proteins, in order to encompass sufficient plasticity at the host–parasite interface to insure parasite survival. The extensive repertoires of parasite membrane surface and secreted proteins can be viewed as 'incubators' harboring diversity and mutability that facilitate genetic selection and evolution. This chapter will describe the catalog of predicted EC proteins encoded within the *Toxoplasma gondii* genome sequence – proteins that likely regulate a variety of cellular processes and host–parasite interactions, such as maintenance of cellular and surface membrane structures; recognition and response to host tissues and cell surfaces; host tissue and cell invasion; and evasion and manipulation of host-immune responses. Despite great progress in recent years, few members of the *Toxoplasma* EC protein repertoire have been studied at the
laboratory bench; therefore, as a reference source, it will be attempted here to distill the catalog into classifications of proteins having similar characteristics. It is hoped that this discussion will also serve as a reference companion to Chapter 11, which describes *Toxoplasma* invasion of host cells and the post-translational proteolytic modifications of surface proteins.

17.1.1 Apicomplexan genomes

Complete genome sequence information is available for species within several apicomplexan genera, including Plasmodium, Cryptosporidium, Theileria, and Toxoplasma, and it is now possible to perform whole genome sequence comparisons to characterize both ancient and lineage-specific parasitic adaptations within the Apicomplexa. High coverage genome sequence sampling is underway for dinoflagellates; the early branching dinoflagellate, Perkinsus; and gregarines; thus allowing comparisons within the alveolate clade. Complete genome sequence information is also available for the kinetoplastids *Leishmania*, Trypanosoma brucei, and T. cruzi, and the diplomonad, Giardia. Whole genome sequence comparisons with these distantly related organisms will allow predictions of protozoan-specific inventions versus molecular, metabolic, and cellular features that are common to eukaryotic or prokaryotic organisms. Genome sequence information for free-living protozoans is thus far lacking, although this type of analysis was recently given a considerable boost by the completion of the genome sequence for the ciliate Tetrahymena thermophila (Eisen et al., 2006). Comparison of free-living with parasitic protozoans might indicate adaptations specific to parasitism, and also provide a greater understanding of ancient molecular mechanisms that have been co-opted to underpin these adaptations.

The genome sequence information is largely complete for *Toxoplasma gondii* (Kissinger *et al.*, 2003), although gap closure is ongoing and fragmentation persists. At the time of writing, contig sequence information has not been anchored to telomeres and it is therefore not possible to examine the putative partitioning of the encoded proteome into telomeric versus internal gene neighborhoods. Thus it is not known whether Toxoplasma sequesters highly diverse gene families to telomeric regions, such as occurs for the var and rifin gene families in Plasmodium. The analyses presented in this chapter have predominantly relied on the TgTwinScan gene predictions and the associated genome sequence browser at the ToxoDB BLAST server website (www.toxodb.org; Kissinger et al., 2003). Gene prediction has been a heroic undertaking for Toxoplasma, largely because it has a greater number of introns than Plasmodium and, in part, due to assembly gaps remaining within the sequence dataset. Errors in gene prediction have undoubtedly been introduced into the following discussions, particularly with regard to defining the ends of genes (relevant to signal peptide and GPI anchor signal predictions) and estimations of the number of genes within large gene families - for example, the srs gene superfamily; the apple domain-containing family; and the mucins. Gene predictions and annotations arising from the T. gondii genome sequence project have not yet been submitted to GenBank, and the following analyses will cite preliminary gene prediction identifiers whenever GenBank gene identifiers are not available. These preliminary gene identifiers should allow retrieval of gene information from the genome project website (www.toxodb.org), and may also be used as queries once GenBank submission occurs.

17.1.2 Themes in the origin of protozoan surface proteins

The genes comprising the *Toxoplasma* EC protein repertoire have heritages that can be attributed to protozoan, alveolate, apicomplexan, coccidian, or lineage-specific provenance (Figure 17.1). In comparison with other apicomplexans, the *Toxoplasma* catalog differs due to the loss of vestigial genes, and the acquisition of new functions via either lateral transfer of genetic information or sequence divergence. For the alveolate clade, lateral transfer may arise following endosymbiotic events (such as the engulfment of

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FIGURE 17.1 The varieties of heritages for *Toxoplasma* EC proteins. The phylogenetic tree is approximate. Specific examples of EC protein and domains are shown in Figure 17.5.

the cyanobacterial-like apicoplast organelle in an ancient alveolate ancestor), or via an as yet unknown mechanism of DNA 'sampling' that mediates lateral transfer from the host or organisms within the environmental niche.

EC protein sequence divergence is facilitated by the duplication of genes, or amplification into

paralogous gene families (for example, the *Toxoplasma* Apple domain proteins; Figure 17.2), thus allowing evolvability while sheltering critical gene product functions. For other pathogenic protozoans, such as *Trypanosoma brucei* and *Plasmodium falciparum*, it is known that divergence within antigenically variant protein families



FIGURE 17.2 Examples of Toxoplasma lineage-specific expansions: the Apple domain proteins.

is accelerated via mechanisms of gene recombination, gene conversion, and perhaps the involvement of mutagenic DNA polymerases during targeted DNA 'repair' (Aravind et al., 2003). These events might be isolated from having deleterious effects on bystander invariant genes by sequestering variant genes within telomeric or sub-telomeric regions. The above themes are repeated in many but not all pathogenic protozoa, and the underlying molecular mechanisms generating diversity are linked either by vertical inheritance or by convergent evolution. Now that complete genome sequence information is available for many protozoan organisms, it should be pursued to complement these datasets with isolate-specific genome sequences (e.g. multiple Toxoplasma isolates), as aids to studies addressing evolutionary hypotheses on the diversification of genome structures and their encoded proteomes.

17.2 THE EC PROTEIN REPERTOIRE OF TOXOPLASMA GONDII

The following sections seek to introduce the repertoire of T. gondii cell-surface and secreted proteins. The sections are grouped primarily according to similarities in protein structure, but are guided wherever possible by examples of cellular localization, life-cycle stage of expression, function, and phylogenetic affinities. This organization is logical because the majority of the genes that are discussed here have not been described and were isolated via a variety of computational screens of the T. gondii genome sequence database. As introduced above, a dominant theme in this chapter will be the description of the phylogenetic origin of T. gondii EC proteins - that is, those of likely ancient origin versus proteins having an apicomplexan-specific, coccidian, or lineage-specific provenance. Finally, as a disclaimer, the identification of Toxoplasma lineage-specific EC proteins is most informative in the context of a whole genome annotation, cluster analyses (BLAST screening of the predicted proteome against itself) to identify gene families, and construction of phylogenetic trees for select proteins – all analyses which were not performed in support of the following discussions.

17.2.1 SAG surface coat antigens

An ancient feature common to all protozoans is abundant surface coat proteins that are associated with the membrane via glycosylphosphatidylinositol (GPI) linkages. The GPI-linked variant surface glycoproteins (VSG) of Trypanosoma brucei are the prototypic examples, and in fact lipid-linkages were first characterized in studies of this pathogen (reviewed in Ferguson, 1999). The surface proteins themselves do not appear to be conserved across phyla, and superficial similarities, such as the presence of cysteine-rich globular domains, are likely to be the result of convergent evolution. Toxoplasma possesses several families of predicted surface-coat proteins, the best-studied of which is the GPI-linked surface antigen (termed SAG; Nagel and Boothroyd, 1989; Bulow and Boothroyd, 1991) within a superfamily of related proteins (termed SRS; Manger et al., 1998; Lekutis et al., 2000, 2001; Jung et al., 2004). The computational analysis presented here indicates that the srs family comprises greater than 40 genes in the T. gondii genome, many of which are localized within gene clusters (Spano et al., 2002; Tinti et al., 2003), including a locus containing seven or more genes on chromosome 11 (i.e. 83.m05678). Some adjacent genes have nearly identical amino-acid sequences, indicating recent duplication events. The sum repertoire of SRS genes remains preliminary, and there might in fact be over 150 SRS genes (Jung et al., 2004) - a difference that is perhaps due to data-mining techniques. Many SRS proteins are conserved in coccidians such as Neospora and Sarcocystis (Howe et al., 1998, 2005), and may include examples of orthologous relationships.

SRS proteins are not identified by reiterative BLAST screens of the *Cryptosporidium* genome sequence. The absence in *Cryptosporidium* is notable because the SRS proteins share a likely vertical relationship with the 6-Cys domain family in *Plasmodium* (Gerloff *et al.*, 2005), which has over 10 family members and includes the gametespecific surface proteins P48/45 and P230, and the sporozoite surface protein, P36 (Ishino *et al.*, 2005; van Dijk *et al.*, 2005). The conservation with *Plasmodium* is supported by sequence similarities, as well as comparisons of predicted domain structures based upon the crystal structure of SAG1 (He *et al.*, 2002). Thus the SRS domain was invented after the split of *Cryptosporidium*, or was eliminated from *Cryptosporidium* via gene loss, or is conserved in *Cryptosporidium* but has diverged such that its relationship is not detectable by reiterative PSI-BLAST screening.

SRS family members show stage specificity in their expression, similar in theme to the Plasmodium 6-Cys proteins, although this analysis is incomplete and only a portion of the SRS genes has been described. The prototypic protein, SAG1, as well as SAG2A/B (Lekutis et al., 2000, 2001), SRS3 (Manger et al., 1998), and SAG3 (Cesbron-Delauw et al., 1994), are expressed on the surface of tachyzoites (Nagel and Boothroyd, 1989), whereas SAG2D (Lekutis et al., 2000) and BSR4 (Manger et al., 1998) are bradyzoite-specific proteins. Recently the surface coat of the sporozoite stage was characterized, and a SAG-related protein, SporoSAG, was shown to be abundantly expressed on the surface of sporozoites (Radke et al., 2004). It is not known whether any SRS family members are expressed in gamonts, or whether they additionally show gender-specificity within these stages. Given the breadth of the SRS family, the stage-specificity of expression for all family members should now be determined, along with the serology and antigenic diversity of family members between isolates, as well as the possibility of antigenic switching. For example, stage-specific switching of SRS expression might be a means by which the parasite avoids an adaptive immune response (Kim and Boothroyd, 2005). The abundance of the SRS at the parasite surface suggests that they might be involved in forming a structural surface coat, or function as a component of host adhesive events. In support of a role in adhesion, the disruption of the SAG3 gene resulted in decreased host-cell adhesion, invasion, and virulence in mice (Dzierszinski et al., 2000). Moreover, the SAG1 crystal structure is composed of a homodimer that forms a positively charged groove between each

subunit that may participate in binding negatively charged target molecules (He *et al.*, 2002), such as host sulfated proteoglycans in the case of SAG3 (Jacquet *et al.*, 2001).

17.2.2 Oocyst wall proteins

As a coccidian, Toxoplasma shares with Neospora, Eimeria, and Sarcocystis, as well as the more distantly related apicomplexan pathogen, Cryptosporidium, the unifying feature of transmission via an environmentally durable sporulated oocyst stage. The coccidians possess common ancient metabolic pathways governing the synthesis of storage polysaccharides, such as trehalose and amylopectin, and they additionally share organelles and proteins involved in the formation of the oocyst wall structures. Coccidian oocysts are formed within the host intestinal epithelium following the zygotic fusion of micro- and macrogametes. Oocyst formation begins with structures that form within the mature macrogametes including two related granules termed the wall-forming bodies 1 and 2. Few studies have addressed coccidian oocyst development at molecular and cellular levels, and only two families of coccidian molecules are known to participate in the formation of the oocyst wall; namely, the large oocyst wall protein (OWP) family that has been described in Cryptosporidium (COWP; Spano et al., 1997; Templeton et al., 2004a), and two related gametocyte antigens of Eimeria (Belli et al., 2003a, 2003b). The former family is also present in Toxoplasma (Templeton et al., 2004a) and the gregarines (Omoto et al., 2004), and thus its origin likely predates the radiation of the Apicomplexa, if not of a more ancient provenance within the cyst stages of alveolates. In contrast, the Eimeria gametocyte antigens are not found in Toxoplasma or Cryptosporidium, following BLAST-based screening of the Cryptosporidium and Toxoplasma genome sequence information. Thus they are either specific to Eimeria, or they have diverged to the extent that they are not identifiable (discussed further below).

The OWP protein family is expanded within the *Toxoplasma* genome, similar to *Cryptosporidium*, with eight or more members (Table 17.1). The extent

Protein family	T. gondii proteins ^a
SRS domain SAG1-like ^b	SAG1 (P30; 44.m00009; AAB20292.1); AAC47762.1 (44.m00008); SAG1-related sequence 2 (AAC48329.1: 44.m00010); SAG1-related sequence 6 (AAF63326.1, 641.m01560); BSR4 (AAK82886, 641.m01561); AAP86768.1 (641.m01567); 42.m05851; AAP86766.1 (42.m05852); AAP86770 (42.m05854); 59.m03655; 59.m03664; AAK25764 (162.m00310); 162.m00312; SAG5A (AAC27649.1; 80.m05039); 57.m01839; 57.m01840; 57.m01841; 645.m00317; AAM27530 (641.m01562); SAG3 (AAK26628; 551.m00001); BSR4-related antigen (AAP86769; 645.m00001); 645.m00002; 162.m00309; AAK25763 (162.m00311); 42.m00002; SAG1-related sequence 3 (AAC48328.1, 583.m00001); 50.m03429; 641.m01566; 641.m01568; SAG5D (AAP06247.1; 80.m02361)
SRS domain SAG2-like ^b	SAG2 (P22; AAA30144; 59.m00008); AAQ63827.1 (23.m00149); SAG2B (AAD21974; 25.m00004); SAG2C (AAD21975; 23.m00152) SAG2D (AAD21976.1; 23.m00015); SAG2E (AAG28794; 33.m00004); 50m.03098; 76.m01625; 63.m00338; 583.m05676; 583.m05287; 583.m05680; 49.m03128; 63.m00336; 583.m05285; 55.m10260; 59.m00008; 55.m04826; 583.m05678; 583.m00693; 63.m00337; 49.m03103; 26.m00238; 583.m05678; 583.m05677; 583.m09139; 33.m01267; 74.m00446; 26.m00239; 49.m03112; 76.m03409; 583.m05672
Mucins and paralogs ^c	49.m03322; 49.m03324; 49.m03325 ; 49.m03331; 57.m01785; 65.m01165; 44.m04609; 65.m01149; 65.m01168; 49.m03326; 59.m07764; 49.m03330; 49.m03329; GRA8 (52.m00002); 49.m03327; 65.m01173; 65.m02531; 65.m01163; 65.m01171; 44.m02529; 80.m00008; BAG protein (55.m00009); 44.m02755; 125.m00069; 80.m02284; 55.m04862; Notch protein (76.m01612); GRA2 (P13404)
TRAP family ^d	MIC2 (AAB63303; 20.m00002); MIC6 (AAD28185.1; 38.m00003); MIC8 (AAK19757; 50.m00002); 57.m01872; 55.m04840; 49.m03396; 76.m01679; 25.m01853
EGF-like domain-containing	MIC3 (AAM33361; 641.m00002); MIC6 (AAD28185; 38.m00003); MIC7 (AAK35070; 83.m01260); MIC8 (AAK19757; 50.m00002); 49.m03396; 583.m05688; 583.m05690; 64.m00337; 49.m03093; 55.m00014; 20.m03716
TSP1 domain-containing	MIC2 (AAB63303; 20.m00002); AAM09953 (38.m00001); 641.m02555; 65.m01138; 42.m03281; 25.m01843
Apple domain-containing	MIC4 (AAD33906; 25.m00006); 25.m01853; 25.m01899; 46.m01644; 46.m01639; 76.m01642; 52.m01652; 8.m00177; 76.m00178; 44.m04666; 50.m03279; 41.m02938
Notch domain-containing	25.m02918; 55.m04618; 52.m01621; 57.m01872; 25.m02918; 25.m02921; 42.m03281; 76.m01612; 27.m00847; TgTwinScan_2704 (ToxoDB preliminary ID)
Oocyst wall proteins (OWPs)	AAR28668 (20.m03865); 27.m00833; 76.m01650; 50.m05652; 59.m03377; 25.m01825; 41.m01378; TgTwinScan_7129 (ToxoDB preliminary ID)
ROP nomenclature rhoptry proteins	ROP1 (AAA69859); ROP2 (CAA85377); ROP4 (AAU87405); ROP5 (AAZ73240); ROP6 (AAV58859); ROP8 (AAC47797);

 TABLE 17.1
 Select gene families that encode Toxoplasma gondii surface proteins

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THE EC PROTEIN REPERTOIRE OF TOXOPLASMA GONDII

Protein family	T. gondii proteins ^a
	ROP10 (AAZ31062); ROP11 (AAZ29607); ROP12 (AAZ38159); ROP13 (AAZ38160); ROP14 (AAZ38165); ROP15 (AAZ38161); ROP16 (AAZ73239); rhoptry neck protein 1 (AAZ38162); rhoptry neck protein 2 (AAZ38163); rhoptry neck protein 3 (AAZ38164); rhoptry neck protein 4 (RON4; GRA3; AAZ38166); rhoptry neck protein 4-like protein (52.m01582)
GRA nomenclature dense-granule proteins	GRA1 (p24; P13403; 59.m00089); GRA2 (28-kDa antigen; AAA30138; 42.m00015); GRA3 (Q27914; 42.m00013); GRA4 (Antigen H11; Q27002; 583.m11414); GRA5 (Q07828; 76.m00004); GRA6 (Q27003; 63.m00002); GRA7 (p29; O00933; 20.m00005); GRA8 (AAD55381; 52.m00002); GRA9 (CAA11451; 50.m00019); GRA10 (AAV36002; 59.m03415)

TABLE 17.1 Select gene families that encode Toxoplasma gondii surface proteins—cont'd

^aCommon names and GenBank accession numbers are indicated, if known. ToxoDB identifiers are given for all proteins, particularly those that have not been described.

^bThe SRS domain proteins are divided into two approximate paralogous groups, SAG1-like and SAG2-like, as determined by preliminary BLAST analyses rather than analysis of molecular phylogenies.

^cThe mucins are classified by the presence of a signal peptide plus serine- or threonine-rich regions. Proteins having paralogous relationship to mucin-like proteins are also included.

^dPredicted TRAP family proteins involved in motility and invasion. Criteria for inclusion in the family are discussed in the text, and family members are shown in Figure 17.4.

of orthologous relationships with Cryptosporidium COWPs, versus lineage-specific expansions, is not known. The OWP proteins are composed of a 'periodic cysteine' structure in which cysteine residues are spaced approximately every 10-12 residues. The cysteines likely participate in disulfide bonds to create periodic globular structures, or form intermolecular bonds that stabilize multi-protein complexes. The COWP proteins also have abundant histidine-rich motifs - a feature that is not conserved in the Toxoplasma OWP proteins. All OWP proteins possess signal peptides but lack transmembrane (TM) domains, suggesting that they are secreted and possibly aggregate to form the rigid cyst wall. It is not known why Toxoplasma and Cryptosporidium require large families of OWP proteins; it is possible that the proteins differentially participate in the inner and outer cyst walls, or underpin a system of antigenic diversity.

The *Eimeria* gametocyte wall-forming bodies and oocyst wall contain abundant tyrosine-rich proteins, gam56, gam82, and gam230 (Belli *et al.*, 2003a, 2003b; Ferguson et al., 2003). Lower molecular weight proteins have also been identified (wp8, wp10, and wp12), some of which appear to be processed products of the gam proteins (Belli et al., 2003a). It is proposed that these proteins participate in formation of the rigid oocyst wall via formation of intermolecular di-tyrosine crosslinks (Belli et al., 2003a). BLAST screening of the Toxoplasma genome sequence using gam56 and gam82 as queries does not identify any orthologs, although it does identify three anonymous low molecular weight proteins (74.m00761, 583.m05759, and 641.m01540) that have signal peptide sequences and a similar tyrosine-rich character. These proteins, as well as gam56 and gam82, are not conserved in Cryptosporidium, and thus it is possible that the coccidians have diverged in certain molecular aspects of the oocyst wall such that there is conservation between Toxoplasma and Eimeria, versus Cryptosporidium.

Toxoplasma is also transmissible via infectious bradyzoites following ingestion of tissue cysts.

The molecular and cellular distinctions between the excreted and tissue-cyst stages are poorly understood, and it is likely that the rigid double walls of external oocysts are composed of a different set of proteins than the elastic wall of tissue cysts. Toxoplasma is unique in comparison with Plasmodium, Theileria, and Cryptosporidium in that it has amplified a large family of Apple domain-containing proteins, with up to 11 family members (Table 17.1; Figure 17.2). Of these proteins only MIC4 has been characterized (Ferguson et al., 2000; Brecht et al., 2001; Reiss et al., 2001; Tomley et al., 2001); it is found associated with dense bodies within mature macrogametes and, ultimately, associated with the oocyst cell wall (Ferguson et al., 2000). Recent proteomic analysis of tachyzoite secretory proteins identified the Apple domain protein, 8.m00179, and paralogs (Zhou et al., 2005). It is therefore important to determine the life-cycle stage of expression for all Apple domain family members, to determine whether these proteins play multiple roles, perhaps in invasion or adhesion, or in the formation of the tissue cysts, rather than participation in the oocyst cell wall.

17.2.3 Mucin-like proteins

Toxoplasma shares with Cryptosporidium the capacity for O-linked glycosylation, and the predicted biosynthetic pathways have been described in some detail and appear to be similar to those found in animals (Odenthal-Schnittler et al., 1993; Wojczyk et al., 2003; Stwora-Wojczyk et al., 2004a, 2004b). Each of the two pathogens possesses four proteins, absent in Plasmodium, that have a glycosyltransferase domain fused to one or more ricin domains plus, in some members, N-terminal TM domains. Cryptosporidium possesses two additional enzymes that may confer lineage-specific protein glycosylation modifications (Templeton et al., 2004b). The first protein is a galactosyltransferase that is homologous to the animal Fringe protein, fused to a C-terminal domain related to the bacterial WcaK-like glycosyltransferase (cgd6 1450; Reeves et al., 1996), whereas the second enzyme is a standalone version of the WcaK-like glycosyltransferase (cgd7_2580). *Toxoplasma* lacks both the Fringe and WcaK-like glycosyltransferase domains, and thus it will be of interest to determine the distribution of these domains within the alveolate clade.

The Cryptosporidium genome sequence contains a diverse catalog of lineage-specific proteins, including multiple families of paralogs, that likely represent O-linked highly glycosylated mucin-like proteins (Barnes et al., 1998; Cevallos et al., 2000; Templeton et al., 2004b). The proteins possess signal peptides plus tracts of Thr and Ser residues and, in some instances, predicted GPI anchor signal sequences. Perhaps the most remarkable of the Cryptosporidium mucins is an 11 696-aa long protein (cgd3_720; GenBank Accession no. EAK89192) that is largely composed of 17 repeats of a roughly 600-residue-long C. parvum-specific all-β-strand globular 12-cysteine domain and numerous Thr repeats, including a stretch of 360 Thr and Ser residues.

In contrast to Cryptosporidium, the predicted mucins of T. gondii do not have pronounced contiguous arrays of Thr or Ser residues; rather, the proteins listed in Table 17.1 are included based upon the presence of shorter stretches of Thr and Ser residues, as well as a general Thr- and Ser-rich character. One example is the dense-granule protein GRA2 (Zinecker et al., 1998), which has a stretch of five Thr and Ser residues. A second dense-granule protein, GRA8 (Carey et al., 2000), is largely composed of proline-rich regions plus two stretches of five and six Thr residues each. BLAST screening of the Toxoplasma genome sequence database reveals a paralogous family of greater than 15 additional proteins that possess signal peptides and predicted GPI-anchor signal sequences. The life-cycle stages of expression and cellular localizations of these proteins have not been determined, and their functional relationship to the GPI-linked SRS family proteins is not known. The majority of the coccidian mucins are either lineage-specific or have diverged such that sequence similarities are undetectable. However, Cryptosporidium and Toxoplasma likely share a few orthologs of highly O-glycosylated proteins, with one example being a predicted secreted protein that lacks a TM or GPI-anchor signal sequence and possesses an array of six Notch receptor-like domains (Figure 17.3; *T. gondii*, 76.m01612; *C. parvum*, cgd8_2800). Of note, the *T. gondii* version contains a Ser-rich region near the N-terminal portion of the protein, whereas the *C. parvum* version has a stretch of 49 Thr residues within the C-terminal region.

17.2.4 Cysteine repeat modular (CRM) proteins

Annotation of the Plasmodium genome sequence revealed a family of four large proteins that have multiple TM domains plus a predicted extracellular region that is composed, in part, of tandem motifs similar to EGF, EGF Ephrin receptor, or TNFR domains. One conserved member of the family (i.e. P. falciparum PFI0550w, Aravind et al., 2003; T. gondii 55.m04919, see Figure 17.3) additionally possesses a single Kringle domain. To date, the cellular and functional aspects of the protein family have not been described for any apicomplexan system. The protein family appears to be widely conserved in the Apicomplexa, including versions within Theileria, Cryptosporidium, and Toxoplasma; however, the extreme divergences within the family make it difficult to ascribe orthologous versions other than the Kringle domain protein.

BLAST analyses of GenBank (nr) additionally identify possible versions in the ciliate Paramecium (e.g. CAH03413) and a large gene family within Dictyostelium (i.e. DDB0218653) that share similarities within the block of TM domains and an N-terminal adjacent EGF-like domain. Conservation with Dictyostelium suggests that the family is the product of an ancient protozoan invention having a conserved function, and predates the split of the alveolate lineage. Plasmodium and Theileria each possess four members of the CRM protein family, whereas the number of family members in Toxoplasma is difficult to determine because of incomplete gene predictions for long, multi-exon genes. Nonetheless, the pathogen appears to possess at least two members of the CRM protein family,

namely the Kringle, domain-containing protein (55.m04919) and a protein that may contain an extended amino terminal cysteine-rich domain (80.m02347). It will be important to determine whether the cellular localization and stage-specificity of expression for the CRM proteins is conserved across the Apicomplexa.

17.2.5 Lineage-specific proteins

The majority of apicomplexan predicted EC proteins are lineage-specific in their provenance, and likely represent adaptations to parasitic niches. A general mechanism underlying this process is gene duplication or amplification, and divergence of the progeny genes by functional or host immune selective pressures. Heretofore, orthologous relationships were defined by sensitive reiterative BLAST analyses, and highly divergent relationships were either left to speculation or implied following modeling of predicted secondary structures.

Crystal structures are increasingly being described for apicomplexan EC domains, and these studies have resulted in new understanding of the origin of apicomplexan surface proteins. As introduced above, the *Toxoplasma* SRS proteins and the 6-Cys module within at least 10 *P. falciparum* proteins is one example of a vertical relationship that is not well-supported by BLAST analyses but is now indicated by using the SAG1 crystal structure to thread molecular models of *P. falciparum* 6-Cys modules.

In a second example, the crystal structure for the AMA-1 protein reveals a two-domain structure that has a protein fold similar to PAN-Apple domains (Bai *et al.*, 2005; Pizarro *et al.*, 2005). Thus, the AMA-1 protein, although of apicomplexan origin with regard to its architecture, is composed of a highly divergent PAN-Apple domain. Whereas AMA-1 is critical for *Toxoplasma* invasion of host cells (Alexander *et al.*, 2005; Mital *et al.*, 2005), *Cryptosporidium* lacks AMA-1 proteins, and this might be attributed to either gene loss or acquisition/invention of the protein following the radiation of the *Cryptosporidium* lineage.



FIGURE 17.3 Representative EC protein architectures and heritages. This figure is reproduced in color in the color plate section.

The absence of additional domains in *Cryp*tosporidium, such as the MAC/Perforin, SRS/6-Cys, and extracellular vWA domains, suggests that this genus branched early in the Apicomplexa, as additionally supported by one phylogenetic analysis indicating a close relation with the gregarines (Carreno *et al.*, 1999). In this phylogenetic model it would be logical to propose that the absence of the OWP family, Notch and Apple domains, and the meprin domain plus diamine oxidase (MAM + DAO) fusion (Figure 17.3; 76.m01681) in *Plasmodium* and *Theileria* are due to gene loss, perhaps a result of the internalization of the oocyst stage within the insect vector.

17.2.6 Role of lateral transfer and 'mix and match' modules

The apicomplexans are unique among the pathogenic protozoa in the degree to which they have acquired animal- and bacterial-like domains that are used in the formation of multi-domain EC proteins. The 'mix and match' character of apicomplexan EC domains was first described in a review by Tomley and Soldati (2001), and has been elaborated upon recently using whole genome and comparative sequence information (Aravind et al., 2003; Templeton et al., 2004b). The abundance of these domains in the Apicomplexa, and their relative absence in other protozoans, indicates that lateral transfer is the most likely mechanism to account for their origin, and that the apicomplexans may uniquely possess a sophisticated secretory apparatus that is capable of assimilating animal-like multi-domain EC proteins.

Completion of the genome sequence information for free-living alveolates, such as the ciliate *Tetrahymena thermophila* (Eisen *et al.*, 2006; an opportunistic fish pathogen), and genome sequence surveys for the dinoflagellates may indicate whether the origin of animal-like EC modules predates dedicated parasitism. Initial analysis of the *Tetrahymena* proteome suggests that the surface proteins are largely lineage-specific cystein rich domains rather than EC domains. Comparison of complete genome sequence information from *Plasmodium*, *Cryptosporidium*, *Theileria*, and *Toxoplasma* reveals that few multi-domain proteins are conserved as orthologs. In fact, even known functional orthologs such as the MIC2/TRAP family of invasion and motility micronemal proteins do not share identical architectures across species boundaries; for example, the *Toxoplasma* and *Plasmodium* TRAP family members contain vWA domains, whereas EC versions of this domain are absent in the *Cryptosporidium* genome sequence.

17.3 MICRONEME, RHOPTRY, AND DENSE-GRANULE PROTEINS

Toxoplasma has many advantages as an experimental apicomplexan, one of which is the ability to obtain preparations of cellular fractions and organelles having high purity and yield. Proteomic analyses of unique apicomplexan organelles, such as the secretory dense granules, and the apical secretory micronemes and rhoptries, have revealed proteins that greatly supplement our understanding of the biology of secreted and parasite surface proteins. Many of these proteins are typical of the 'mix and match' domain architectures (Tomley and Soldati, 2001) described above, although other proteins – particularly the densegranule proteins – appear to be lineage-specific inventions.

17.3.1 Microneme proteins

MIC2 (micronemal protein 2) is the prototypic micronemal protein, and mediates tachyzoite locomotion and cell invasion via extracellular adhesion and intracellular interaction with an actinomyosin motility apparatus. MIC2 is described in greater detail in Chapter 11, and has also been recently reviewed (Brossier and Sibley, 2005; Huynh *et al.*, 2006). MIC2 shares functional and architectural similarity with the *Plasmodium* vWA and TSP domain-containing proteins, TRAP and CTRP. The hallmarks of TRAP family proteins include extracellular predicted adhesive domains, including TSP1, vWA, and Apple (the latter domain in the case of the *Cryptosporidium* protein TRAP-C1, AAC48311); a TM domain; and a short, acidic cytoplasmic domain that contains a C-terminal proximal tryptophan residue. The tryptophan residue has been shown to be essential for interaction with aldolase, as a bridge with actin within the parasite cytoskeletal motility network (Buscaglia et al., 2003; Jewett and Sibley, 2003).

All apicomplexan genera appear to have multiple proteins possessing these features, and Toxoplasma is no exception. Table 17.1 and Figure 17.4 list candidates for Toxoplasma TRAP/MIC2-family proteins, such as two proteins, MIC6 (Reiss et al., 2001; Saouros et al., 2005) and MIC8 (Cerede et al., 2002), that are proposed to serve as micronemal escorter proteins (Meissner et al., 2002). MIC2 is also closely associated as a heterohexameric complex with an approximately 30-kDa protein, M2AP (Huynh et al., 2003; Jewett and Sibley, 2004), that is perhaps a chaperone or microneme trafficking subunit (Huynh et al., 2006). M2AP is thus far specific to Toxoplasma and Neospora, and might represent a clade-specific invention.

Few of the micronemal proteins are conserved as orthologs between Toxoplasma and Plasmodium, although this is likely due in part to the dynamic nature of the 'mix and match' domain architectures and their capacity to underpin functional homologs that do not have true orthologous relationships



FIGURE 17.4 The TRAP/MIC2 superfamily.

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(e.g. MIC2 and TRAP). Many micronemal proteins exhibit lineage-specific provenances; for example, Toxoplasma lacks the dbl domain family of micronemal proteins that are found in Plasmodium, such as the proteins EBA-175 and BAEBL that participate in erythrocyte binding. Other micronemal proteins show a wider phylogenetic distribution in the Apicomplexa, such as AMA1, which is essential for cell invasion (Hehl et al., 2000; Mital et al., 2005). AMA1 (AAB65410; 55.m00005) has two paralogs in Toxoplasma (583.m09144 and 145.m00588), although their gene structures, domain architectures, and functions have not been confirmed. Plasmodium and Theileria each have single copies of the gene, suggesting that AMA1 was amplified in the coccidian lineage. In contrast, Cryptosporidium lacks an AMA1 ortholog, and this likely reflects a fundamental distinction for this coccidian in its molecular mechanism of host-cell recognition or invasion.

17.3.2 Rhoptry proteins

Relatively less is known regarding the biological role of the rhoptries, which release their contents subsequent to micronemal release, once a junction with the host cell has been formed and invasion has commenced (Carruthers and Sibley, 1997). Rhoptries consist of a sac-like organelle that is filled with electron-dense arrays of proteins and lipids, and a duct-like neck at the conoid process of the apical complex. In an elegant recent study a molecular marker was identified, the rhoptry neck protein 4 (RON4), which distinguishes the rhoptry neck during the process of cell invasion (Lebrun *et al.*, 2005).

Recent strides have been made in characterizing the complement of rhoptry proteins, including 38 proteins that were identified in a recent proteomic survey (Bradley *et al.*, 2005). In keeping with the nomenclature of the MIC micronemal proteins (and GRA dense-granule proteins, below), the predicted rhoptry proteins are designated ROP or RON (for rhoptry neck proteins). Numerous of the predicted rhoptry secreted proteins have divergent, and possibly inactive, kinase domains, including ROP2 (CAA85377), ROP4 (AAU87405), ROP5 (AAZ73240), ROP8 (AAC47797), ROP11 (AAZ29607), and ROP16 (AAZ73239). ROP2 and ROP4 have been shown to be secreted into the parasitophorous vacuole, and are phosphorylated (Carey *et al.*, 2004). Their role during invasion or in the formation of the parasitophorous vacuole is not known; and they do not have recognizable homologs in other apicomplexans on which to base analogies of function.

In fact, few of the *Toxoplasma* rhoptry proteins appear to be conserved in other apicomplexans; and in those instances the amino-acid identities are less than 30 percent, frequently being relegated to single domains within large proteins such as the rhoptry neck proteins. It is difficult to discern orthologs versus highly divergent proteins having common ancestry (and thereby perhaps conserved functions). There are likely to be substantial lineagespecific distinctions in the catalogs of rhoptry proteins, and it will be interesting to determine the phylogenetic distribution of the catalogs within the Apicomplexa.

The rhoptry neck proteins 1 through 4 are a case in point; for example, RON1 appears to be conserved in Plasmodium, Theileria, and Cryptosporidium, particularly within a C-terminal region sushi domain, whereas RON3 is shared only with Plasmodium, with similarity within an N-terminal domain. Both RON3 and RON4 have possible paralogs in Toxoplasma (20.m03905 and 52.m01582, respectively), and the latter protein shares an N-terminal domain with possible orthologs in Plasmodium and Theileria. RON2 is found in Plasmodium and Theileria but not Cryptosporidium, and possesses a four-cysteine domain that is also found in the RhopH1/Clag proteins. However, as a complex, Plasmodium RhopH1/clag plus RhopH2 and RhopH3 (Kaneko et al., 2005) does not have recognizable orthologs in Toxoplasma, nor in Theileria and Cryptosporidium, although this conclusion should be approached with caution due to possible extreme sequence divergence of protein homologs.

17.3.3 Dense-granule proteins

The dense granules of *Toxoplasma* are distinct from the apical-secreted micronemes and rhoptries, and

appear to contain a unique complement of secreted proteins. Dense granules are exocytosed during and following invasion of host cells, to release their contents into the newly formed parasitophorous vacuole. The dense-granule proteins, termed GRA proteins, are thought to participate in the modification of the parasitophorous vacuole, although the function of none of the GRA proteins is known. The cellular biology of the dense granules is discussed in detail in Chapter 11, as well as in a recent review (Mercier *et al.*, 2005), and will not be discussed here; rather, brief structural and phylogenetic observations will be given for the GRA proteins (Table 17.1).

None of the GRA proteins shares paralogous relationships, nor are they composed of known domains, and their inclusion in a family is based upon their common cellular localization. All GRA proteins possess signal peptide sequences, and several additionally contain TM domains (namely GRA3, GRA4, GRA5, GRA6, GRA7, and GRA8). The orthologous relationships that are observed for the GRA proteins appear to be restricted to the coccidians Toxoplasma, Neospora, and Sarcocystis. Orthologs are not found in Plasmodium or Theileria and, moreover, the coccidian Cryptosporidium also appears to lack examples of the GRA proteins. It is possible that the sum catalog of GRA proteins defines a taxonomic difference in cellular architecture; that is, Plasmodium and Cryptosporidium may lack equivalents of the dense-granule organelles.

17.3.4 Protein trafficking in *Toxoplasma*

Trafficking motifs regulating targeting to rhoptry, microneme, and dense granules are little understood, and it is difficult to identify repertoires of proteins based upon specific targeting motifs (reviewed in Kaasch and Joiner, 2000; Joiner and Roos, 2002). Targeting to secretory organelles might be based upon conserved eukaryotic mechanisms that involve recognition of tyrosine residue-based motifs within the carboxy-terminal region of proteins (Hoppe *et al.*, 2000); features within transmembrane domains (Karsten *et al.* 2004); and association with escorter proteins (Rabenau *et al.*, 2001; Meissner *et al.*, 2002; Huynh *et al.*, 2006).

Notably, Toxoplasma lacks the Pexel/VTS motif that mediates targeting of Plasmodium falciparum proteins across the parasitophorous vacuole to the infected erythrocyte (Hiller et al. 2004; Marti et al., 2004), and it is not known whether Toxoplasma harbors an analogous repertoire of host-targeted proteins. It is beyond the scope of this chapter to describe trafficking motifs in further detail; however, in summary, the complete annotation of expanded gene families, and the prediction of organellar and secreted proteins within Toxoplasma, will benefit from screening for the above identified motifs, as well as screens to identify new motifs that might direct protein traffic within the parasite and host cell.

17.4 THE LCCL DOMAIN-CONTAINING PROTEINS

A family of LCCL domain-containing apicomplexan-specific proteins is remarkable for the complexity of its multi-domain architectures, and the orthologous conservation throughout the apicomplexan clade (Figure 17.3). The proteins are composed of both animal-like and bacterial-like domains, which together appear to confer manifold adhesive activities recognizing sugar, lipid, and protein moieties. The domain architectures of three members of the family (variously termed by different laboratories; herein CCp1, CCp2, and CCp3) are conserved in *Plasmodium, Cryptosporidium, Theileria*, and *Toxoplasma*, thus making them the most highly conserved multi-domain EC proteins in the Apicomplexa.

CCp1 and CCp2 are paralogs whose origin via gene duplication likely preceded the radiation of the Apicomplexa, inferred from their widespread conservation. CCp3 is present as a single copy gene in *Plasmodium*, *Cryptosporidium*, and *Theileria*, but is duplicated within the *Toxoplasma* genome sequence (TgSR1 and TgSR2; AAZ94422 and AAZ94423). The function of the CCp proteins is unknown, although their conservation suggests that their cellular localization and function are

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conserved. Targeted gene disruption in *Plasmodium* has revealed a requisite function during sporozoite development (Claudianos *et al.*, 2002; Pradel *et al.*, 2004), and this correlates with proposed sporozoite stage expression in *Cryptosporidium* (Tosini *et al.*, 1999, 2004). The protein family members are also expressed in *Plasmodium* gametocytes (Delrieu *et al.*, 2002; Pradel *et al.*, 2004), and thus the proteins may serve functions in multiple lifecycle stages. It is of interest to determine whether the CCp family members, or any of the component domains, are conserved in other alveolates, such as within the gregarines, *Perkinsus*, ciliates, or dinoflagellate species.

17.5 THE ARTICULINS

Although this chapter concerns events at the parasite membrane surface, it is worthwhile diverging slightly to discuss a little recognized group of cytoskeletal proteins termed the articulins. These proteins were initially described in the ciliates (Huttenlauch et al., 1995, 1998a, 1998b; Huttenlauch and Stick, 2003; Kloetzel et al., 2003), and are composed of proline- and valine-rich repeats. The articulins appear to be specific to the alveolate clade, but may have a widespread (Marrs and Bouck, 1992), and therefore ancient, distribution. All apicomplexans have multiple members of the family (Templeton et al., 2004b), and there are likely eight or more genes in Toxoplasma. It is not possible conclusively to identify orthologs, due to the extreme low complexity of the articulins. The articulins likely show stage-specific expression in all apicomplexans, as indicated by predicted macrogametocyte- (Pfs77; Baker et al., 1995) and sporozoite-specific (*Pb*IMC1a; Khater *et al.*, 2004) transcripts in Plasmodium. Three articulins have been described in Toxoplasma - IMC-1 (AAK39634;



FIGURE 17.5 Phylogenetic provenance of select apicomplexan proteins and domains. The protein and domain examples are discussed in the text. OWP, oocyst wall protein; MAM, meprin-like predicted adhesive domain; DAO, monoamine/diamine oxidase; CRMP, cysteine-rich modular protein family; CCp family, multi-domain proteins united by the presence of an LCCL domain; HINT domain, the carboxy-terminal autoprocessing domain of the animal hedgehog proteins; perforins, a domain in common with animal proteins such as the macrophage attack complex (MAC) perforin; vWA, EC versions of the von Willebrand factor A-like domain; 6-Cys domain, found in the *Plasmodium* P48/45 and P230 superfamily, and a highly divergence version in the *Toxoplasma* SAG family proteins and anonymous versions in *Theileria*. AMA-1, apical membrane antigen 1. SCP, $\alpha\beta$ -domain of the PR-1 superfamily fold.

44.m00004; see Mann and Beckers, 2001); IMC-3 (AAT49041; 35.m01595; see Gubbels *et al.*, 2004); and IMC-4 (41.m00021; see Hu *et al.*, 2006) – and exhibit a cellular localization consistent with a subpellicular cytoskeletal network. The ToxoDB identifiers for the additional members of the family are 44.m00020, 39.m00350, 52.m01590, 44.m00031, 42.m03481, 64.m00327, 49.m03173, and 55.m04893.

17.6 CONCLUSIONS

Complete annotation of the predicted Toxoplasma gondii proteome will provide a first estimate of the repertoire of EC proteins involved in a landscape of host-parasite interactions. This chapter attempts an initial distillation of these proteins into classes that can be refined and then addressed via determinations of life-cycle stages of expression, cellular localization, and studies of function. Whole genome comparisons will allow us to perform, at a molecular level, what taxonomists have labored upon for decades at an ultrastructural level: the phylogenetic organization of organisms according to phenotypic criteria (Figures 17.1 and 17.5). In this endeavor all Apicomplexan become 'model organisms', in that they are mutually revealing in the biology of this phylum of pathogenic protozoans.

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Comparative Aspects of Nucleotide and Amino-acid Metabolism in *Toxoplasma gondii* and other Apicomplexa

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18.1 INTRODUCTION

Nucleotides and amino acids are of fundamental importance in the replication and development of *Toxoplasma gondii* and other apicomplexan parasites. Nucleotides are in particular essential for replication of DNA and transcription of RNA in rapidly dividing stages. DNA synthesis relies on an ample supply of pyrimidine (dCTP, dTTP) and purine (dATP, dGTP) deoxynucleotide 5'-triphosphates, while RNA synthesis utilizes the ribonucleotides ATP, CTP, and GTP, as well as uridine 5'-triphosphate (UTP). Nucleotides are also essential in providing the cellular energy sources (ATP and GTP), and are involved in numerous other metabolic roles. Nucleotides provide precursors of more complex molecules such as folate, serve as nucleotide-based enzyme cofactors such as NAD+ or FAD, serve in regulatory roles as intracellular messengers such as cAMP, and also play roles in controlling metabolic and gene regulation. Nucleotides are either synthesized from small molecules and amino acids, or they are acquired via salvage pathways from preformed host-derived nucleobases and nucleosides. The apicomplexan parasites considered in this chapter, *Toxoplasma gondii, Plasmodium falciparum*, and *Cryptosporidium parvum*, are important pathogens of humans that cause significant morbidity and mortality. Notably, current treatment strategies in human infections caused by *T. gondii* or *P. falciparum* are based on blocking the accumulation of nucleotides. This validated approach to chemotherapy highlights the significance of further research to dissect details of nucleotide metabolism in the Apicomplexa as well as the potential for this research to lead to the development of new treatments.

Apicomplexa are obligate intracellular lower eukaryotic single-cell organisms that are only capable of replication when they properly associate with their parasitized host cell. They may exist in a viable form extracellularly for short periods of time, but are incapable of even a single cell division. A comparative approach examining three selected apicomplexan pathogens that differ greatly in their interactions with the human host was undertaken to begin to address the possible relationships between the host cell and environment occupied by each parasite, and the strategy that each parasite has adopted to ensure the delivery of a sufficient supply of nucleotides and amino acids to support rapid parasite replication. The recent completion of the genome sequences for T. gondii (http://toxodb.org), P. falciparum (http://plasmodb. org), and C. parvum (http://cryptodb.org) enables new predictions about parasite biology, opens new opportunities for development of chemotherapy directed at selected parasite targets, and permits a retrospective comparison of newly identified orthologs of genes involved in nucleotide and amino-acid metabolism with previous biochemical, cell biological, and genetic studies of nucleotide and amino-acid metabolism.

The study of nucleotide and amino-acid metabolism in Apicomplexa is extraordinarily complicated by the obligatory presence of the host cell. Human host cells are much larger than their apicomplexan inhabitants, are themselves rich in nucleotides and amino acids, and also possess extensive metabolic capacities to transport, synthesize, interconvert, and catabolize nucleotides and amino acids. Nucleotide and amino-acid metabolism of the obligate intracellular parasites still largely remains a mystery due to the complex interaction of the parasite and its host cell. While studies have examined the extracellular form of the parasite in regard to the transport, synthesis,

and interconversion of nucleotides, the isolation of free parasites without contaminating host material is uncertain. Consequently, genetic studies have been a particularly informative approach to examine the phenotype of engineered parasites lacking or gaining a gene involved in nucleotide or aminoacid metabolism, or alternatively, in examining the interaction of normal or mutant parasites in a mutant host cell. Most of the genetic studies have been performed in T. gondii due to the more rapid and robust genetic models available for manipulating this parasite (Kim and Weiss, 2004). Several important genetic selection models developed for T. gondii are also based on parasite nucleotide and amino-acid metabolism. Genetic models based on nucleotide metabolism are available in *P. falciparum*, but these models are not yet developed for C. parvum.

18.2 PURINES

Purines are crucial to all cells as required components of nucleic acids, a cellular energy source, and cofactors or substrates for specific aspects of cellular metabolism. All aspects of the apicomplexan life style, including motility-dependent invasion of host cells, is powered by nucleotides (Kimata and Tanabe, 1982). Remarkably, parasitic protozoa, with the exception of Acanthamoeba (Hassan and Coombs, 1986), lack the ability to synthesize the purine ring de novo (Marr et al., 1978; Berens et al., 1981; Wang and Simashkevich, 1981; Fish et al., 1982; Miller and Linstead, 1983; Wang and Aldritt, 1983; Wang, 1984,). Since T. gondii and other apicomplexan parasites lack the machinery to synthesize the purine ring de novo, they rely on essential capture, transport, and salvage machinery to steal purines from their hosts for incorporation into the parasite nucleotide pools (Perotto and Keister, 1971; Schwartzman and Pfefferkorn, 1982; Krug et al., 1989; Chaudhary et al., 2004; Ting et al., 2005). The integration of these pathways in Apicomplexa is likely to reflect the specific needs of each parasite, as well as the differing purine resources potentially available in various host cells, tissues, and environments inhabited by each parasite.

Although incapable of replication extracellularly even in the richest medium, early investigations in *T. gondii* demonstrated that the parasite could obtain purines and meet all other demands for normal intracellular replication even in host cells that are blocked in protein synthesis (Pfefferkorn and Pfefferkorn, 1981), and in enucleated host cells that are blocked in host RNA and DNA synthesis (Jones, 1973; Sethi *et al.*, 1973), as well as in host cells that are operating glycolytically due to deficient mitochondrial function (Schwartzman and Pfefferkorn, 1981).

The capture, transport, and salvage pathways necessary for purine acquisition have long been viewed as a significant weakness of the parasite that may be targeted for chemotherapy, and significant efforts have been directed towards this objective (Avila and Avila, 1981; Berens et al., 1984; Fish et al., 1985; Avila et al., 1987; Marr and Berens, 1988; Gero et al., 1989, 2003; Pfefferkorn et al., 2001). Current models propose that parasite purine transporters on the parasite plasma membrane are a required mechanism of purine acquisition from the host (Carter et al., 2003; de Koning et al., 2005). For apicomplexan parasites such as T. gondii or P. falciparum, this model suggests that host-cell-derived purines either passively or actively accumulate in the parasitophorous vacuole space. Therefore the purines acquired from the host by the intracellular parasite could be derived from some combination of potential resources, including de novo purine synthesis in the parasitized host cell, existing purine pools within the host cell, host-cell purine catabolism, or purine transport flux into the parasitized host cell. Few studies have experimentally addressed whether there is any requirement for host-cell purine tranporters. Significant progress has been made in understanding the purine transport capability of the apicomplexan parasites, as well as the parasite enzymes that facilitate interconversion, salvage, and incorporation of host purines into the parasite purine nucleotide pools. The host menu of purine compounds, the purine capture and transport capabilities of the parasite, and the specific activity levels of purine salvage and interconversion enzymes will ultimately determine the potential flux of purines from the host to the parasite.

18.2.1 Capture and transport

The details of purine transport capacity and purine transporters have been recently elucidated from several protozoans, with the majority of work being reported on transporter functions in Leishmania sp. and Trypanosoma sp. (Landfear et al., 2004; de Koning et al., 2005). Nucleoside and nucleobase transporters fall into two general groups; sodium-dependent concentrative nucleoside transporters (CNT), and non-concentrative equilibrative nucleoside transporters (ENT). Mammals and bacteria possess CNT and ENT transporters, while the protozoa only have ENT-type transporters (Landfear et al., 2004; Chaudhary, 2005; de Koning et al., 2005). Other genes for nucleobase transporters in bacteria, fungi, and plants are classified as the plant purine related transporter family, or the microbial purine related transporter family, or the nucleobase/ ascorbate transporter family (de Koning and Diallinas, 2000). Genes corresponding to these three groups of purine transporters have not been identified among the protozoa.

18.2.1.1 Genome analysis of purine transporters in Apicomplexa

The complete genome sequences of Toxoplasma gondii, Plasmodium falciparum, and Cryptosporidium parvum have identified putative ENT-type transporter genes, putative channels, and other transporters that may play a role in purine transport. The genome of C. parvum revealed remarkable transport capacities and putative purine transporters which await functional characterization (Abrahamsen et al., 2004; Xu et al., 2004). The *P. falciparum* genome encodes a previously characterized purine transporter, PfNT1, as well as three additional putative ENT orthologs (Bahl et al., 2002, 2003; Chaudhary, 2005; Kirk et al., 2005; Martin et al., 2005). Similarly, the T. gondii genome reveals TgAT1 and three additional ENT orthologs designated as TgNT1, TgNT2, and TgNT3 (Kissinger et al., 2003; Li et al., 2003; Chaudhary, 2005).

Recent work has demonstrated that TgAT1, TgNT1, and TgNT3 are expressed in tachyzoites,

bradyzoites, and sporulated oocysts, whereas TgNT2 is not (Chaudhary, 2005). TgAT1 and TgNT3 proteins are localized to the plasma membrane of intracellular tachyzoites, while TgNT1 was localized as a punctate labeling of the parasite cytosol within a compartment that did not co-localize with any other marker of a known *T. gondii* organelle (Chaudhary, 2005). Other than *T. gondii* TgAT1 and *P. falciparum* PfNT1, the functional role of each of these recently identified ENT orthologs is currently unknown.

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18.2.1.2 An early model of purine acquisition in T. gondii

The first working model of purine acquisition by T. gondii was proposed to be from host ATP, the most abundant host purine present at greater than 4 mM in host cell cytosol (Plagemann, 1986; Plagemann et al., 1988; Traut, 1994). The hypothesis that T. gondii used host cell ATP as its purine source was based on data from dual-label experiments showing that extracellular T. gondii readily incorporated the nucleoside component of ATP or AMP into nucleic acids, but did not incorporate the phosphate moiety (Schwartzman and Pfefferkorn, 1982). At this time it was also appreciated that the parasitophorous vacuole surrounding the intracellular tachyzoite was covered in a layer of host-cell mitochondria, an organelle rich in ATP (Jones et al., 1972). The identification of the parasitophorous vacuole surrounding intracellular tachyzoites as a passive permeation barrier suggested that the high concentration of cytosolic- or mitochondrial-derived host ATP potentially could permeate into the parasitophorous vacuole space and equilibrate at mM concentrations (Schwab et al., 1994).

The discovery of a remarkably abundant nucleoside triphosphate hydrolase (NTPase) activity secreted into the parasitophorous vacuole space suggested that host ATP may be hydrolyzed to AMP within the vacuolar space (Bermudes *et al.*, 1994; Sibley *et al.*, 1994; Asai *et al.*, 1995). Consequently, it was postulated that *T. gondii* might obtain its purine requirement from the flux created through permeation of ATP into the vacuolar space, conversion of ATP to AMP by vacuolar NTPase

activity, and conversion of AMP to adenosine by a putative parasite plasma membrane 5'-ectonucleotidase. Since the pool of host-cell adenosine is very small, at ~1 µM (Plagemann, 1986; Plagemann et al., 1988; Traut, 1994), utilization of host ATP pools was an attractive model to explain how host adenosine may be concentrated within the parasitophorous vacuole space. The first adenosine transporter characterized for T. gondii, TgAT1, was described as a non-concentrative low-affinity $(K_m \sim 120 \,\mu M)$ adenosine transport system that likely would require a higher concentration of adenosine than the 1 µM present in host-cell cytosol for physiological significance (Schwab et al., 1995). Subsequent work has demonstrated that intracellular tachyzoites as well as the parasitophorous vacuole space, and membrane, have no detectable 5'-ectonucleotidase activity (Ngo et al., 2000). Therefore, T. gondii has no significant access to intracellular pools of host cell nucleotides.

18.2.1.3 Functional properties of purine transporters in Apicomplexa

Functional studies of purine transporters in *C. parvum* are not yet available. By contrast, several purine transporters have been functionally characterized in *T. gondii*. TgAT1 is a low-affinity transporter for adenosine, guanosine, and inosine (Schwab *et al.*, 1995; Chiang *et al.*, 1999). Low-affinity transport of adenosine by TgAT1 is blocked by dipyridamole and inhibited by excess inosine, formycin B, or hypoxanthine, but not pyrimidines, suggesting a role in transport that is purine-selective and broad spectrum.

The subsequent selection and characterization of adenine arabinoside (ara-A) resistant mutants identified mutations at a genetic locus corresponding to a gene, designated as TgAT, and characterized as a putative 11-membrane-spanning region protein of the ENT family (Chiang *et al.*, 1999). Expression of TgAT in *Xenopus laevis* oocytes reconstituted low-affinity adenosine transport function similar to the previously characterized TgAT1, suggesting that TgAT is likely to represent the cloned gene of TgAT1 (Schwab *et al.*, 1995; Chiang *et al.*, 1999). Disruption of the TgAT one gene caused a complete loss of all adenosine transport function in extracellular tachyzoites (Chiang *et al.*, 1999).

Additional high-affinity nucleoside and nucleobase transporters were identified in extracellular tachyzoites of T. gondii (de Koning et al., 2003). This study characterized a low-affinity adenosine (K_m ~105 μ M) and inosine (K_m ~134 μ M) transporter equivalent to the previously characterized TgAT1 (TgAT). Kinetic evidence revealed the presence of a high-affinity adenosine ($K_m \sim 0.49 \ \mu M$) and inosine ($K_m \sim 0.77 \ \mu M$) transport system that was also a high-affinity and broad-spectrum transporter capable of transporting a large number of different purine and pyrimidine nucleosides. This second adenosine transport system was designated as TgAT2 (de Koning et al., 2003). The high affinity and broad specificity of TgAT2 suggested it could be an efficient route for uptake of various therapeutic nucleosides (de Koning et al., 2003). The same work also revealed a third transport system, TgNT1, which is the first purine transporter that has been demonstrated to selectively transport purine nucleobases. Based on competition kinetics, TgNT1 has a high affinity for hypoxanthine $(K_m \sim 0.91 \,\mu\text{M})$ as well as guanine and xanthine.

The presence of TgAT2 and TgNT1 on the tachyzoite plasma membrane of extracellular tachyzoites suggests an extraordinary capacity of T. gondii to salvage exceedingly low concentrations of purine nucleobases and nucleosides from within the parasitophorous vacuole space in the intracellular environment. This extraordinary capacity to scavenge purines may in part explain the remarkable ability of T. gondii to replicate in virtually any mammalian cell type. Yet while the T. gondii parasitophorous vacuole membrane is proposed to be a molecular sieve that permits passive permeation of neutral or charged molecules up to 1300-1900 Da between the host-cell cytoplasm and the parasitophorous vacuole space (Joiner et al., 1994, 1996; Schwab et al., 1994), experimental studies specifically addressing permeation or concentration of nucleobases, nucleosides, or nucleotides in the T. gondii parasitophorous vacuole space have not been reported.

P. falciparum expresses an 11 membranespanning ENT family nucleoside transporter designated PfNT1 (or PfENT1) which is localized to the parasite plasma membrane (Carter *et al.*, 2000; Parker *et al.*, 2000; Rager *et al.*, 2001). PfNT1 is expressed in all life stages and is markedly upregulated in young trophozoites, corresponding with an increased need for purines in replication. PfNT1 expression in *Xenopus laevis* oocytes confers high-affinity adenosine transport (K_m ~ 13 μ M) and low-affinity inosine transport (K_m ~ 253 μ M). Inhibition studies revealed PfNT1 is broad spectrum for a number of purine and pyrimidine nucleosides and, unlike its mammalian host, can transport both D- and L-nucleosides.

18.2.2 Purine transport in the parasitized host cell

The *P. falciparum*-parasitized erythrocyte exhibits remarkable alterations in transport functions compared with the normal erythrocyte (Kirk et al., 1994; Gero et al., 2003). Nucleosides enter parasitized erythrocytes through at least three parasiteinduced permeation pathways, including a saturable high-affinity adenosine transport system (Upston and Gero, 1995), through the tubulovesicular membrane (TVM) network connecting the parasitophorous vacuole to the erythrocyte periphery (Lauer et al., 1997), and through a non-saturable, anion-selective-type channel that also has capacity to transport L-nucleosides, amino acids, sugars, and cations (Kirk et al., 1994). P. falciparum has a TVM network extending from the parasitophorous vacuole as well as the requisite machinery to enable regulated protein secretion from the parasite cytoplasm to the vacuolar space, the erythrocyte cytoplasm, and the erythrocyte periphery (Lauer et al., 1997; Kyes et al., 2001).

The ability of parasitized erythrocytes selectively to transport L-nucleosides was recently used to elegantly deliver therapeutic agents to intracellular *P. falciparum* parasites, selectively killing them (Gero *et al.*, 2003). Conjugating 5-fluorouridine to L-adenosine or L-thymidine selectively delivered cytotoxic 5-fluorouridine to intracellular *P. falciparum*, whereas these hybrid molecules showed no transport or cytotoxicity in mammalian cells. It is unknown whether the new transport functions of the parasitized erythrocyte are due to modification or incorporation of new transporters, channels, ducts, or TVM near the erythrocyte periphery.

While mammalian cells lack any ability to transport the mammalian adenosine transporter inhibitor 6-thiobenzylthioinosine (NBMPR), *T. gondii*-parasitized host cells selectively transport NBMPR (Al Safarjalani *et al.*, 2003). NBMPR is selectively cytotoxic to intracellular *T. gondii* (el Kouni *et al.*, 1999). The activation of NBMPR cytotoxicity is associated with the ability of *T. gondii*-derived enzyme extracts, but not host-cell derived enzyme extracts, to phosphorylate NBMPR to its nucleoside 5'-monophosphate. Adenosine kinase (AK) deficient *T. gondii* fail to phosphorylate NBMPR to its nucleoside 5'-monophosphate, showing AK to be the major pathway to selective incorporation and cytotoxicity (Al Safarjalani *et al.*, 2003; Rais *et al.*, 2005).

Similar to P. falciparum-parasitized erythrocytes, T. gondii-parasitized host cells also selectively transport non-physiological β-L-enantiomers of purine nucleosides, β-L-adenosine, β-Ldeoxyadenosine, and β-L-guanosine. Uninfected host cells do not transport NBMPR or the β-Lnucleosides. NBMPR also inhibits the transport function of the host-cell nucleoside transporter ENT1 (es) (Gupte et al., 2005). Dipyridamole, another inhibitor of nucleoside transport, inhibited transport of NBMPR and β-L-nucleosides into parasitized host cells. Transport of NBMPR and β-L-nucleosides in the parasitized host cell required a functional TgAT1 transporter (Chiang et al., 1999; Al Safarjalani et al., 2003). While these observations explain a requirement for transport into the intracellular tachyzoite from the parasitophorous vacuole space, these studies do not specifically address why the T. gondii-parasitized host cell selectively transports these compounds. Therefore, infection with T. gondii confers parasitespecific transport mechanisms to the host cell.

The novel transport capacity of parasitized host cells opens a new avenue towards developing chemotherapeutic approaches, as well as addressing other biological modifications of the parasitized host. The novel transport mechanisms specific to the *T. gondii*-parasitized host cell may entail numerous possibilities, including the recently reported

equilibratory high-affinity adenosine transporter (de Koning *et al.*, 2003), a concentrative ion-dependent channel, a tubulovesicular membrane (TVM) system interconnecting the parasitophorous vacuole to the host cell periphery, and a duct for transport of macromolecules that bypasses the host-cell membrane (Gero *et al.*, 2003).

The elegant electrophysiological description of the mechanism of parasitophorous vacuole formation in T. gondii suggested that, after T. gondii invasion and vacuole formation, a fission pore remnant is left on the host-cell surface (Suss-Toby et al., 1996). Shortly after invasion of the host cell, a protein- and membrane-rich intravacuolar network derived from electron-dense granules is formed in the parasitophorous vacuole space (Sibley et al., 1995; Mercier et al., 1998, 2002). Three-dimensional imaging of T. gondii within recently formed vacuoles revealed fibrous and tubular material that connects the parasite plasma membrane on intracellular tachyzoites within the parasitophorous vacuole to the remnant of the fission pore at the host-cell plasma membrane (Schatten and Ris, 2004). Collectively, these observations suggest that the transport of nutrients such as purines to the tachyzoite within the parasitophorous vacuole may be facilitated by additional mechanisms beyond simple diffusion of nutrients within host-cell cytosol through proposed pores in the parasitophorous vacuole membrane. Further studies are necessary to assign functional roles to the ENT gene orthologs identified in T. gondii, the requirement for hostcell transporters, and the potential role of the tubulovesicular network in the parasitophorous vacuole space, or to understand other mechanisms that promote permeation of host purines to the intracellular parasite.

18.2.3 Purine interconversion and salvage pathways in Apicomplexa

Early studies in non-replicating extracellular tachyzoites demonstrated that the purine ring precursor, glycine, was poorly incorporated into *T. gondii* nucleic acids (Perotto and Keister, 1971). When *T. gondii* were grown in a mutant host cell deficient in *de novo* purine synthesis, glucose and formate precursors of the guanine and adenine ring were also poorly incorporated by intracellular *T. gondii* (Schwartzman and Pfefferkorn, 1982). A third study demonstrated that *T. gondii* could not synthesize the purine nucleotides *de novo* from formate, glycine, or serine (Krug *et al.*, 1989). Collectively, these studies clearly demonstrated that *T. gondii* cannot synthesize purines *de novo* and therefore strictly relies on stealing a supply of purines from the host.

The potential of targeting the purine auxotrophy of apicomplexan parasites for inhibition of parasite replication was also illustrated in early studies. Replication of *T. gondii* is efficiently inhibited by adenine arabinoside (ara-A), and the establishment of parasite mutants resistant to ara-A demonstrated that a parasite adenosine kinase (AK) activity is required for activation and incorporation of ara-A (Pfefferkorn and Pfefferkorn, 1976, 1978).

Most of the reported work on purine auxotrophy in apicomplexan parasites has focused on the machinery responsible for interconverting purines and incorporating host purine nucleobases and nucleosides into the parasite nucleotide pools. This work is fairly extensive, and has included studies on:

- · enzyme activities
- gene cloning, expression, kinetic analysis, structure determination
- · regulation and cellular localization
- gene knockouts
- mutant host cells
- · genome and evolutionary analysis.

T. gondii has presented the most amenable model for pursuing all of these varied approaches to deciphering purine acquisition in Apicomplexa.

T. gondii possesses significant machinery for purine salvage, and this extensive machinery may enable this parasite to survive and replicate in an extensive range of mammalian cell types. By contrast, *C. parvum* possesses the most diminished purine salvage machinery of the apicomplexans, and this diminished capacity is likely to be related to the nutrient-rich, but restricted, niche of this parasite. The *P. falciparum* purine pathways are only slightly less robust than pathways present in *T. gondii.* Nonetheless, *Plasmodium* sp. is atypical in infecting and replicating within erythrocytes, and this apicomplexan parasite possesses novel adaptations in the purine pathways that are not observed in other parasites.

18.2.3.1 Purine salvage pathways in T. gondii

Recent genetic studies in *T. gondii* have complemented biochemical approaches to more clearly define the transport and purine salvage capacity of this parasite. In early studies, {³H} hypoxanthine labeling of *T. gondii*-infected Lesch-Nyhan mutant human host cells, deficient in hypoxanthineguanine phosphoribosyltransferase activity, demonstrated that only intracellular parasites were labeled with no detectable incorporation into hostcell nucleic acids (Pfefferkorn and Pfefferkorn, 1977a). Therefore, the flux of purines is unidirectional from the host cell to the parasite.

The only comprehensive biochemical investigation of parasite activities involved in salvage, interconversion, and incorporation of host purines in T. gondii was assessed in viable extracellular tachyzoites (Krug et al., 1989). To some degree, host-cell membranes and host purine metabolism enzymes may contaminate preparations of extracellular tachyzoites (Ngo et al., 2000). This study also used high concentrations of radiolabeled purines to maximize transport and incorporation of purines in extracellular tachyzoites, and such high concentrations of purines are unlikely to be available to the intracellular tachyzoite. Extracellular tachyzoites also are not replicating organisms, and this model may not accurately reflect the complexity of the purine interactions between intracellular parasites and the host cell.

The purine bases hypoxanthine, xanthine, guanine, and adenine were incorporated and indicated the presence of a hypoxanthine-xanthineguanine phosphoribosyltransferase (HXGPRT) activity as well as an adenine phosphoribosyltransferase (APRT) activity (Krug *et al.*, 1989). Adenine was incorporated one-half as efficiently as hypoxanthine. Guanine was incorporated at 55 percent, and xanthine at 67 percent of the rate at which hypoxanthine was incorporated (Krug *et al.*, 1989). Subsequent studies have demonstrated that *T. gondii* has no APRT gene or activity (Chaudhary *et al.*, 2004).

The purine nucleosides adenosine, inosine, guanosine, and xanthosine were incorporated into nucleic acids. Adenosine was incorporated more than 12-fold as well as any other purine nucleoside or nucleobase, and suggested a parasite adenosine kinase (AK) to be the major route to AMP. By contrast, hypoxanthine was incorporated at 8.3 percent, inosine at 8.2 percent, xanthine at 5.6 percent, guanosine at 2.5 percent, adenine at 3.9 percent, guanosine at 2.5 percent, and xanthosine at 0.3 percent of the rate at which adenosine was incorporated (Krug *et al.*, 1989). Correspondingly, in parasite protein extracts prepared from extracellular tachyzoites, AK activity was greater than 15-fold more active than the next most active enzyme.

Guanine, guanosine, xanthine, and xanthosine labeled only guanylate nucleotides. Therefore, *T. gondii* has no pathway from guanylate to adenylate nucleotides (Krug *et al.*, 1989). Adenosine, inosine, and hypoxanthine labeled adenylate and guanylate nucleotide pools at approximately equal ratios (Krug *et al.*, 1989; Pfefferkorn *et al.*, 2001).

Purine nucleoside phosphorylase (PNP) activities were detected only for inosine and guanosine. Deaminase activities were detected for guanine (GUAD), adenine (ADE), adenosine (ADA), and AMP (AMPD) (Krug et al., 1989). While the reported GUAD may be present in T. gondii, this activity is not shown on the current model of purine pathways (Figure 18.1A) because this activity was low; there is abundant host GUAD that may contaminate tachyzoite preparations, and no gene ortholog is yet detected for a T. gondii GUAD (Chaudhary et al., 2004). While T. gondii ADA activity was demonstrated in tachyzoites grown in a mutant host cell deficient in host adenosine deaminase (Krug et al., 1989), the AK pathway is by far the most significant pathway for incorporation of adenosine due to the high specific activity of AK. While no putative gene ortholog for ADA has been yet identified, this pathway is present and may be significant, particularly to parasites lacking AK activity (Chaudhary et al., 2004).

A recent study reported that adenine was variably but generally poorly incorporated into *T. gondii* nucleic acids during infection of normal host cells, but in host cells that are deficient in APRT activity the adenine incorporation was significantly less than previously reported in Krug et al. (1989) (Chaudhary et al., 2004). While a putative gene ortholog for T. gondii ADE has been identified, the ADE pathway to hypoxanthine appears to be a minor pathway for this parasite (Krug et al., 1989; Chaudhary et al., 2004). Hypoxanthine is converted to inosine 5'-monophosphate (IMP) by HXGPRT. Once IMP is available, AMP can be made in two steps by adenylosuccinate synthetase (ADSS) and adenylosuccinate lyase (ADSL), and GMP can be made in two steps by IMP dehydrogenase (IMPDH) and GMP synthetase (GMPS). Therefore, interconversion of nucleotides occurs only in the direction of adenylate to guanylate nucleotides via AMPD (Figure 18.1A) (Krug et al., 1989; Pfefferkorn et al., 2001).

There is no GMP reductase, and other than AK no other nucleoside kinase or phosphotransferase activities are present (Krug *et al.*, 1989). Therefore, *T. gondii* possesses a minimum of 10 enzymes involved in the interconversion and salvage of host purines. Gene orthologs have been reported for nine of these enzymes in *T. gondii* (ADE, PNP, AK, HXGPRT, ADSS, ADSL, AMPD, IMPDH, and GMPS) (Chaudhary *et al.*, 2004). *T. gondii* can transport and salvage the host nucleosides adenosine, inosine, and guanosine, as well as the host nucleobases adenine, hypoxanthine, xanthine, and guanine (Figure 18.1A). The parasite can incorporate host purines into the parasite nucleotide pool by two major routes, via AK and HXGPRT.

Adenosine kinase and hypoxanthine-xanthineguanine phosphoribosyltransferase Incorporation of host adenosine into the AMP pool by AK appears to be the most significant purine salvage pathway (Krug *et al.*, 1989; Ngo *et al.*, 2000; Chaudhary *et al.*, 2004). Yet resistance to ara-A due to mutation and disruption of parasite AK was described even in early studies (Pfefferkorn and Pfefferkorn, 1976, 1978). Subsequently, a genome-wide insertional mutagenesis screen was used to select ara-Aresistant mutants. One class of isolated mutants was disrupted in the AK gene and activity, demonstrating



FIGURE 18.1 Model of purine transport and salvage pathways in (A) T. gondii; (B) C. parvum;



FIGURE 18.1 cont'd (C) P. falciparum.

The host cell cytosol is shown outside of the parasitophorous vacuole membrane. The parasitophorous vacuole space and the parasite plasma membrane are indicated. Purine transporters are shown as cylinders resting in the parasite plasma membrane. Purine compounds accessible to each parasite are shown inside the parasitophorous vacuole space. Inside the parasite cytosol the enzymic machinery managing purine interconversion and salvage of host purine nucleobases and nucleosides into the adenylate (AMP) and guanylate (GMP) nucleotide pools is shown. Solid lines and arrows depict active pathways. Where information on purine flux is available the weighting of the pathway is emphasized by the weight of the lines and arrows. The weighting of pathways described in Figure 18.1 reflects the most likely predictions from available data; however, the weightings shown in these diagrams are only hypothetical and the purine flux of host purine to the parasite, as well as interconversion and incorporation within the intracellular parasite, remains to be experimentally tested. Substrates of each enzyme activity are shown on the side of the solid line, and the product(s) of each enzyme activity are shown on the arrowhead side. The enzyme activity responsible for each interconversion step is shown in capital italicized text beside the arrowhead line. Adenosine kinase (AK) and hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) represent the major pathways for salvage and incorporation into the nucleotide pool, and these key activities, if present, are shown. ADA, adenosine deaminase; ADE, adenine deaminase; PNP, purine nucleoside phosphorylase; ADSS, adenylosuccinate synthetase; ADSL, adenylosucccinate lyase; AMPD, AMP deaminase; IMPDH, inosine 5'-monophosphate dehydrogenase; GMPS, GMP synthetase; SS, spermidine synthetase; MTA, 5'-methylthioadenosine; MTI, 5'-methylthioinosine; PRPP, 5-phosphoribosyl-1-pyrophosphate. Enclosed triangles: PS, polyamine synthesis; S, spermidine.

again that a parasite with disrupted AK function still replicates normally, most likely by salvage through the HXGPRT (Sullivan *et al.*, 1999).

T. gondii AK has been expressed in *E. coli* for biochemical, kinetic, and structural studies that have revealed significant differences between the parasite AK and the mammalian AK, which may be exploited for drug design (Darling *et al.*, 1999; Schumacher *et al.*, 2000a, 2000b). Subversive substrates of *T. gondii* AK have been shown to be selectively toxic to the parasite (Pfefferkorn and Pfefferkorn, 1976; Iltzsch *et al.*, 1995; el Kouni *et al.*, 1999). Interestingly, the AK gene from *C. parvum* complements AK-deficient *T. gondii*. This complementation system may present a useful high-throughput model by which to screen for potential inhibitors of *C. parvum* AK (Striepen *et al.*, 2004).

HXGPRT represents the second major route of incorporation of host purines. Yet, as for AK, disruption of T. gondii HXGPRT has no significant effect on replication or viability of tachyzoites (Pfefferkorn and Borotz, 1994; Donald et al., 1996). The unique xanthine phosphoribosyltransferase activity of HXGPRT, which is absent in the human host, may be exploited for drug design using toxic analogs of xanthine. This approach has been validated in studies using 6-thioxanthine, a compound with selective toxicity to T. gondii (Pfefferkorn and Borotz, 1994; Pfefferkorn et al., 2001). The crystal structure and enzyme mechanisms of T. gondii HXGPRT have been determined, and the parasite HXGPRT is a potential drug target (Schumacher et al., 1996; Heroux et al., 1999a, 1999b).

Parasites completely deficient in HXGPRT or AK are viable, suggesting that either pathway can suffice for purine incorporation and parasite replication (Donald *et al.*, 1996; Sullivan *et al.*, 1999). In the *T. gondii* HXGPRT knockout parasite, host adenosine would be required for parasite replication and would be incorporated into adenylate nucleotides by AK, and then into guanylate nucleotides by AK, AMP deaminase, IMPDH, and GMPS (Figure 18.1A). Conversely, in a *T. gondii* AK knockout mutant, host guanine, guanosine, and xanthine would provide guanylate nucleotides, but no adenylate nucleotides could be formed from these purines. In this mutant host adenine, adenosine, inosine, or hypoxanthine could potentially satisfy the parasite's demand for both adenylate and guanylate nucleotides (Figure 18.1A). All of the potential host purine precursors would funnel through hypoxanthine into the IMP pool, which can go to guanylate nucleotides through IMPDH and GMPS, and to adenylate nucleotides through ADSS and ADSL.

Therefore, unlike P. falciparum and C. parvum (discussed below), T. gondii possesses a functionally redundant purine salvage pathway (AK and HXGPRT) with the capacity to meet the purine requirement by using an assortment of potential host-cell purine nucleobases and nucleosides. This feature of T. gondii may help to explain how the parasite is capable of replicating in such a wide variety of host cells and tissues that are likely to present quite varied potential purine resources. The complex purine salvage pathway of T. gondii also suggests that this pathway is likely to be subjected to complex regulatory mechanisms (Figure 18.1A). For instance, parasites that are growth-inhibited by treatment with 6-thioxanthine incorporate fourfold more hypoxanthine and xanthine into nucleic acids than do untreated control parasites. This increase in salvage of hypoxanthine and xanthine is not due any increase in specific activity of HXGPRT, but involves some other aspect of the salvage pathways (Pfefferkorn et al., 2001).

Recent genetic studies have been performed on *T. gondii* AK and HXGPRT that help to clarify previous observations. HXGPRT and AK activities cannot be simultaneously disrupted in *T. gondii*, suggesting that these are the only functional routes to purine nucleotides in the parasite (Chaudhary *et al.*, 2004). Consequently, it is feasible to knock out the parasite AK and HXGPRT activities as long as at least one functional pathway to purine nucleotides is provided in *trans*. Low activities of APRT activity are detected in lines complemented by a functional APRT gene from *Leishmania donovani*, but simultaneous deletion of the genes for AK and HXGPRT is still not possible (Chaudhary *et al.*, 2004).

This genetic study also demonstrated that single gene knockouts of parasite AK or HXGPRT have

small but detectable defects in fitness, as determined by growth rate of tachyzoites. AK-deficient parasites exhibit a fitness defect in growth rate of 7.6 percent per generation, while HXGPRTdeficient parasites exhibit a fitness defect in growth rate of 3.7 percent per generation (Chaudhary *et al.*, 2004). This significant finding suggests that purine acquisition may be rate-limiting to parasite growth rate. In the case of the AK knockout parasite the same host supply of adenosine would be available as in the wild-type parent, thus the flux of adenosine, when diverted by lack of AK activity, to inosine to hypoxanthine to IMP, then to guanylate and adenylate nucleotides, is not sufficient to support fully the normal parasite growth rate.

Therefore, parasite transport and incorporation of host adenine, adenosine, inosine, hypoxanthine, guanosine, guanine, and xanthine through parasite HXGPRT is insufficient to fully support normal parasite replication (Figure 18.1A). Similarly, in an HXGPRT knockout, transport and incorporation of host adenosine through the high-activity AK pathway is insufficient to fully support parasite replication, suggesting the host supply of adenosine itself is not quite sufficient to fully support the maximum parasite replication rate. Collectively, these observations suggest multiple host purine nucleobases and/or nucleosides, and both pathways of incorporation of host purines into the nucleotide pools of T. gondii are likely to be required for supporting a maximum replication rate. Considering the very high specific activity of parasite AK (Krug et al., 1989), the bottleneck for purine flux to the replicating intracellular tachyzoite is most likely due to a limited availability of host purines, or a limited transport capacity of the parasitophorous vacuole and intracellular tachyzoite.

Multiple isoforms and localization of HXGPRT A novel feature of the *T. gondii* purine salvage pathway is the expression of two forms of HXGPRT from a single gene locus by alternatively spliced mRNA (Donald *et al.*, 1996; White *et al.*, 2000). The two isoforms differ by a 49 amino-acid segment comprising an extra exon in isoform II. Both isoforms behave in a kinetically similar manner, although isoform II is slightly less efficient in

recognizing guanine as a substrate (White *et al.*, 2000). The cellular compartmentalization of the two HXGPRT isoforms is different. Isoform I is cytosolic, while the longer version isoform II is localized to the inner membrane complex (IMC) of the tachyzoite (Chaudhary *et al.*, 2005). The 49 amino-acid insert at the N-terminus of isoform II is required for the localization to the IMC. The mechanism of IMC localization was identified to be palmitoylation, which occurred at three adjacent cysteine residues within a 49-amino-acid insert. Mutation of these three cysteines blocked palmitoylation and localization to the IMC (Chaudhary *et al.*, 2005).

The biological basis of functional redundancy of HXGPRT in *T. gondii* is not obvious because both isoforms are functionally competent HXGPRT activities (Donald *et al.*, 1996; Donald and Roos, 1998). It is possible that this functional redundancy in HXGPRT enables *T. gondii* to grow in a wider variety of cell types where purines may be limiting and additional mechanisms may be required for purine transport and salvage. Alternatively, this novel dual localization of HXGPRT may reflect an economy of purine metabolism within the parasite itself, or perhaps some unknown aspect of purine regulation.

Studies on T. gondii purine nucleoside phosphorylase The T. gondii PNP gene has been cloned and expressed in E. coli. Similar to human and P. falciparum PNP, the recombinant T. gondii PNP enzyme recognizes inosine and guanosine as good substrates, and adenosine and xanthosine as poor substrates. Both T. gondii PNP and malaria PNP are unusual in that deoxynucleosides are poor substrates (Chaudhary et al., 2006). This unusual substrate property of the parasite PNP suggests that the intracellular parasite may have access to the host pool of guanosine and inosine, but would poorly incorporate host deoxyguanosine or deoxyinosine transported by the parasite. T. gondii PNP demonstrated no activity against 5'-methylthioadenosine (MTA) or 5'-methylthioinosine (MTI), and was insensitive to inhibition by methylthio (MT)-immucillin-H (Chaudhary et al., 2006). Immucillin-H is a strong nM inhibitor of the parasite enzyme in vitro (Chaudhary et al., 2006).

While the replication of wild-type T. gondii parasites is completely unaffected by immucillin-H in vitro, an AK knockout mutant of T. gondii is inhibited by immucillin-H (Chaudhary et al., 2006). The growth inhibition of immucillin-H in the AK knockout background is largely reversed by providing excess hypoxanthine to the in vitro culture medium (Chaudhary et al., 2006). If host adenine and hypoxanthine were being supplied in sufficient amounts to the replicating parasite, this purine supply should confer resistance to immucillin-H even in an AK knockout mutant (Figure 18.1A). Therefore, based on the growth inhibition of an AK knockout by immucillin-H, it is possible that the supply of host adenosine and inosine in the intracellular environment exceeds the potential supply of host adenine and hypoxanthine, suggesting that host nucleosides rather than host nucleobases are the more important purine pool (Figure 18.1A). A caveat to this interpretation of the inhibition of parasite PNP by immucillin-H is the possibility that hypoxanthine may, partly, antagonize inhibition of PNP, or that host PNP is also inhibited by immucillin-H, thus reducing host purine pools (Kicska et al., 2002a). Nonetheless, these studies further validate the proposed pathways for purine incorporation and salvage in T. gondii (Figure 18.1A).

Genetic selection based on T. gondii HXGPRT Early studies established the parasite HXGPRT as both a potential drug target and a gene that would be amenable for both positive and negative genetic selection in T. gondii (Pfefferkorn and Borotz, 1994). The mechanism of 6-thioxanthine inhibition is based on activation of 6-thioxanthine to 6-thioxanthine 5'-monophosphate by parasite HXGPRT (Pfefferkorn et al., 2001). Unlike mercaptopurine in mammals (Elion, 1989a, 1989b), 6-thioxanthine and its nucleotide product 6-thioxanthine 5'-monophosphate are not a substrate for T. gondii GMPS and are not incorporated into nucleic acids. The mechanism of inhibition is parasitostatic, and primarily involves inhibition of parasite IMPDH by 6-thioxanthine 5'-monophosphate (Pfefferkorn et al., 2001).

T. gondii mutants deficient in HXGPRT are completely resistant to the toxic effects of

6-thioxanthine (Pfefferkorn and Borotz, 1994). Once 6-thioxanthine resistance was selected by knockout of HXGPRT, parasites with a functional HXGPRT could be positively selected by growth in mycophenolic acid (MPA) with supplements of xanthine or guanine (Pfefferkorn and Borotz, 1994). This selection scheme is based on the ability of MPA specifically to inhibit IMPDH, blocking the conversion of IMP to XMP (Figure 18.1A). Thus parasites with a non-functional HXGPRT cannot be rescued with xanthine or guanine when IMPDH is inhibited, whereas parasites with a functional HXGPRT will be rescued by xanthine or guanine supplementation of growth medium, and this pathway will bypass the inhibition at IMPDH. The biochemical description of this selection strategy also proved that T. gondii is perfectly capable of obtaining adenylate nucleotides by the AK pathway, and guanylate nucleotides by HXGPRT salvage of xanthine to XMP, or salvage of guanine to GMP (Pfefferkorn and Borotz, 1994).

The identification of the HXGPRT gene enabled a test of this biochemical prediction and resulted in the establishment of a robust genetic selection scheme for positive and negative selection using the selection principles described above (Donald et al., 1996). The HXGPRT selection scheme established the first genetic system for hit-and-run mutagenesis in T. gondii, where a stable pseudodiploid can be established during positive selection, then negative selection used to force out the HXGPRT gene to create a subtle or major mutation within the gene locus of interest (Donald and Roos, 1998). The HXGPRT genetic model has been extensively used to generate knockout mutants in non-essential parasite genes as well as in genetic studies of parasite development. This genetic system was also adapted for functional cloning studies and has resulted in the initial identification of the ε-proteobacterium-type IMPDH gene from C. parvum (Striepen et al., 2002).

18.2.3.2 Purine salvage pathways in C. Parvum

Experimental work on purine pathways in *C. parvum* has been limited by the lack of robust

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systems for culturing this parasite, the lack of any genetic models, and the difficulty of obtaining purified parasites for biochemical analysis. The Cryptosporidium genomes have given the first detailed insights into the strategy this parasite has adopted to satisfy its appetite for host-cell purines (Abrahamsen et al., 2004; Striepen et al., 2004; Xu et al., 2004). In addition to its limited ability to synthesize amino acids and pyrimidines (discussed below), C. parvum also possesses a very limited repertoire of purine salvage and interconversion enzymes compared to other apicomplexans (Chaudhary et al., 2004; Striepen et al., 2004). While an early study using crude parasite extracts suggested that C. parvum expressed HXGPRT and APRT activities (Doyle et al., 1998), C. parvum lacks any gene ortholog for APRT or HXGPRT, and the parasite is insensitive to the 6-thioxanthine (Pfefferkorn et al., 2001; Chaudhary et al., 2004; Striepen et al., 2004).

Remarkably, adenosine is the only host purine that is of any physiological significance to C. parvum, and is supplied by the gut environment or host cells occupied by C. parvum (Figure 18.1B). Similar to T. gondii and P. falciparum, C. parvum possesses an adenosine transporter gene ortholog that is likely to be responsible for transport of host adenosine into the parasite cytosol (Abrahamsen et al., 2004). When host adenosine is available in the parasite cytosol, the parasite can incorporate adenosine into the nucleotide pool by the AK pathway to AMP. Once the parasite has a nucleotide pool of AMP, this pool meets the entire demand for all adenylate and guanylate nucleotides (Abrahamsen et al., 2004). AMP is first deaminated to IMP by AMPD. IMP is then converted to XMP by IMPDH, and XMP is converted to GMP by GMPS (Figure 18.1B). Since there is no reverse pathway from IMP to AMP, the nucleotide flux extends only from AMP to GMP. The C. parvum IMPDH gene was acquired by horizontal gene transfer from an ε-proteobacterium (Striepen and Kissinger, 2004; Striepen et al., 2002, 2004). The parasite possesses only these four enzymes to direct the unidirectional flow of transported host adenosine to parasite adenylate and guanylate nucleotides (Figure 18.1B).

Potential drug targets in C. parvum purine salvage pathways Unlike T. gondii, each of the C. parvum purine pathway enzymes (AK, AMPD, IMPDH, and GMPS) individually represents a potential drug target. The C. parvum AK activity has been validated as a drug target in genetic experiments using an AK-deficient mutant of T. gondii. C. parvum AK complements the AK deficiency in T. gondii, restoring significant AK activity (Striepen et al., 2004).

IMPDH inhibition using drugs such as ribavirin, mizoribine, and MPA has been pursued as a strategy to induce guanylate nucleotide depletion for therapy in cancer, immunosuppressive chemotherapy, and infectious diseases (Allison and Eugui, 2000, 2005; Pockros et al., 2003). The ability of humans and many pathogens to phosphoribosylate guanine to GMP may dilute the potential therapeutic effect of IMPDH inhibition by providing a second route to GMP. The absence of this second route to GMP in C. parvum suggests guanylate starvation can be effectively induced via inhibition of IMPDH or GMPS, as well as AK and AMPD. Ribavirin and MPA were shown in early work to inhibit C. parvum development (Woods and Upton, 1998). Recent work has shown that C. parvum development is inhibited in a dose-dependent manner by these IMPDH inhibitors (Striepen et al., 2004).

18.2.3.3 Purine salvage pathways in P. falciparum

The purine salvage and interconversion pathways are more diverse in *P. falciparum* than in *C. parvum*, and may be slightly less robust than the pathways present in T. gondii. Yet P. falciparum (and Plasmodium sp.) uniquely possess enzymes of purine metabolism that are highly adapted to the intracellular challenges faced by this parasite. Within the Apicomplexa, *Plasmodium* sp. is unique in surviving and replicating within erythrocytes. The erythrocyte is a highly differentiated cell type that has lost many capabilities normally present in other mammalian cell types, and therefore has very limited nucleotide requirements. The human erythrocyte also has no nucleus, does not synthesize DNA, RNA, or protein, and lacks both purine and pyrimidine de novo synthetic pathways.

To fulfill its nucleotide requirements, P. falciparum appears to be capable of transporting the purine nucleobases hypoxanthine, xanthine, and guanine, as well as the purine nucleosides, adenosine, inosine, and guanosine. P. falciparum possesses appropriate enzymic machinery for the interconversion and incorporation of any of these six potential purine sources (Figure 18.1C) (Chaudhary et al., 2004). P. falciparum has no reverse pathway from guanylate to adenylate nucleotides, and while the host-supplied xanthine, guanine, or guanosine, collectively, could meet the parasite's demand for guanylate nucleotides, these precursors could not supply any adenylate nucleotides. Host adenosine, inosine, and hypoxanthine, individually or collectively, are therefore likely to be necessary for meeting the parasite's demand for adenvlate and guanylate nucleotides. While the parasite can transport additional purine compounds, their physiological relevance, if any, is not clear at this time. For example, adenine is transported by P. falciparum, yet is not incorporated because the parasite lacks APRT and ADE genes and activities (Chaudhary et al., 2004).

Similar to T. gondii, P. falciparum can convert adenosine to inosine via ADA, and inosine is then converted to hypoxanthine by parasite PNP (Figure 18.1C). Similar to human and T. gondii PNP, P. falciparum PNP does not recognize either xanthosine or adenosine as a good substrate (Kicska et al., 2002a). Hypoxanthine is incorporated into the nucleotide pool as IMP by HXGPRT. Once IMP is available, the parasite can meet its entire demand for adenylate and guanylate nucleotides. Notably, P. falciparum lacks any detectable AK gene or activity. IMP is converted to AMP in two steps by sequential reactions of ADSS and ADSL. This is the only pathway to AMP. The parasite can balance IMP and AMP pools in the reverse reaction of AMP deamination to IMP (Figure 18.1C).

Since *P. falciparum* has no direct route to AMP from adenosine, the most important purine for incorporation into purine nucleotides becomes hypoxanthine. Hypoxanthine is the only purine compound that can completely satisfy the parasite's demand for both adenylate and guanylate nucleotide pools. As also seen in both *T. gondii* and

C. parvum (Figure 18.1), IMP is converted to XMP by parasite IMPDH, and subsequently to GMP by parasite GMPS. XMP and GMP are also incorporated in *P. falciparum* through the phosphoribosylation of xanthine and guanine, respectively. Thus the parasite ultimately incorporates all purine nucleotides via parasite HXGPRT activity, and possesses a minimum of seven distinct purine interconversion or incorporation activities (Figure 18.1C).

Potential drug targets in the P. falciparum *purine salvage pathway* The machinery used by *P. falciparum* to satisfy its purine auxotrophy suggests that purine transporters, parasite HXGPRT, certain purine interconversion enzymes, or a recently discovered novel purine recycling or salvage pathway may all be amenable targets for drug development. Due to the absence of AK activity in *P. falciparum*, ADA and PNP activities were recognized as potential drug targets in early studies (Daddona *et al.*, 1984, 1986; Webster *et al.*, 1984).

Hypoxanthine could be directly transported by the parasite from the parasitized host cell or, alternatively, hypoxanthine could be supplied from transported inosine and adenosine via ADA and PNP activities. Host erythrocyte hypoxanthine pools may increase during infection from host catabolism of ATP in deteriorating parasitized erythrocytes. Host erythrocyte ATP is catabolized sequentially, via ADP, AMP, adenosine, and inosine, to produce hypoxanthine via host PNP (Ting et al., 2005). It is known that depletion of hypoxanthine effectively inhibits P. falciparum replication in vitro (Berman et al., 1991; Berman and Human, 1991); thus the physiological source of hypoxanthine is a critical question to be answered for optimizing strategies to inhibit purine acquisition in Plasmodium infections. If the bulk of incorporated hypoxanthine is derived strictly in the parasite cytosol from transported host purines, the parasite ADA and PNP activities may be the optimal drug targets. Complicating this analysis, however, are recent studies that demonstrate P. falciparum to be extremely unusual in possessing a pathway from 5'-methylthiopurines to hypoxanthine. If P. falciparum can transport 5'-methylthiopurines from the erythrocyte environment, the parasite

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can potentially salvage additional hypoxanthine by this novel pathway (discussed below).

Inhibition of PNP in P. falciparum P. falciparum possesses a structurally novel PNP enzyme that is inhibited by immucillin compounds (Kicska et al., 2002a). Several immucillin compounds bind P. falciparum PNP with low nanomolar inhibition constants and inhibit enzyme activity. Human PNP is also the target of immucillins, with subnanomolar inhibition constants, and currently these compounds are being investigated for therapy in cancer and immunosuppression chemotherapy. Immucillin-H, an immucillin that inhibits both human and P. falciparum PNP, induced purine-less death in P. falciparum infection (Kicska et al., 2002b). The parasite growth inhibition induced by immucillin-H is reversed by supplementing culture medium with high doses of hypoxanthine, suggesting that the effect of immucillin-H is primarily to block acquisition of essential purines, resulting in purine-less death (Kicska et al., 2002b). Crystal structure and energetic mapping studies of P. falciparum PNP and immucillin interactions are under investigation to identify more selective inhibitors of the parasite PNP (Shi et al., 2004; Lewandowicz et al., 2005).

Inhibition of HXGPRT in P. falciparum Early studies on inhibitors of *P. falciparum* growth support the parasite HXGPRT activity for drug development (Queen *et al.*, 1990). *P. falciparum* growth *in vitro* is inhibited by purine analogs 6-mercaptopurine, 6-thioguanine, and 8-azaguanine. Studies have not been performed to establish whether the mechanism of 6-mercaptopurine inhibition in *P. falciparum* is similar to the mechanism in mammals, which requires activation by phosphoribosylation via host HGPRT, followed by recognition of GMPS to incorporate the toxic analog into the guanylate nucleotide pool for its toxic incorporation into nucleic acids (Elion, 1989a, 1989b).

Early studies characterized the enzymatic properties and the gene encoding the *P. falciparum* HXGPRT (Queen *et al.*, 1988; Vasanthakumar *et al.*, 1989, 1990). The crystal structure of the *P. falciparum* HXGPRT revealed unique structural features of the parasite enzyme compared to the structure of the human enzyme (Shi et al., 1999a, 1999b). Additional studies have investigated enzyme mechanisms, and features directing substrate specificity and lead transition state inhibitors have been reported (Sarkar et al., 2004; Thomas and Field, 2002; Shi et al., 1999a; Li et al., 1999). Based on the purine incorporation pathways elucidated for P. falciparum, selectively blocking incorporation of hypoxanthine by inhibition of HXGPRT has been generally predicted to induce purine-less death in P. falciparum (Figure 18.1C). Screens have been established to identify selective substrates of the P. falciparum HXGPRT (Shivashankar et al., 2001). It is possible that the xanthine phosphoribosyltransferase activity of P. falciparum HXGPRT, which is absent in the human host, may also support a mechanism for incorporating toxic xanthine analogs to block parasite replication, analogous to inhibition of T. gondii replication by 6-thioxanthine (Pfefferkorn et al., 2001).

Inhibition of guanylate nucleotide pools in P. falciparum Guanylate nucleotides could arise from guanine supplied by the host, guanine derived from host guanosine by the parasite PNP, and xanthine via HXGPRT. The erythrocyte host of P. falciparum lacks a guanosine kinase activity, and biochemical evidence suggests that the host guanine and guanosine pools available to P. falciparum are limited (Reves et al., 1982). P. falciparum growth in vitro is inhibited by psicofuranine, an inhibitor of bacterial GMPS (McConkey, 2000). Psicofuranine inhibition of parasite growth is antagonized by supplying additional guanine to parasite growth medium (McConkey, 2000). These observations provide strong evidence that the primary route to GMP is through GMPS, and demonstrates that during in vitro cultivation the potential supply of host guanine and guanosine is limited (Figure 18.1C).

Unfortunately, the possible importance of host xanthine in GMP formation is not clarified by these studies. Xanthine can by incorporated into the GMP pool by the sequential reactions of HXGPRT and GMPS. Human erythrocytes are reported to have a concentration of xanthine of ~3.6 μ M,

which may be sufficient for physiological transport and incorporation by HXGPRT and GMPS (Traut, 1994). MPA, a highly specific inhibitor of IMPDH, blocks *P. falciparum* replication at micromolar doses *in vitro* (Queen *et al.*, 1990). While these observations suggest that host xanthine pools are not sufficient to bypass the inhibition at parasite IMPDH, these results cannot be conclusively interpreted because MPA also inhibits host IMPDH and depletes host guanylate nucleotide pools.

Inhibition of adenylate nucleotide pools in P. falciparum The only pathway to AMP is from the IMP nucleotide pool through sequential reactions catalyzed by ADSS and ADSL. *P. falciparum* ADSL has been cloned and expressed in *E. coli* for kinetic analysis, and a crystal structure is now available (Jayalakshmi *et al.*, 2002; Eaazhisai *et al.*, 2004). A unique reaction mechanism has been recently described for this essential parasite enzyme (Raman *et al.*, 2004). Targeting either ADSS or ADSL, or both, is predicted to completely deplete *P. falciparum* of essential adenylate nucleotides and inhibit parasite replication (Figure 18.1C).

18.2.3.4 A novel alternative purine Pathway in Apicomplexa

Of the apicomplexans considered in this chapter, only P. falciparum possesses a unique alternative pathway to purines via recycling of 5'-methylthiopurines generated during polyamine metabolism (Ting et al., 2005) (Figure 18.1C). While earlier studies hinted at the existence of this novel pathway (Sufrin et al., 1995), recent studies have helped to illustrate an important physiological role for the pathway, and in vitro studies have validated components of the pathway to be drug targets. This is an exciting and emerging area of biology in P. falciparum and Plasmodium sp., and will require further detailed studies to determine the physiological relevance of these newly described pathways to optimize strategies for drug development. The growth inhibition achieved by treating parasite-infected erythrocytes with immucillins (Kicska et al., 2002a, 2002b) may be multifaceted, because the P. falciparum PNP plays a dual role

both in conversion of inosine to hypoxanthine, and in recycling 5'-methylthioinosine to hypoxanthine (Ting *et al.*, 2005). Therefore, *P. falciparum* has a novel pathway to hypoxanthine. Current evidence suggests this novel pathway plays an important role in parasite metabolism during replication in erythrocytes (Ting *et al.*, 2005).

The intersection of polyamine and purine metabolism in P. falciparum Erythrocytes do not synthesize polyamines, and *P. falciparum*, unlike *T. gondii*, must synthesize its own polyamines. Therefore, *P. falciparum* replication can be blocked by difluoromethylornithine (DFMO), a mechanism-based inhibitor of ornithine decarboxylase (ODC) involved in conversion of ornithine to putrescine in polyamine synthesis (Muller *et al.*, 2001). The polyamine biosynthesis pathway forms a molecule of 5'-methylthioadenosine (MTA) for each molecule of spermidine, or spermine, that is synthesized. In humans and other organisms, the MTA is typically recycled to regenerate both adenine and methionine pools.

Surprisingly, the genes associated with purine salvage (APRT) and recycling of MTA into the methionine pool are absent in the P. falciparum genome (Gardner et al., 2002a, 2002b; Chaudhary et al., 2004). However, P. falciparum ADA was recently discovered to have the novel ability to recognize MTA as a substrate and convert MTA to 5'-methylthioinosine (MTI) (Ting et al., 2005). Additionally, P. falciparum PNP was found to have the unique ability to recognize MTI as a substrate and convert MTI to hypoxanthine. This novel pathway was proven to be functional when exogenously supplied MTI was incorporated into P. falciparum nucleic acids (Ting et al., 2005). Therefore, the unique substrate properties of P. falciparum ADA and PNP comprise a novel metabolic pathway from MTA to MTI to hypoxanthine for selective recycling of purines from polyaminine biosynthesis (Figure 18.1C).

Early studies suggested that *P. falciparum* expressed very abundant levels of ADA, PNP, and HXGPRT when compared to corresponding activities present in the host erythrocyte, therefore indicating a potentially important secondary role
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beyond their role in normal purine pathways (Reyes *et al.*, 1982). Although *P. falciparum* grows normally in PNP- and ADA-deficient host erythrocytes (Daddona *et al.*, 1984, 1986), inhibitors of parasite and host ADA such as coformycin, deoxyco-formycin, and L-ribosyl analogs of coformycins block *P. falciparum* replication (Daddona *et al.*, 1984; Gero *et al.*, 2003). Selective inhibition of *P. falciparum* PNP, but not host PNP, with MT-immucillin-H blocked parasite replication nearly as efficiently as immucillin-H (Ting *et al.*, 2005).

The efficacy of an MT-immucillin-H against malaria indicates that P. falciparum PNP is a critical parasite activity. It is unlikely that MT-immucillin-H inhibits the polyamine pathway by product inhibition at MTA, since P. falciparum ADA converts MTA to MTI (Figure 18.1C) and, significantly, exogenously supplied hypoxanthine reverses the in vitro inhibition achieved by treatment with MTimmucillin-H or immucillin-H (Kicska et al., 2002b; Ting et al., 2005). These observations suggest that the host pool (or flux) of hypoxanthine alone is likely to be insufficient to support parasite growth during in vitro cultivation. Thus the host inosine pool, the host adenosine pool, or hypoxanthine recovered in the novel purine recycling pathway is likely to be required for normal replication of P. falciparum (Figure 18.1C). These in vitro observations currently validate the P. falciparum enzymes ADA and PNP for further drug discovery, as well as further validating enzymes involved in polyamine biosynthesis (Kicska et al., 2002b; Ting et al., 2005).

Recycling and/or salvage? A key question remaining to be answered is whether the novel purine recycling pathway in *P. falciparum* plays any direct role in purine salvage. Exogenously supplied MTI is transported by *P. falciparum* and incorporated into parasite nucleic acids (Figure 18.1C) (Ting *et al.*, 2005). Any supply of host MTA transported by *P. falciparum* could be salvaged to hypoxanthine and incorporated into the IMP nucleotide pool (Figure 18.1C). MTA may represent a significant source of purines for *P. falciparum* if MTA is abundant in erythrocytes and the parasite has a transporter capable of stealing this molecule from the host cytosol/parasitophorous vacuole space. Alternatively, it is also plausible that *P. falciparum* may either secrete ADA into the erythrocyte cytosol or parasitophorous vacuole space to convert host MTA to MTI, or secrete PNP into the erythrocyte cytosol or parasitophorous vacuole space to convert host MTI to hypoxanthine. Both of these strategies could increase the supply of purines to P. falciparum because both MTI and hypoxanthine are transported and incorporated into parasite nucleic acids (Figure 18.1C) (Ting et al., 2005). Therefore, studies of the localization of P. falciparum ADA and PNP in parasitized erythrocytes, as well as the capabilities of MTI and MTA transport, are necessary to fully resolve the major physiological function of this novel pathway.

18.2.3.5 *The source of polyamines in Apicomplexa*

Plasmodium falciparum Host erythrocytes do not synthesize polyamines. By contrast, P. falciparum growth in vitro is efficiently blocked by the ODC inhibitor DFMO (Muller et al., 2001). ODC catalyzes the conversion of ornithine to putrescine in the first step in the synthesis of spermidine and spermine (Tabor and Tabor, 1985). Incorporation of radiolabeled glutamine into the ornithine pool in P. falciparum was minor, suggesting that the parasite possesses another major route to acquire or synthesize ornithine (Gafan et al., 2001). In organisms that synthesize polyamines, the other major routes are through enzymes for arginase and ODC, or arginine decarboxylase (conversion of arginine to agmatine) and agmatinase. Arginase directly converts arginine to ornithine, while agmatinase bypasses ornithine entirely by converting agmatine to putrescine (Wu and Morris, 1998).

A *P. falciparum* arginase gene has been cloned and the recombinant enzyme, when expressed, possessed arginase activity with no detectable agmatinase activity (Muller *et al.*, 2005). As discussed in sections 18.3 and 18.4 (pyrimidines and amino acids), all of the apicomplexan parasites are incapable of *de novo* arginine synthesis and therefore must acquire arginine from the host.

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Arginine taken from the host by *P. falciparum* is utilized in protein synthesis as well as polyamine biosynthesis. The protozoan parasite *Leishmania* sp. also utilizes an arginase activity that is essential for polyamine biosynthesis (Satriano, 2003; Roberts *et al.*, 2004).

Sensitivity of P. falciparum to DFMO suggests that the ODC activity is essential. P. falciparum has a novel ODC activity that exists as a bifunctional enzyme with S-adenosylmethionine decarboxylase. This bifunctional enzyme enables a well-balanced synthesis of putrescine from ornithine without involving domain-domain interactions (Krause et al., 2000; Muller et al., 2000; Wrenger et al., 2001; Birkholtz et al., 2004). Putrescine and deoxyadenosylmethionine are combined by spermidine synthase, generating a single molecule of spermidine and a molecule of MTA, which is recycled to MTI by ADA, and then to hypoxanthine by PNP (Figure 18.1C) (Muller et al., 2000; Chaudhary, 2005; Ting et al., 2005). P. falciparum also transports host spermidine (Muller et al., 2001). No P. falciparum gene ortholog for a spermine synthase has been identified (Chaudhary, 2005).

Cryptosporidium parvum In contrast with *P. falciparum, C. parvum* appears to biosynthesize polyamines by the pathway predominantly present in bacteria and plants involving arginine decarboxylase and agmatinase (Yarlett *et al.*, 1996). Based on the extraordinary transport capacities of *C. parvum*, it is also likely that this parasite may also transport putrescine or other polyamines from the rich environment of the human gut. *C. parvum* ADC activity is inhibited by difluoromethylarginine, and is unaffected by DFMO (Keithly *et al.*, 1997). Since *C. parvum* lacks any gene ortholog or activity for PNP (Figure 18.1B), this parasite is incapable of recycling 5'-methylthiopurines back into the parasite purine pool.

Toxoplasma gondii The origin of polyamines in *T. gondii* is not clearly resolved at this time, although the weight of current evidence suggests that this parasite is auxotrophic for polyamines and must inhabit host cells that can supply polyamines.

The parasite appears to have transporters capable of transporting ornithine, putrescine, or other polyamines (Chaudhary, 2005). *T. gondii* appears to lack ODC activity (Seabra *et al.*, 2004), and growth of tachyzoites is unaffected by DFMO (Chaudhary *et al.*, 2006).

The T. gondii genome does reveal the presence of a gene ortholog with homology to the ADC/ODC gene family (Kissinger et al., 2003). If functional, this gene ortholog would represent a potential ADC based on lack of sensitivity to DFMO. Currently, no gene ortholog can be identified for a member of the related arginase/agmatinase gene family, or any other gene member of the polyamine biosynthetic pathway (Chaudhary, 2005). Collectively, these data suggest that T. gondii is incapable of polyamine biosynthesis and most likely relies upon direct transport and salvage of preformed polyamines supplied by the host cell. The absence of any gene ortholog for spermidine synthase or spermine synthase suggests that the parasite does not produce MTA. Even in the highly unlikely case that MTA or MTI were present in T. gondii, 5'-methylthiopurines could not be recycled into the parasite purine pool. T. gondii PNP cannot utilize MTI as a valid substrate (Figure 18.1A) (Chaudhary et al., 2006).

Adaptation of the polyamine and purine pathways in Apicomplexa The selective adaptation of P. falciparum ADA and PNP enzymes to recycle 5'-methylthiopurines to hypoxanthine may have arisen in response to the loss of AK activity (Ting et al., 2005). The potential purine flux is reflected by both the demands of the parasite and the purine limitations of the host erythrocyte. Replication of P. falciparum occurs by schizogony, where DNA replication and nuclear division are coordinated in a narrow window of time. Schizogony, where many daughter nuclei are rapidly formed, may demand an increased requirement for purine flux into P. falciparum. By contrast, T. gondii replicates by endodyogeny and creation of daughter parasites approximately every 7 hours. Endodyogeny, where one daughter nucleus is formed from one mother nucleus, may inherently require a lower flux of purines to sustain replication.

18.3 PYRIMIDINES

Pyrimidines are essential components of nucleic acids, and are involved in other aspects of cellular metabolism as well. Most apicomplexan parasites have retained the ability for *de novo* synthesis of the parental pyrimidine molecule, uridine 5'-monophosphate (UMP). The apicomplexan parasites that rely on the *de novo* synthetic pathway are therefore dependent on an appropriate supply of precursor molecules for the biosynthesis of pyrimidines. These precursor molecules include small molecules such as bicarbonate, amino acids, and purine nucleotides. Consequently, acquisition of amino acids and satisfying the purine auxotrophy of apicomplexan parasites are necessary for pyrimidine biosynthesis.

Most apicomplexans, including T. gondii and P. falciparum, have functional pathways for de novo pyrimidine synthesis. T. gondii is unusual in also possessing pyrimidine salvage activities. However, the pyrimidine biosynthetic pathway is essential for replication of both T. gondii and P. falciparum. Some apicomplexans, such as C. parvum, have lost the ability to synthesize UMP by the de novo pathway, and completely rely on salvage pathways to acquire required pyrimidines from the host. In these apicomplexans, acquisition of pyrimidines is completely dependent on transport and salvage of preformed pyrmidine nucleobases and nucleosides from the host. C. parvum, uniquely, has acquired several pyrimidine salvage activities not observed in any other apicomplexan parasite.

Relatively little experimental work has been performed on the associated transport pathways essential for pyrimidine biosynthesis, or the relationships and cross-regulatory talk likely to occur between the pyrimidine and purine pathways. Interestingly, pyrimidine starvation is one of the triggers used to experimentally induce *in vitro* stage differentiation in *T. gondii* from the tachyzoite to the bradyzoite stages (Bohne and Roos, 1997; Roos *et al.*, 1997). Recent studies have clarified the functional organization of pyrimidine metabolism in the Apicomplexa, and have revealed new strategies to target essential pyrimidine acquisition pathways in apicomplexan parasites.

18.3.1 *De novo* pyrimidine synthesis in Apicomplexa

The human host of *T. gondii*, *P. falciparum*, and *C. parvum* is capable of significant salvage and biosynthesis of pyrimidines, such that either pathway can supply the pyrimidine requirements. Early work in nucleotide metabolism determined that the Apicomplexa are generally capable of synthesizing pyrimidines from amino-acid precursors glutamine and aspartic acid by the same six-step pathway present in their hosts. While *C. parvum* cannot synthesize pyrimidines *de novo*, *T. gondii* and *P. falciparum* possess the intact pyrimidine biosynthetic pathway (Figure 18.2) (Hill *et al.*, 1981a, 1981b; O'Sullivan *et al.*, 1981; Schwartzman and Pfefferkorn, 1981; Reyes *et al.*, 1982; Asai *et al.*, 1983).

The pyrimidine biosynthetic pathway starts with carbamoyl phosphate synthetase II (CPSII), which combines two molecules of ATP, L-glutamine, and bicarbonate in a sequence of elegant chemical reactions at multiple active sites to produce a molecule of carbamoyl phosphate (Holden et al., 1999; Thoden et al., 1999; Kothe et al., 2005). Carbamoyl phosphate is then fused with L-aspartate in the second step by aspartate carbamoyltransferase (ATC) to produce carbamoyl aspartate. Dihydroorotase (DHO) then converts carbamoyl aspartate to dihydroorotate in the third step. In the fourth step of the pathway, dihydroorotate dehydrogenase (DHODH) creates orotate from dihydroorotate, and in doing so also creates electrons to CoQ in the mitochondrion. Orotate is combined with 5-phosphoribosyl-1-diphosphate (PRPP) in the fifth step by orotate phosphoribosyltransferase (OPRT) to produce orotidine-5'-monophosphate. Uridine-5'-monophosphate (UMP), the parent pyrimidine mononucleotide and the precursor of all other pyrimidine nucleotides, is finally produced in the sixth step via the decarboxylation of orotidine-5'monophosphate (OMP) by orotidine-5'-monophosphate decarboxylase (OMPDC).





PYRIMIDINE BIOSYNTHESIS in T. gondii & P. falciparum

PYRIMIDINE SALVAGE in C. parvum

FIGURE 18.2 Pyrimidine biosynthesis and salvage pathways in *T. gondii, P. falciparum*, and *C. parvum*. Pathways are shown for pyrimidine biosynthesis in *T. gondii* and *P. falciparum* (top), for pyrimidine salvage in *T. gondii* (bottom left), for pyrimidine salvage in *C. parvum* (bottom right). Solid lines with arrowheads depict active pathways. Substrates of each enzyme are shown to the side of the solid lines, and the product(s) of each enzyme activity are shown on the arrowhead side. The enzyme activity responsible for each conversion step is shown in capital italicized text beside the arrowhead lines. CPSII, carbamoyl phosphate synthetase II; ATC, aspartate carbamoyltransferase; DHO, dihydroorotase; DHODH, dihydroorotate dehydrogenase; OPRT, orotate phosphoribosyltransferase; URDP, uridine phosphorylase; dURDP, deoxyuridine phosphorylase; CYTD, cytidine deaminase; dCYTD, deoxycytidine deaminase; UPRT-UK, uracil phosphoribosyltransferase; UMP, uridine 5'-monophosphate; CMP, cytidine 5'-monophosphate; CMP, cytidine kinase; TK, thymidine kinase; PRPP, 5-phosphoribosyl-1-pyrophosphate; CoQ, mitochondrial coenzyme Q.

In all Apicomplexa, UMP is phosphorylated to UTP in two sequential steps by UMP kinase and nucleoside diphosphate kinase. UTP is converted to CTP by CTP synthase (CTPS) in a rate-limiting step in all organisms. CTPS is the only known route for *de novo* synthesis of cytidine nucleotides. *T. gondii, P. falciparum,* and *C. parvum* all express a CTPS gene (Hendriks *et al.,* 1998; Kissinger *et al.,* 2003; Abrahamsen *et al.,* 2004; Xu *et al.,* 2004). Conversion of ribonucleotides to deoxyribonucleosides occurs

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PYRIMIDINE SALVAGE in T. gondii

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at the level of nucleoside diphosphate, and is catalyzed by a ubiquitous ribonucleotide reductase (RNR).

18.3.1.1 Unique architecture, organization and regulation of CPSII in Apicomplexa

The key regulatory enzyme in the apicomplexan pyrimidine biosynthetic pathway is the first step encoded by CPSII. CPSII catalyzes the rate-limiting step and controls the flux through the pathway (Jones, 1980; Evans and Guy, 2004). The architecture of CPSII enzymes from T. gondii and P. falciparum is unique to the Apicomplexa and other protozoan parasites. Leishmania sp., Trypanosoma sp., Babesia bovis, Plasmodium sp., and T. gondii encode a novel glutamine-dependent CPSII activity fused with an N-terminal glutamine amidotransferase (GAT) activity (Aoki et al., 1994; Flores et al., 1994; Chansiri and Bagnara, 1995; Gao et al., 1998, 1999; Nara et al., 1998; Fox and Bzik, 2003). By contrast, mammalian pyrimidine-specific CPSII is contained on the CAD gene encoding a multifunctional protein possessing the GAT domain fused via linkers of various lengths in order with CPSII, DHO, and ATC (Mori and Tatibani, 1978; Davidson et al., 1993). In Saccharomyces cerevisiae a multifunctional protein contains, in order, GAT, CPSII, and ATC domains, but is missing the DHO activity found on mammalian CAD (Davidson et al., 1993). A different strategy was taken by plants, eubacteria, and archebacteria, which express a gene containing the monofunctional CPS, and a separate gene encoding GAT (Jones, 1980; Zhou et al., 2000).

While the *P. falciparum* CPSII gene possesses no introns, the *T. gondii* CPSII is encoded by a single gene with a complex organization of 37 exons and 36 introns specifying a polypeptide of 1687 amino acids (Fox and Bzik, 2003). CPSIIs from *T. gondii*, *P. falciparum* (2391 amino acids), and *Babesia bovis* (1645 amino acids) are also much larger polypeptides than are CPSIIs from other species (Flores *et al.*, 1994; Chansiri and Bagnara, 1995; Fox and Bzik, 2003). Relative to other CPS and GAT domains, the apicomplexan CPSII enzymes have unique sites of insertion within GAT domains, within CPS domains, and in the linker region fusing the two CPS halves, as well as within the C-terminal allosteric regulatory domain (Fox and Bzik, 2003).

18.3.1.2 CPSII activity required for de novo pyrimidine synthesis is a validated drug target

Conceptually, inhibition of UMP synthesis by the pyrimidine biosynthetic pathway is likely to be a more potent strategy than blocking only thymine nucleotide synthesis and DNA synthesis indirectly through inhibition of folate metabolism (discussed below), because inhibition of pyrimidine biosynthesis will cause starvation for UTP, CTP, and TTP nucleotides essential for both RNA and DNA synthesis. The apicomplexan CPSII enzymes exhibit several differences from the mammalian CPSII that provides a basis for chemotherapy. The novel and large amino-acid insertions in T. gondii and P. falciparum CPSII may provide parasite-specific targets for inhibiting CPSII and de novo pyrimidine synthesis in chemotherapy. Targeting ribozymes to a site corresponding to a novel P. falciparum CPSII insertion blocked replication of P. falciparum in vitro (Flores et al., 1997). A rapid microassay has been developed for P. falciparum CPSII activity that may be amenable to high throughput assays (Flores and Stewart, 1998). The GAT activity of T. gondii CPSII has also been shown to be a possible target of acivicin (Fox and Bzik, 2003).

Mammalian CPSII is an allosterically regulated enzyme with activity activated by 5-phosphoribosyl-1-pyrophosphate (PRPP), and activity suppressed by UTP (Jones, 1980). P. falciparum CPSII is activated by PRPP, and high UTP concentrations cause CPSII inhibition (Gero et al., 1984). Remarkably, T. gondii CPSII is insensitive to activation by PRPP, but is inhibited by UTP (Asai et al., 1983). The regulatory domain controlling allosteric regulation of CPS activity is contained within the C-terminal ~150 amino acids of the polypeptide (Mora et al., 1999; Fresquet et al., 2000; Evans and Guy, 2004). Both the P. falciparum and the T. gondii CPSII enzymes possess significant amino-acid insertions in the allosteric regulatory domain, as well as divergent amino-acid composition (Flores et al., 1994; Fox and Bzik, 2003). The allosteric regulatory domain is the most divergent domain within the entire CPSII, suggesting that that regulation of CPSII is unique in apicomplexans (Fox and Bzik, 2003).

Other enzymes in the *de novo* pathways of *T. gondii* and *P. falciparum* are also under investigation as potential drug targets. *T. gondii* ATC catalyzes the second step and is a cytosolic monofunctional enzyme (Asai *et al.*, 1983). Recombinant *T. gondii* ATC product has been produced and characterized (Mejias-Torres and Zimmermann, 2002). The lack of any observed regulation on *T. gondii* ATC further supports the key regulatory role of CPSII in the *de novo* pathway of pyrimidine biosynthesis (Asai *et al.*, 1983; Mejias-Torres and Zimmermann, 2002).

T. gondii DHO catalyzes the third step and is a cytosolic monofunctional enzyme which is unresponsive to any nucleotide (Asai et al., 1983). DHODH from T. gondii has been cloned and expressed (Sierra Pagan and Zimmermann, 2003). Several biosynthetic enzymes from P. falciparum, including DHO, OPRT, and OMPDC, have been cloned, expressed, and recombinant enzymes characterized (Christopherson et al., 2004; Krungkrai et al., 2004a, 2004b). In T. gondii and P. falciparum OPRT and OMPDC are present on separate genes (Krungkrai et al., 2004b). Potential inhibitors of apicomplexan CPSII, DHO DHODH, OPRT, and OMPDC have been studied (Niedzwicki et al., 1984; Seymour et al., 1994; Flores et al., 1997; Javaid et al., 1999; Fox and Bzik, 2003; Boa et al., 2005; Krungkrai et al., 2005).

Genetic inactivation of the pyrimidine biosynthetic pathway by knockout of the CPSII gene produced a strain of T. gondii that is auxotrophic for uracil (Figure 18.2) (Fox and Bzik, 2002). The T. gondii uracil auxotroph replicates at a normal growth rate in vitro in the presence of high concentrations of exogenously supplied uracil. By contrast, if uracil is omitted from the in vitro culture medium then this mutant will invade host cells normally, but has no detectable growth rate. In murine infections with the T. gondii uracil auxotroph, this mutant displayed severe defects in virulence (Fox and Bzik, 2002). Compared with its virulent parental strain RH (Sabin, 1941), the uracil auxotroph was at least 10 million-fold less virulent, or was completely avirulent, suggesting that

pyrimidines are not at high enough concentration in the mammalian host to support significant salvage capability. Consequently, the pyrimidine *de novo* synthetic pathway presents a key target for drug development in *T. gondii* and *P. falciparum*.

18.3.1.3 Indirect inhibition of pyrimidine biosynthesis

The pyrimidine biosynthetic pathway of apicomplexans is indirectly inhibited by atovaquone. Atovaquone, a naphthoquinone derivative, in combination with proguanil (MalaroneTm) is clinically used to treat human malaria infections. Atovaquone is a structural analog of coenzyme Q (CoQ; ubiquinone) in the mitochondrial electron transport chain. Atovaquone collapses the membrane potential of Plasmodium sp. by inhibition of cytochrome b in the bc_1 complex (complex III) of the parasite electron transport chain (Srivastava et al., 1999; Korsinczky et al., 2000). Blocking electron flow inhibits mitochondrial membraneassociated enzymes such as DHODH, which requires electron transfer to CoQ when it oxidizes dihydroorotate to orotate in the fourth step of the pyrimidine biosynthetic pathway (Figure 18.2).

Resistance of *Plasmodium* sp. to atovaquone is associated with specific mutations within the parasite cytochrome *b* (Srivastava *et al.*, 1999; Korsinczky *et al.*, 2000). Atovaquone treatment of *P falciparum in vitro* causes major accumulations of carbamoyl aspartate and dihydroorotate, demonstrating that the breakdown in electron flow disrupts DHODH and causes significant substrate accumulation leading to starvation of UMP (Seymour *et al.*, 1994).

Atovaquone is also an approved drug for treatment of acute toxoplasmosis. As with *P. falciparum*, the DHODH of *T. gondii* is most likely associated with mitochondrial membranes. DHODH purifies from *T. gondii* in the particulate fraction of tachyzoites, and is inhibited by respiratory chain inhibitors (Asai *et al.*, 1983). The DHODH gene of *T. gondii* is most similar to the family of DHODH enzymes linked to the respiratory chain in mitochondria for their catalytic redox force (Sierra Pagan and Zimmermann, 2003). *T. gondii* mutants resistant to atovaquone can be selected *in vitro* (Pfefferkorn *et al.*, 1993), and these mutants possess mutations within the *T. gondii* cytochrome *b* gene, suggesting this function to be the target of atovoquone (McFadden *et al.*, 2000).

18.3.2 Pyrimidine salvage in Apicomplexa

While *de novo* pyrimidine synthesis is essential for *T. gondii* and *P. falciparum*, *C. parvum* lacks all six enzymes required for synthesis of UMP (Figure 18.2). Therefore, pyrimidine salvage in *C. parvum* presents a key target for drug development.

18.3.2.1 Salvage of pyrimidines in C. parvum

The recent genome sequence of C. parvum and Cryptosporidium hominis demonstrated that Cryptosporidium sp. does not retain any of the six enzymes comprising the pyrimidine biosynthetic pathway (Figure 18.2) (Abrahamsen et al., 2004; Puiu et al., 2004; Striepen et al., 2004; Xu et al., 2004). Thus C. parvum is completely dependent on the host for supplying pyrimidines that must be salvaged to meet the pyrimidine demand of the parasite. Remarkably, C. parvum has three potentially significant pyrimidine salvage activities expressed by three genes. C. parvum encodes a thymidine kinase (TK), a monofunctional uracil phosphoribosyltransferase (UPRT), and a bifunctional polypeptide having UPRT and uridine kinase (UPRT-UK) activities (Striepen et al., 2004). With this repertoire of salvage activities, C. parvum can potentially convert thymidine (TK), cytidine and uridine (UK), and uracil (UPRT) to their respective pyrimidine-5'-monophosphates (Figure 18.2).

Biochemical evidence supports the expression of active TK and UK enzymes in *C. parvum* infection based on incorporation of bromodeoxyuridine and cytosine-arabinoside, respectively (Woods and Upton, 1998; Striepen *et al.*, 2004). However, the proposed UPRT gene activity has not yet been experimentally validated either by enzyme assay or by demonstrating the incorporation of uracil during *C. parvum* infection. The presence of TK and UK activities in *C. parvum* is highly unusual when compared with their absence in other apicomplexan parasites such as *T. gondii* and *P. falciparum*. Phylogenetic analysis indicates that genes involved in nucleotide metabolism in *C. parvum* were incorporated by horizontal gene transfer from bacterial (TK), algal (UK-UPRT), or protozoan sources (Abrahamsen *et al.*, 2004; Huang *et al.*, 2004; Striepen and Kissinger, 2004; Striepen *et al.*, 2004). *C. parvum* may use the parasitized host cell, the gut environment itself, or both, as the source of the pyrimidine nucleobase/ nucleoside precursors.

C. parvum growth in vitro is inhibited by cytosine-arabinoside, a prodrug that is activated by UK (Pfefferkorn and Pfefferkorn, 1976; Woods and Upton, 1998). The UPRT as well as the bacterialtype TK activity of C. parvum potentially could be similarly exploited for selectively targeting incorporation of toxic analogs. Thus the genome sequence of C. parvum has revealed the lack of biosynthetic capability and several newly identified essential salvage activities that may be amenable for drug development. An important goal will be to determine experimentally whether each gene of pyrimidine salvage (UPRT-UK, UPRT, or TK) is individually capable of fully supporting parasite replication in vivo, or whether multiple activities are required.

18.3.2.2 Salvage of pyrimidines in P. falciparum

P. falciparum appears to be highly restricted in its ability to salvage pyrimidines marking the de novo primidine synthetic pathway a key drug target. Even though salvage is highly restricted, P. falciparum is capable of significant transport and accumulation of pyrimidine nucleobases and nucleosides (Lauer et al., 1997). For example, P. falciparum is capable of accumulating thymidine and can incorporate exogenously supplied orotic acid and cytotoxic 5-fluoroorotic acid (Rathod et al., 1989; Lauer et al., 1997). The poor ability of P. falciparum to salvage pyrimidines is related to the absence of genes encoding the salvage enzymes UPRT, TK, and UK that are required for any incorporation of pyrimidine nucleobases or nucleosides into the nucleotide pool (Gardner et al., 2002a, 2002b; Striepen et al., 2004).

18.3.2.3 Salvage of pyrimidines in T. gondii

The pyrimidine salvage capabilities of T. gondii, P. falciparum, and C. parvum are summarized in Figure 18.2. Unlike P. falciparum or C. parvum, T. gondii has the six-step de novo synthetic pathway yet has also retained potentially significant salvage activities. Early labeling studies demonstrated that uracil was well incorporated into intracellular or extracellular tachyzoites, but did not label host-cell nucleic acids (Pfefferkorn and Pfefferkorn, 1977b). Importantly, these initial studies demonstrated efficient and selective labeling of T. gondii nucleic acids by uracil, and made the significant biological observation that the host cell has no access to the parasite pyrimidine nucleotide pools. Uracil incorporation is a method still in use today to assess parasite replication.

A parasite mutant was first selected to be resistant to 5-fluorodeoxyuridine (FUDR), and both intracellular (or extracellular) tachyzoites of this mutant were found to be deficient in their ability to incorporate uridine, deoxyuridine, and uracil (Pfefferkorn and Pfefferkorn, 1977b, 1977c). Correspondingly, this mutant was also determined to be co-resistant to 5-fluorouracil and 5-fluoruridine. In pioneering work, the basis of the FUDR-resistant mutant was identified as a biochemical defect in the parasite UPRT activity (Pfefferkorn, 1978), thus demonstrating that the UPRT activity was non-essential to the tachyzoite stage. This in turn suggested that the de novo pyrimidine synthesis pathway was fully capable of supporting parasite replication. Carbondioxide starvation was used to suppress T. gondii de novo pyrimidine synthesis (blocking step 1, CPSII) and growth of wild-type parasites could be rescued with uracil supplementation, whereas growth of the FUDR-resistant mutant was not rescued. These observations suggested that T. gondii is fully capable of growth in vitro by salvage of uracil in the absence of a functioning pyrimidine biosynthetic pathway to UMP (Pfefferkorn, 1978).

T. gondii salvage capacities were comprehensively investigated using biochemical measurements of enzyme activities present in protein extracts prepared from isolated tachyzoites. *T. gondii* possesses salvage activities enabling the parasite to salvage a variety of pyrimidines, including deoxycytidine, deoxyuridine, cytidine, uridine, and uracil (Figure 18.2) (Iltzsch, 1993). *T. gondii* can also recover pyrimidines arising from degradation of parasite nucleic acids through degradation of dUMP, dCMP, and CMP to their corresponding nucleosides by nucleoside 5'-monophosphate phosphorylase (Iltzsch, 1993). The parasite cannot directly obtain phosphorylated nucleotides from the host cell, but can transport and incorporate pyrimidine nucleobases and nucleosides (Iltzsch, 1993; Pfefferkorn and Pfefferkorn, 1977b, 1977c).

Uracil is the only pyrimidine compound that can be directly incorporated into the pyrimidine pool by conversion to UMP by the major UPRT activity. All other pyrimidine compounds are first catabolized to uracil mediated by parasite activities for uridine phosphorylase, deoxyuridine phosphorylase, cytidine deaminase, and deoxycytidine deaminase (Figure 18.2). This unique salvage strategy has been described by E.R. Pfefferkorn as a salvage funnel to uracil (Pfefferkorn, 1978). The *T. gondii* genome sequence suggests that the uridine/ deoxyuridine/thymidine phosphorylase activities, and the cytidine/deoxycytidine deaminase activities are likely to be present on single polypeptides (Iltzsch, 1993; Kissinger *et al.*, 2003).

Although *T. gondii* has retained the ability to interconvert thymine and thymidine, the parasite lacks any TK activity and is incapable of salvaging thymidine. However, these compounds can be incorporated into *T. gondii* which has been transformed with a TK gene derived from herpes simplex virus (Fox *et al.*, 2001). Similarly, *T. gondii* cannot salvage cytosine unless the parasite has been transformed with a bacterial cytosine deaminase (CD) gene (Fox *et al.*, 1999).

Potential inhibitors of pyrimidine salvage in T. gondii Genetic studies on the pyrimidine salvage pathway have been performed in *T. gondii*. In early mutagenesis and biochemical studies, UPRT was demonstrated to be dispensable and therefore UPRT could be used as a target for genetic inactivation (Pfefferkorn, 1978). Integration of plasmid DNA into the UPRT locus was selected using 5-fluorouracil (Donald and Roos, 1995). Mutants selected to be resistant to 5-fluorouracil compounds were defective in UPRT activity. Isolation of plasmid transgenes and surrounding chromosomal sequences demonstrated that integration of plasmid in these mutants occurred at the UPRT locus, which was cloned and characterized in the same study.

Therefore, direct inhibition of salvage enzymes in T. gondii is unlikely to perturb parasite replication. Nonetheless, parasite enzymes may have unique substrate properties, and potential inhibitors of T. gondii uridine phosphorylase have been assessed (Iltzsch and Klenk, 1993; el Kouni et al., 1996). UPRT activity expressed by T. gondii is largely absent in the mammalian host, and this suggests another pathway of drug development. In addition to its natural substrate uracil, T. gondii UPRT also recognizes 2,4-dithiouracil and incorporates this analog into parasite nucleic acids. Interestingly, this property of UPRT has been adapted to enable cellspecific microarray analysis of mRNA synthesis and decay (Cleary et al., 2005). UPRT also recognizes 5'-fluorouracil compounds as well as other uracil analogs that suggest a pathway of drug development based on selective incorporation of toxic analogs by parasite UPRT. This approach has been validated in studies using 5'-fluorouracil, 5'-fluorouridine, FUDR, and emimycin (Pfefferkorn and Pfefferkorn, 1977b, 1977c; Pfefferkorn, 1978; Pfefferkorn et al., 1989). 5'-fluorouracil compounds are incorporated by UPRT then ultimately become an inhibitor of thymidylate synthase that prevents synthesis of thymine nucleotides. The crystal structure of T. gondii UPRT has been determined, and efforts are underway to identify potential analogs that may be selectively incorporated into parasite nucleic acids to block replication of tachyzoites (Iltzsch, 1993; Iltzsch and Tankersley, 1994; Carter et al., 1997; Schumacher et al., 1998, 2002).

18.3.3 Assessment of pyrimidine synthesis and salvage pathways related to the parasite niche

18.3.3.1 P. falciparum and C. parvum

The different approaches used by *P falciparum* and *C. parvum* to acquire their pyrimidines is likely to be related to their particular niche within the

human host. *C. parvum* resides in the gut, an environment rich in a nutrient supply, whereas *P. falciparum* resides initially in hepatocytes and subsequently during pre-clinical and clinical stages within erythrocytes – a host cell that loses its ability to synthesis pyrimidines and is not engaged in the business of transcription or replication. The erythrocyte niche of *P. falciparum* has a low abundance of pyrimidines. *P. falciparum* has compensated over time by abandoning the enzymes, if initially present, that can salvage pyrimidines. *P. falciparum* has retained a robust *de novo* pyrimidine synthetic pathway fully capable of supporting rapid replication.

By contrast, *C. parvum* has no component of the pyrimidine biosynthetic pathway. *C. parvum*, as a gut pathogen, discovered free food and acquired enzymes via horizontal transfer from other organisms to take advantage of pyrimidine nucleobases and nucleosides present in the environment. The *C. parvum* genome reveals in general that this pathogen is rich in genomic bias with a plethora of transporters, including putative transporters of nucleobases and nucleosides (Abrahamsen *et al.*, 2004; Puiu *et al.*, 2004).

18.3.3.2 T. gondii

By contrast to P. falciparum and C. parvum, T. gondii can invade and replicate in most human tissues and cells. Correspondingly, it has more significant capabilities, possessing both pyrimidine biosynthesis and salvage pathways. T. gondii can rely strictly on the de novo pathway, and this pathway is required for virulence in mammals (Fox and Bzik, 2002). These observations demonstrate that T. gondii can readily obtain the amino-acid precursors and the necessary ATP from purine salvage to synthesize pyrimidines de novo in the wide variety of cells and tissues that this parasite can infect. Surprisingly, T. gondii tachyzoites blocked in the pyrimidine biosynthetic pathway can grow strictly on the salvage pathway in vitro with uracil supplements (Pfefferkorn, 1978; Fox and Bzik, 2002) - yet complete disruption of the salvage pathway through mutation and disruption of UPRT has no detectable effect on tachyzoite growth or virulence (Pfefferkorn, 1978; Donald and Roos, 1995).

These observations raise the puzzling question of why *T. gondii* has retained the capacity to grow on either pathway, whereas *C. parvum* and *P. falciparum* have not.

Potential roles of T. gondii UPRT It remains a mystery why uracil is so well incorporated into nucleic acids in tachyzoites and why the UPRT activity of the parasite is such a major activity (Pfefferkorn, 1978). The retention of a non-essential gene for the parasite UPRT and other salvage activities suggests that some advantage may be conferred by its expression. T. gondii UPRT recognizes uracil as its only natural substrate for pyrimidine compounds that are normally available in the mammalian host (Pfefferkorn, 1978; Carter et al., 1997). It is possible that the expression of UPRT confers some minor advantage to intracellular parasites by enabling the recovery and reincorporation of pyrimidines into the UMP pool that are catabolized from T. gondii or host nucleic acids and nucleotides.

The ability of T. gondii to grow or survive on uracil alone in the absence of the *de novo* pyrimidine pathway may be essential during another life stage of the parasite other than the tachyzoite stage. Because UPRT is an enzyme that is absent in mammals but commonly found in bacteria and plants, it is plausible that uracil may be a nutrient that is present in certain non-mammalian environmental niches. Within these niches, the environmentally stable oocyst stage may have some access to uracil. Indirect biochemical evidence suggests that T. gondii UPRT may interact with the soil environment. The naturally occurring antibiotic emimycin, produced by Streptomyces sp. (primarily an inhabitant of soil), is an equivalent substrate to uracil for T. gondii UPRT (Terao et al., 1960; Terao, 1963; Pfefferkorn et al., 1989). Emimycin is a selective inhibitor of T. gondii growth and nucleic acid synthesis, and parasite mutants with disrupted UPRT activity are resistant to emimycin. Emymycin is incorporated into emimycin 5'-monophosphate (EMP) by T. gondii UPRT, and further into EDP and ETP. However, ETP is not well incorporated into nucleic acids, and the inhibition of T. gondii RNA and DNA synthesis may occur at the level of parasite RNA and DNA polymerases (Pfefferkorn *et al.*, 1989).

It is also possible that the T. gondii UPRT activity was retained because it plays an important role in another aspect of cell biology or metabolism. The structure and biochemical properties of UPRT demonstrated that in the absence of substrates or its activator GTP, the enzyme behaves as a homodimer composed of two identical subunits. In the presence of GTP, GTP binding stabilizes an active tetrameric structure of UPRT exhibiting high enzyme activity compared to the homodimer (Schumacher et al., 2002). Based on these observations, it was suggested that T. gondii UPRT may play some role in balancing purine and pyrimidine pools in T. gondii (Schumacher et al., 2002). If UPRT is involved in balancing pyrimidine and purine pools this balance may be best achieved in the nonmammalian environment, because UPRT cannot substantially contribute to parasite pyrimidine nucleotide pools in infected mammalian cells or animals (Fox and Bzik, 2002).

18.3.4 Folate pathways and synthesis of thymine nucleotides

For decades the folate pathway has been a key target for antimicrobial agents directed against T. gondii and P. falciparum, and it is under current investigation for targeting C. parvum (Anderson, 2005). Thymine nucleotides are formed during the thymidylate cycle by the methylation of dUMP to produce dTMP in a reaction mediated by thymidylate synthase (TS) in folate metabolism (Figure 18.3). During conversion of dUMP to dTMP, a molecule of tetrahydrofolate is oxidized to dihydrofolate. During the thymidylate cycle, T. gondii, P. falciparum, and C. parvum rely on TS to produce dTMP from dUMP, on DHFR for recycling of dihydrofolate to tetrahydrofolate, and on serine hydroxymethyltransferase to produce 5,10-methylene-tetrahydrofolate from serine (Figure 18.3). Therefore, thymidine starvation can be induced in T. gondii and P. falciparum, as well as humans, by reducing the pool of tetrahydrofolate via inhibition of DHFR and recycling of dihydrofolate to tetrahydrofolate. C. parvum is unusual in



FIGURE 18.3 Thymine nucleotide synthesis and pathways to folate in Apicomplexa. Pathways present in *T. gondii* (Tg), *P. falciparum* (Pf), and *C. parvum* (Cp) are indicated in rectangles. The thymidylate cycle is enclosed by a large triangle. Potential synthetic or slavage sources of folate entering the thymidylate cycle are shown. Solid lines and arrows depict active pathways present. Substrates of several enzyme activities are shown at the start of the solid lines and the product(s) are shown on the arrowhead side. Several key enzymes are indicated in capital italicized text beside the arrowhead lines. All pathways shown appear to be present as indicated; however, the significance of the folate salvage and pABA salvage pathways is unclear in Tg and Pf and is indicated with a question-mark. Polyglutamated forms of folate are not shown. dTMP synthesis is shown at the bottom of the thymidylate cycle. Salvage of thymidine in Cp is shown. DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthetase; SHMT, serine hydromethyltransferase; DHFR, dihydrofolate reductase; TS, thymidylate synthetase; TK, thymidine kinase.

that it also expresses a TK activity that grants this parasite a potentially significant salvage pathway to dTMP (Figures 18.2, 18.3) (Abrahamsen *et al.*, 2004; Striepen *et al.*, 2004). It is currently unknown whether inhibition of DHFR will inhibit *C. parvum* replication.

18.3.4.1 Biosynthesis of folates in Apicomplexa

The primary precursors for the *de novo* synthesis of folates are *para*-aminobenzoic acid and guanosine

5'-triphosphate (GTP), a purine nucleotide that protozoan parasites cannot synthesize *de novo*. Humans lack the ability to synthesize folates *de novo*, and rely on folate transport from dietary sources. By contrast, several of the apicomplexans possess an endogenous folate biosynthetic pathway that is susceptible to antifolate inhibitors. *T. gondii* and *P. falciparum* possess the full complement of genes encoding all seven enzymic steps in the *de novo* biosynthesis of 7,8-dihydrofolate (Figure 18.3). By contrast, genome analysis indicates that *C. parvum* lacks any identifiable gene ortholog for folate

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biosynthetic enzymes, and is apparently dependent on folate salvage to obtain 7,8-dihydrofolate for the thymidylate cycle (Figure 18.3) (Abrahamsen *et al.*, 2004; Striepen *et al.*, 2004).

P. falciparum, C. parvum, and T. gondii possess gene orthologs for putative folate transporters, indicating that these parasites have the potential to salvage folates from the host (Striepen et al., 2004; Chaudhary, 2005). Both the salvage pathway to para-aminobenzoic acid and the de novo synthesis of 7,8-dihydrofolate from GTP and paraaminobenzoic acid are functional in P. falciparum because labeled precursors are incorporated into end products (Wang et al., 2004a). Functional data on folate salvage in T. gondii and C. parvum are not available. In addition to salvage from the host, a second pathway to para-aminobenzoic acid exists as a product of the shikimate pathway via chorismate. While C. parvum possesses none of the genes involved in this pathway, T. gondii and P. falciparum encode several of the enzymes of this pathway (Figure 18.3) (Roberts et al., 1998; McConkey et al., 2004).

18.3.4.2 Antifolate chemotherapy and antifolate resistance

For several decades, antifolates such as pyrimethamine (PYR) and proguanil (PG), which target DHFR and folate recycling, and sulfa drugs, which target dihydropteroate synthase (DHPS), have been in clinical use for the treatment of P. falciparum and T. gondii infections (Figure 18.3) (Brooks et al., 1987; Gregson and Plowe, 2005). While humans depend upon a monofunctional dihydrofolate reductase (DHFR) for DNA replication, in Apicomplexa the DHFR activity is present on a bifunctional polypeptide with TS activity (DHFR-TS) (Bzik et al., 1987). Sulfa drugs target DHPS, which is a monofunctional enzyme in humans but is also a bifunctional enzyme in T. gondii and P. falciparum with hydroxymethylpterin pyrophosphokinase activity (Triglia and Cowman, 1994; Pashley et al., 1997).

Mutations in *P. falciparum* DHPS have been correlated with resistance to sulfa drugs (Triglia *et al.*, 1998; Wang *et al.*, 2004b). Genetic studies have documented that various mutations in DHPS correlate with resistance to sulfa drugs *in vivo* (Triglia *et al.*, 1998).

Resistance to antifolates that target DHFR arose rapidly in *P. falciparum*. Current antifolate therapy is based on synergism achieved in combination therapy of a DHFR inhibitor combined with sulfa drugs (Gregson and Plowe, 2005). The current treatment for *T. gondii* infection employs a similar strategy using PYR and sulfadiazine. While *T. gondii* has not developed resistance to antifolates, long-term use of this therapy to treat toxoplasmosis in AIDS patients has proven to be difficult due to significant adverse clinical reactions (Haverkos, 1987; Leport *et al.*, 1988).

Antifolates are also under investigation as potential inhibitors of *C. parvum* (Anderson, 2005). However, genome data from *C. parvum* indicate the presence of a TK gene that could bypass inhibition by antifolates if the TK activity is physiologically relevant. The atypical presence of a TK gene in *C. parvum*, as well as the lack of a DHPS gene, the target of sulfa compounds, suggests that directly targeting *C. parvum* DHFR to inhibit replication may be a difficult objective (Striepen *et al.*, 2004).

Antifolate resistance in P. falciparum and C. parvum In early work, mutation of DHFR in bifunctional DHFR-TS was identified as the basis of resistance to PYR in P. falciparum (Inselburg et al., 1987, 1988). In laboratory isolates, gene duplication and chromosomal changes have also been reported as a mechanism of resistance to PYR (Inselburg et al., 1987; Tanaka et al., 1990a, 1990b). Early studies of PYR resistance in isolates of P. falciparum identified mutations in key amino-acid residues (codons 51, 59, and 108) that were associated with high-level resistance to PYR (Cowman et al., 1988; Inselburg et al., 1988; Peterson et al., 1988). The crystal structure of wild-type and mutant P. falciparum DHFR-TS suggests that these key resistance mutations cause steric interactions with inhibitors or weaken the binding of inhibitors in the active site (Yuvaniyama et al., 2003). Several new antifolates that inhibit current drug-resistant alleles of P. falciparum DHFR are currently in clinical trials (Anderson, 2005; Gregson and Plowe, 2005).

The crystal structure of *Cryptosporidium hominis* DHFR-TS was recently determined (O'Neil *et al.*, 2003a, 2003b), and has revealed some new insights into the natural resistance of *C. parvum* DHFR to antifolates (Vasquez *et al.*, 1996). Since no current genetic selection model is available for *C. parvum*, the identification of strong inhibitors of *C. parvum* DHFR or TK may be a useful approach to define the most amenable targets for blocking dTMP accumulation and DNA replication in *C. parvum* infection.

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Antifolate resistance in T. gondii Resistance to pyrimethamine has not been frequently observed in clinical treatment of toxoplasmosis. Even low-level resistance to PYR has been difficult to select under drug pressure in vitro, but has been demonstrated in a genetic model of direct mutagenesis of a plasmidborne T. gondii DHFR-TS followed by transfection of PYR-sensitive T. gondii and growth selection under PYR in vitro (Reynolds et al., 2001). By modeling *P. falciparum* resistance mutations at codons equivalent to 59 and 108 (Bzik et al., 1987), high-level PYR resistance was obtained in T. gondii DHFR-TS (Donald and Roos, 1993; Roos, 1993). Plasmids conferring high-level PYR resistance in T. gondii have been useful in genetic dissection of evolution and mechanisms associated with PYR and cycloguanil resistance (Reynolds and Roos, 1998). This approach was adapted to investigate the evolutionary fitness of DHFR-TS mutations associated with PYR resistance. This study revealed subtle, but potentially significant, effects on fitness of mutant DHFR-TS enzymes in vitro that appear to be associated with the natural appearance, or non-appearance, of these mutants in vivo (Fohl and Roos, 2003).

Modeling studies of *T. gondii* DHFR-TS have suggested that the long linker domain connecting DHFR to TS domains in Apicomplexa donates a helix that crosses to the second DHFR domain of the homodimer complex and contacts the outer shell of the DHFR active site (O'Neil *et al.*, 2003b; Belperron *et al.*, 2004). Genetic studies of *T. gondii* DHFR-TS revealed that various mutations within the linker domain inactivated pyrimethamine resistance and enzyme activity *in vitro* and *in vivo* (Belperron *et al.*, 2004).

18.3.5 Genetic selection models based on enzymes of pyrimidine metabolism

High-level PYR resistance based on mutant DHFR-TS was used to establish an early model of positive selection in T. gondii (Donald and Roos, 1993; Roos, 1993). The high efficiency of obtaining PYR-resistant parasites allowed the refinement of methods for efficiently incorporating plasmid DNA into the parasite (Roos et al., 1994). The isolation of both a genomic DNA version as well as a cDNA version of the PYR-resistant DHFR-TS enabled an assessment of general homology requirements for recombination in T. gondii. The cDNA version of T. gondii DHFR-TS lacks numerous introns contained in the genomic DNA version, and randomly incorporates into the genome of the parasite (Donald and Roos, 1993). By contrast, the genomic DNA version was demonstrated primarily to integrate into the homologous DHFR-TS locus (Donald and Roos, 1994, 1995). These studies established methods and tools for random insertional mutagenesis, as well as gene replacement approaches in T. gondii.

Subsequently, the high-level PYR-resistant bifunctional DHFR-TS was converted into a trifunctional enzyme by the incorporation of either herpes simplex virus TK, or bacterial cytosine deaminase (CD). The TK and CD enzymes were inserted as in-frame genes into the linker domain of T. gondii DHFR-TS. The DHFR-CD-TS plasmid conferred high-level resistance to PYR (positive selection), and stable PYR-resistant parasite clones were killed (negative selection) by treatment of parasites with low doses of the normally non-toxic prodrug 5-fluorocytosine (Fox et al., 1999). This study established DHFR-CD-TK as a trifunctional enzyme capable of positive and negative selection in T. gondii. Transgenic T. gondii parasites expressing CD are killed by 5-fluorocytosine by its conversion to 5-fluorouracil, through salvage by T. gondii UPRT to 5-fluorouridine 5'-monophosphate, and conversion to 5-fluoro-dUMP, a suicide inhibitor of TS activity that blocks the accumulation of dTMP and therefore parasite replication (Fox et al., 1999).

Similarly, construction of a plasmid encoding the trifunctional enzyme DHFR-TK-TS on a single

polypeptide enabled positive selection by highlevel resistance to PYR, and negative selection in submicromolar doses of ganciclovir (Fox *et al.*, 2001). The DHFR-TK-TS plasmid was used in positive and negative selection experiments to obtain the avirulent uracil auxotroph mutant by targeted knockout of the CPSII gene (Fox and Bzik, 2002). The *T. gondii* CPSII is the most amenable among the apicomplexans for genetic dissection of CPSII activities and regulation. Cloning of the cDNA for *T. gondii* CPSII enables a genetic scheme for positive selection based on complementation of the uracil auxotroph CPSII knockout mutant. Complementation of CPSII has been demonstrated (Fox and Bzik, 2002).

18.4 AMINO ACIDS

Amino acids are essential in numerous areas of parasite metabolism, and are necessary precursors for nucleotide synthesis. L-aspartate is essential for the synthesis of AMP from IMP in T. gondii and P. falciparum (Figures 18.1A, 18.1C), and the amino acids L-glutamine and L-aspartate are essential precursor molecules for de novo pyrimidine synthesis of UMP in T. gondii and P. falciparum (Figure 18.2). In Apicomplexa, serine is also required for synthesis of dTMP during the thymidylate cycle (Figure 18.3). This section will briefly review acquisition of amino acids in Apicomplexa. Other reviews of amino-acid metabolism in protozoa can be found in previous texts (Gutteridge and Coombs, 1979; North and Lockwood, 1995).

18.4.1 Studies of amino acid auxotrophy in *T. gondii*

Surprisingly, the architecture of CPSII activity in *T. gondii* and *P. falciparum* revealed the presence of a single glutamine-dependent CPSII gene and activity (Flores *et al.*, 1994; Fox and Bzik, 2002, 2003). The existence of a single CPSII in *T. gondii* is highly unusual for a eukaryotic organism. In many prokaryotes a single CPS polypeptide is typically

found, and this CPS activity is responsible for producing carbamoyl phosphate, the precursor molecule for both pyrimidines and arginine. Consequently, disruption of *E. coli* CPS produces a dual pyrimidine and arginine auxotrophy (Beckwith *et al.*, 1962).

In many eukaryotes two distinct CPS genes and activities are found: a glutamine-dependent CPSII linked with pyrimidine biosynthesis, and a mitochondria-associated CPSI dedicated to arginine biosynthesis (Makoff and Radford, 1978; Davis, 1986). The carbamoyl phosphate produced by CPSI in many eukaryotes is sequestered in the mitochondria for immediate conversion to citrulline via ornithine carbamoyltransferase (OCT). Arginine is produced from citrulline in two steps by the sequential actions of argininosuccinate synthetase (AS) and argininosuccinate lyase (AL) (Makoff and Radford, 1978; Davis, 1986).

The availability of a CPSII knockout mutant in T. gondii enabled a functional determination of whether the sole CPSII in Apicomplexa is responsible for pyrimidine and arginine biosynthesis (Fox et al., 2004). This study conclusively demonstrated that the T. gondii CPSII and the carbamoyl phosphate product are dedicated to pyrimidine biosynthesis. T. gondii is a natural arginine auxotroph. The arginine auxotrophy of T. gondii is rescued by supplementing growth media with either arginine or citrulline. Using mutant host cells it was demonstrated that rescue with citrulline was dependent on the presence of host-cell AS and AL activities. These experiments demonstrated the functional absence of any arginine biosynthetic enzyme activity in T. gondii, and this conclusion has been verified by the absence of corresponding gene orthologs in the T. gondii genome (Chaudhary and Roos, 2005).

Other apicomplexans such as *C. parvum* and *P. falciparum* are also natural arginine auxotrophs (see below). The natural arginine auxotrophy and arginine depletion in *T. gondii* infection have been linked with the differentiation of tachyzoites to bradyzoite-containing cysts *in vitro* (Fox *et al.,* 2004). These observations suggest that local depletion of arginine by inducible nitric oxide synthase during the host immune response to *T. gondii*

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infection may promote signaling or formation of slow-growing bradyzoites or cyst development and maintenance (Wu and Morris, 1998; Fox *et al.*, 2004; Grillo and Colombatto, 2004).

Curiously, a second amino-acid auxotrophy of T. gondii has been previously linked to host immune response and control of infection. Early work demonstrated a natural tryptophan auxotrophy of T. gondii, which was also associated with reduced growth rate of tachyzoites during tryptophan depletion elicited by the host immune response to T. gondii infection (Pfefferkorn, 1984, 1986; Pfefferkorn and Guyre, 1984; Pfefferkorn et al., 1986a, 1986b; Khan et al., 1988). In this case, the tryptophan depletion is triggered from downstream responses to the strong induction of interferon gamma during the host immune response to T. gondii infection. Collectively, these studies suggest a fundamental link in host immune responses leading to local depletion of amino acids at sites of T. gondii infection as a general mechanism to slow parasite growth or trigger differentiation of rapidly replicating tachyzoites into slow-growing encysted bradyzoite forms.

18.4.2 Genome studies of aminoacid metabolism in Apicomplexa

Relatively little experimental work has been reported on amino-acid transport, synthesis, interconversion, or catabolic pathways in the Apicomplexa. The genome of T. gondii, P. falciparum, and C. parvum has enabled a predictive comparison of the capabilities of these parasites in regard to amino-acid metabolism. Humans require 9 amino acids in their diet, and can synthesize the remaining 11 amino acids required for protein synthesis. Mammals, primarily in liver tissue, can synthesize arginine de novo from CPSI, OCT, AS, and AL activities, and arginine is now considered to be a non-essential amino acid (Wu and Morris, 1998; Grillo and Colombatto, 2004). In addition to arginine, humans can synthesize alanine, serine, and glycine (derived from glycolysis), glutamic acid, glutamate, proline, aspartic acid and asparagine (derived from the TCA cycle), and tyrosine and cysteine (derived from essential amino acids phenylalanine and methionine, respectively, from the diet).

All of the protozoan and apicomplexan parasites have diminished amino-acid biosynthetic capabilities compared with the human host (Chaudhary and Roos, 2005). C. parvum as a gut pathogen likely has the most access to host amino acids. This is consistent with C. parvum harboring the most diminished amino-acid biosynthetic capability. C. parvum is auxotrophic for tyrosine, alanine, serine, glutamic acid, aspartic acid, asparagine, arginine, and cysteine, and can synthesize only three amino acids (glycine, glutamate, and proline). While C. parvum has acquired an auxotrophy for serine, it has lost an auxotrophy for the essential amino-acid tryptophan that can be metabolized from serine. C. parvum may also retain a salvage pathway for tryptophan. P. falciparum, in the process of digesting host hemoglobin, would have reduced needs for amino-acid biosynthetic capability. Consistent with this available food source, P. falciparum is auxotrophic for tyrosine, alanine, serine, arginine, and cysteine, and can synthesize only six amino acids (glycine, glutamic acid, glutamate, proline, aspartic acid, and asparagine).

T. gondii has the least diminished amino-acid biosynthetic capability, and most resembles the human host. Compared with the human host, T. gondii is auxotrophic only for arginine and cysteine. Uniquely, lysine is not an essential amino acid in T. gondii because the parasite can synthesize lysine from aspartic acid derived in the TCA cycle by the diaminopimelate pathway (Chaudhary and Roos, 2005). The acquired ability of T. gondii to synthesize lysine suggests an increased need for this amino acid, or its limited availability in host tissues inhabited by this parasite. T. gondii may also be capable of salvaging lysine from the host cell, but if the parasite lacks this capability then the diaminopimelate pathway represents a potential target. Collectively, these observations suggest significant adaptations of the different apicomplexan parasites related to their potentially available nutritional amino acid resources.

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Toxoplasma as a Model System for Apicomplexan Drug Discovery

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19.1 INTRODUCTION

As parasitic protozoa, the Apicomplexa encompass a wide range of pathogenic species for which current therapies are either inadequate or threatened by emerging drug resistance. This chapter reviews the productive application of *Toxoplasma* as a model system for apicomplexan drug discovery, in particular for the validation of potential targets. The need for effective validation tools is partly driven by the plethora of potential targets revealed by systematic comparative analysis of completed genomes of *Toxoplasma, Theileria, Plasmodium, Cryptosporidium,* and *Eimeria* (Nene *et al.,* 2000; Gardner *et al.,* 2002, 2005; Abrahamsen *et al.,* 2004; Bahl *et al.,* 2002). However, the novelty alone of a gene or pathway in

the genome of a pathogen compared with the host is not the only criterion for deeming a potential target an attractive one. Indeed, some wellestablished or novel antiparasitic targets are orthologs of enzymes present in the animal host, where subtle evolutionary divergence can be effectively exploited by selective small molecule inhibitors. Of fundamental importance to any discovery endeavor is genetic validation, the demonstration that a potential gene target is essential rather than functionally redundant or (unexpectedly) dispensable in nature. In commercial programs, where a considerable medicinal chemistry investment is typically required to develop suitable pharmacokinetic and safety properties from initial leads, rigorous genetic as well as pharmacological validation of a target

is a pre-requisite. As the most well-developed apicomplexan system for molecular genetic manipulation, Toxoplasma is ideal for this purpose. With a full complement of molecular tools available, molecular genetic interrogation of any gene of interest is now eminently feasible. The ease with which Toxoplasma can be cultivated both in vitro and in rodent models facilitates the evaluation of compounds with desirable efficacy and pharmacological properties. The amenability of both Toxoplasma and its murine host to genetic manipulation can help define the selectivity of a compound for processes in the parasite compared with analogous ones in the host, as well as evaluate the sometimes synergistic interplay between antiparasitic efficacy and the host immune system.

The use of Toxoplasma as a model parasite for investigating various aspects of apicomplexan biology has been the subject of previous reviews in which the utility as well as the limitations of this tractable surrogate have been clearly articulated (Roos et al., 1999a, 1999b; Kim and Weiss, 2004). Where clear-cut orthologs of target enzymes or proteins are present across related species, molecular genetic validation approaches in Toxoplasma are frequently productive. For example, the resistance determinants of several broad-spectrum clinically used drugs, including pyrimethaminesulfur and atovaquone, have been confirmed at the level of target enzymes by mutational analysis in Toxoplasma (McFadden et al., 2001). On the other hand, Toxoplasma and malaria differ so profoundly in key aspects of their life cycle that the primary mechanism of mainstay antimalarials (such as aminoquinolines or artemisenins) cannot be explored in this system, due to the absence of an erythrocytic-stage food vacuole. For closely related coccidians such as Eimeria, Sarcocystis and Neospora, Toxoplasma is likely to be a more consistently useful model as these all share a similar life cycle of sporozoite invasion, sporogeny, merogeny, and oocyst development in the gut epithelium of their definitive hosts. For the evolutionary distant apicomplexans Cryptosporidium and Theileria, however, where there is a much greater degree of metabolic and physiological divergence (Nene et al., 2000; Huang et al., 2004; Striepen and Kissinger, 2004; Striepen, 2006), there is little basis for useful comparison with *Toxoplasma*. In the case of *Cryptosporidium*, comparative analysis of *Toxoplasma* and malaria genomes reveals that such differences are a consequence of a great deal of horizontal gene transfer (from bacteria and algal endosymbionts), genome miniaturization, and loss of plastid and mitochondrial function (Abrahamsen *et al.*, 2004; Huang *et al.*, 2004).

The chapter begins with a brief history of the use of Toxoplasma for dissecting apicomplexan metabolic pathways that are potentially exploitable as chemotherapeutic targets. To minimize redundancy with previous reviews (McFadden and Roos, 1999; McFadden et al., 2001; Coombs and Muller, 2002; Seeber, 2003; Campbell et al., 2004; Kim and Weiss, 2004; Lu et al., 2005), highlights of more recent work will be emphasized or topics indulged where personal experience may provide perspective. A discussion of current strategies for industrial antimicrobial drug discovery programs will follow, which favor the use of cell-based assays for screening compound collections rather than targetbased enzyme assays. A byproduct of this lack of a priori focus is that it can lead to the discovery of novel targets and biological processes that could not have been otherwise predicted by rationale or in silico design. The discovery of a broad-spectrum anticoccidial compound that blocks parasite hostcell invasion through the selective inhibition of a cGMP-dependent protein kinase (PKG) provides an excellent example of the utility of such an approach. It also offers an opportunity to present a case study for how the Toxoplasma model system has provided support and impetus for a medicinal chemistry effort focused on this target in the Eimeria pathogen (Donald et al., 2002; Gurnett et al., 2002; Biftu et al., 2005, 2006). The use of engineered PKG alleleic variants refractory to inhibitors in enzyme assays as well as in transgenic parasites has provided a means to measure the selectivity of compounds during the lead optimization process, as well as a way to identify potential secondary protein kinase targets that might also contribute to compound efficacy. In the last section, the future outlook for Toxoplasma as a validation tool is discussed in the context of recent technological developments such as systems for genetic complementation and the construction of conditional gene knockout strains. The chapter concludes with a pragmatic perspective on the future of apicomplexan drug discovery.

19.2 UNDERSTANDING MECHANISMS OF CURRENT THERAPIES

19.2.1 Antifolate therapies

Synergistic combinations of folate antagonists and sulfa drugs have been used to treat a variety of bacterial and apicomplexan pathogens, including Toxoplasma and Plasmodium species. The pyrimethamine-sulfa regimen remains the most effective treatment for symptomatic toxoplasmosis, and prior to the advent of widespread drug resistance was also a frontline treatment for malaria. Pyrimethamine is a competitive inhibitor of parasite DHFR with narrow (~ six-fold) selectivity for the T. gondii enzyme compared with the mammalian enzyme (Schweitzer et al., 1990; Chio and Queener, 1993). This small therapeutic window can account for some of the toxicity associated with the drug, which is reversible by co-administration of folinic acid. A major problem of antifolate combination therapy is the generally poor tolerance by patients for the sulfa drug component (sulfadoxine or sulfadiazine), which precludes their prophylactic or long-term use. When sulfa drug hypersensitivity arises in toxoplasmosis patients, clindamycin, azithromycin, or atovoquone can be substituted. None of these drugs is very effective as monotherapy, but in combination, synergism with pyrimethamine is observed (Djurkovic Djakovic et al., 1999, 2002; Chirgwin et al., 2002). Atovaquone is also used in combination with the antifolate proguanil (prodrug of cycloguanil) for the treatment of chloroquine-resistant malaria (Malorone). The interaction of all of these drugs with their target enzymes is now well established, in most cases aided significantly through the use of Toxoplasma as an experimental system.

The cloning, expression, and subsequent biochemical analysis of the fused DHFR-TS enzyme of *Toxoplasma* (Donald and Roos, 1993; Roos, 1993; Trujillo et al., 1996) has resulted in a detailed understanding of drug-target interactions and drug-resistance mechanisms that have also served as a useful model for the malarial enzyme. Active recombinant bifunctional T. gondii DHFR-TS is more readily expressed in E. coli and in transgenic parasites than its closely related malarial counterpart, facilitating the modeling of allelic variants associated with malarial field isolates resistant to pyrimethamine-sulfa therapy (Reynolds and Roos, 1998). In this study, Toxoplasma DHFR-TS substitutions (and combinations thereof) analogous to those associated with pyrimethamine and cycloguanil resistance in malaria DHFR-TS residues enabled a reconstruction of the likely phylogeny of resistance mutations that appear to occur in a stepwise fashion in malaria. These predictions could be rationalized at the level of their effects on enzyme kinetics and binding-site architecture, using a similarity model based on the crystal structure of the Leishmania major DHFR-TS. The combinations Arg59 + Asn108 and Arg59 + Ser223 (P. falciparum numbering) are especially resistant to pyrimethamine, resulting in an 80-fold reduction in susceptibility to pyrimethamine for parasites harboring single-copy TgDHFR-TS transgenes compared with untransformed parasites. Fitness costs associated with these drug-resistance alleles were assessed in follow-up in vitro and in vivo competition studies between isogenic T. gondii tachyzoites harboring wild-type and mutant DHFR-TS alleles (Fohl and Roos, 2003). The combination Arg59 + Asn108 had no effect on fitness in cultured parasites, whereas the Arg59 + Ser223 pair imparted a significant fitness defect of >2.8 percent per generation. In infected mice, the former alleleic combination was outcompeted by wild-type more rapidly than was the latter. The results help explain why the Arg59 + Asn108 persists in field isolates of P. falciparum, while the Arg59 + Ser223 combination has only been observed in laboratory strains.

The modeling of malarial pyrimethamine resistance alleles and their use as selectable markers in *Toxoplasma* (Donald and Roos, 1993) led directly to the development of a first generation of vectors for the stable transformation of malaria (Donald and Roos, 1993; van Dijk *et al.*, 1995; Crabb and Cowman, 1996; Wu *et al.*, 1996; Crabb *et al.*, 1997; van der Wel *et al.*, 1997). Subsequently, targeting constructs using the TgDHFR-TS minigene (or analogous *P. berghei* constructs) have been used to generate a variety of gene replacements and gene knockouts in *P. falciparum*, *P. berghei*, and *P. knowlesi* for functional studies (Sultan *et al.*, 1997; Triglia *et al.*, 2000; Tomas *et al.*, 2001; Billker *et al.*, 2004). In this way, early work in *Toxoplasma* that was inspired by the challenge to understand drug resistance in malaria has ultimately contributed significantly to the development of molecular genetics tools for both systems.

Clinical resistance to pyrimethamine-sulfa therapy in Toxoplasma has not been the problem that it was in malaria for several reasons. First, unlike with malaria, clinical drug resistance in Toxoplasma cannot be readily transmitted if it arises in response to treatment. Secondly, in most instances T. gondii infections are self-limiting and asymptomatic, both in the definitive host (felines) and in secondary hosts (other animals). As a result, the primary reservoir of potential infection is never exposed to drug therapy. An effort has been made to screen Toxoplasma clinical isolates by PCR amplification for mutations in DHFR-TS and DHPS genes that might confer resistance to pyrimethamine-sulfadoxine therapy (Aspinall et al., 2002a, 2002b). DHPS is the target of sulfa drugs, and multiple-resistance mutations associated with failed combination therapy have been identified in the malarial enzyme (Dorsey et al., 2004; Ogutu et al., 2005; Omar et al., 2005). No mutations that could be definitively associated with exposure to combination therapy were found in Toxoplasma, although one of four polymorphisms identified in the DHPS gene corresponded to an allele associated with sulfonamide resistance in malaria. This raises the possibility that naturally occurring, drug-resistance conferring DHPS polymorphisms are present in the parasite population in the absence of drug pressure.

19.2.2 Antibiotic protein synthesis inhibitors

The mechanism by which certain macrolide, lincosamide, and thiopeptide antibiotics inhibit

the growth of T. gondii and malaria has long been assumed to be at the level of protein synthesis occurring in plastid or mitochondrial organelles. Toxoplasma is particularly sensitive to azithromycin and clindamycin, while malaria is sensitive to thiostrepton. Protein-labeling experiments with tachyzoites suggest that cytosolic and mitochondrial protein synthesis is unaffected by antibiotic treatment (Beckers et al., 1995). Furthermore, ribosomal DNA sequences corresponding to plastid encoded (but not mitochondrial encoded) rRNAs are predicted to be sensitive to both azithromycin and clindamycin. Confirmation that these antibiotics inhibit plastid function was demonstrated by quantitative DNA hybridization analysis that showed the selective loss of the plastid genome in response to clindamycin treatment (Fichera and Roos, 1997). Ciprofloxacin, a fluoroquinoline inhibitor of bacterial and plant plastid DNA gyrase, also inhibited plastid replication, implicating another associated activity as a potential therapeutic target. Interestingly, a variety of drugs affecting plastid function in this manner all share a peculiar phenotype of delayed efficacy in which the first cycle of growth remains uninhibited (Fichera et al., 1995). Only parasites entering a second cycle of growth following egress from a primary vacuole are affected. This phenomenon has subsequently been observed in malaria with thiostrepton (Sullivan et al., 2000) and with clindamycin or chloramphenicol (Surolia et al., 2004). In contrast, tetracyclines, which also show some efficacy against Toxoplasma and malaria, have a more immediate effect on parasite growth, and are assumed to act primarily on mitochondrial protein synthesis (Beckers et al., 1995; Fichera et al., 1995). However, the high levels of tetracycline required to inhibit Toxoplasma growth in vitro (~100 µM) make it hard to rule out alternative mechanisms, including some level of inhibition of apicoplast translation.

More direct evidence for the inhibition of plastid protein synthesis by clindamycin has been confirmed by the identification of a resistance mutation (following selection in cultured parasites) that maps to the *T. gondii* plastid largesubunit rRNA at a position corresponding to the known clindamycin-binding site in *E. coli* rRNA (Camps *et al.*, 2002). Similarly, the site of binding of thiostrepton within the GTPase domain of malarial plastid 23S rRNA has been confirmed through *in vitro* mutagenesis experiments and binding assays, based on the knowledge of the analogous target in bacteria (Clough *et al.*, 1997; Rogers *et al.*, 1997).

19.2.3 Electron transport inhibitors

Hydroxynaphthoquinones form a class of compounds with broad-spectrum potency against a variety of protozoan parasites (Baggish and Hill, 2002). They include parvaguone and buparvaquone, which are remarkably effective in curing cattle infected with Theileria (McHardy et al., 1985; Hashemi Fesharki, 1991). Atovaguone, a chlorophenyl derivative of parvaquone, is used in various combinations to treat malaria (with proguanil as Malarone) and toxoplasmosis (with pyrimethamine), and also has clinical efficacy against Pneumocystis carinii and babesiosis (with azithromycin). These highly lipophilic compounds closely resemble the structure of ubiquinone, a mitochondrial electron carrier, suggesting a mechanism affecting mitochondrial membrane potential at the level of the cytochrome bc_1 complex. Bioenergetic studies of mitochondria in situ using permeabilized Toxoplasma tachyzoites provided the first direct biochemical evidence that the respiratory chain and oxidative phosphorylation are functional in apicomplexan parasites (Vercesi et al., 1998). These studies also demonstrated that the primary site of atovaquone inhibition is between cytochromes b and c₁, and that parasite respiration is at least 20-fold more sensitive than is mammalian respiration to the drug. As reduced ubiquinone is an essential cofactor of dihydroorotate dehydrogenase, it is also possible that atovaquone indirectly blocks pyrimidine biosynthesis.

The precise molecular interactions of atovaquone and its presumptive cytochrome target have come through an analysis of drug-resistant mutants. Mutagenesis studies with cultured *Toxoplasma* tachyzoites implicated a common mechanism for atovaquone and decoquinate (Pfefferkorn *et al.*,

1993), an aminoquinoline electron transport inhibitor and ubiquinol analog used to treat Eimeria and Toxoplasma infections in livestock (Wang, 1975; Fry et al., 1984). A Toxoplasma mutant selected for resistance to atovaquone in cell culture following chemical mutagenesis was partially cross-resistant to decoquinate (Ato^R-1), and a mutant selected for resistance to decoquinate was hypersensitive to atovaquone (Deq^R-1). Both mutants were resistant to the inhibitory effects of the drugs on respiration, as measured from rates of oxygen consumption by freshly prepared extracellular tachyzoites. An analysis of cytochrome b as the potential site of drug-resistance conferring mutations became possible with the reconstruction of the T. gondii gene by RT-PCR cloning (McFadden et al., 2000), a necessary strategy due to the presence in the T. gondii nuclear genome of numerous highlyfragmented copies of the cytochrome b gene (Ossorio et al., 1991). Two independent mutations (M129L and I254L) mapping to the cytochrome b Q_o domain were identified in atovaquone-resistant clonal isolates derived from mutagenized tachyzoites. When introduced into the yeast cytochrome b gene, the analogous alleles (M139L and I269L) also confer resistance to atovaquone in S. cerevisiae (Kessl et al., 2006). Molecular modeling, based on the crystal structure of the yeast and chicken cytochrome b enzymes, suggests that the mutated residues make contact with atovaquone in a pocket that can accommodate the hydrophobic sidechain of the drug that extends from the quinone core (McFadden et al., 2000; Kessl et al., 2003). Analogous Q₀ domain mutations are found associated with atovaquone-resistant Plasmodium (Srivastava et al., 1999; Syafruddin et al., 1999; Korsinczky et al., 2000; Kuhn et al., 2005). However, the cytochrome b gene of the Toxoplasma atovaquone-resistant mutant Ato^R-1 mutant appears unaffected, suggesting that resistance can arise by other mechanisms. Nevertheless, the Q₀ domain is also a likely determinant for decoquinate resistance in Toxoplasma. The atovaquone-hypersensitive, decoquinate-resistant DeqR-1 mutant (Pfefferkorn et al., 1993) harbors an F260L substitution that maps to the ubiquinol binding pocket according to the chicken

cytochrome b similarity model (McFadden and Boothroyd, 1999), consistent with a role in modulating interaction with both drugs.

19.3 VALIDATION OF SOME POTENTIAL APICOMPLEXAN TARGETS

19.3.1 Nucleotide metabolism

Much of the progress in understanding pyrimidine and purine metabolism in Toxoplasma is attributable to the pioneering work of Elmer Pfefferkorn (Dartmouth College). He developed genetic selection strategies for Toxoplasma mutants defective in pyrimidine and purine salvage pathways with the use of nucleoside or free base analogs including fluorouracil, adenosine arabinoside, and 6-thioxanthine. Antiparasitic activity of these analogs is dependent on their activation by salvage-pathway enzymes. Biochemical and genetic analysis of resistant mutants effectively unraveled the mechanisms by which the parasite scavenges pyrimidine and purine precursors from the host (Pfefferkorn, 1978; Pfefferkorn and Pfefferkorn, 1978; Pfefferkorn et al., 1983, 2001; Pfefferkorn and Borotz, 1994; Schwab et al., 1995). In summary, Toxoplasma scavenges the pyrimidine base uracil, but also makes pyrimidines de novo. Like all parasitic protozoa, Toxoplasma is a purine auxotroph, and salvages purine nucleotide precursors (in this case) through parallel redundant pathways. Subsequent confirmatory work by other groups has resulted in the cloning and detailed characterization of the enzymes involved. They include uracil phosphoribosyltransferase (UPRT) (Donald and Roos, 1995; Carter et al., 1997; Schumacher et al., 2002), hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) (Donald et al., 1996; Schumacher et al., 1996), adenosine kinase (AK) (Darling et al., 1999; Sullivan et al., 1999; Cook et al., 2000; Recacha et al., 2000; Schumacher et al., 2000), IMP dehydrogenase (IMPDH) (Sullivan et al., 2005), and an adenosine transporter (Chiang et al., 1999).

The ability to obtain uncompromised null mutants or gene knockouts in genes encoding

purine salvage enzymes has essentially invalidated the pathways as chemotherapeutic targets in *Toxoplasma* and in closely related coccidians such as *Eimeria*. Furthermore, comparative genomic analysis has revealed surprising diversity in purine salvage pathways among the Apicomplexa (Chaudhary *et al.*, 2004), making it unlikely that a single therapeutic strategy will find broadspectrum application. Malaria lacks AK and appears to salvage purines exclusively through HXGPRT. On the other hand, *Theileria* and *Cryptosporidium* lack HXGPRT altogether, and salvage via AK.

Recently there has been some effort to identify new subversive substrates of an activating adenosine kinase, using the *T. gondii* enzyme for evaluating structure–activity relationships (Yadav *et al.*, 2004; Rais *et al.*, 2005). While there seems to have been some success in finding compounds with greater selectivity for the parasite enzyme compared with the orthologous enzyme of the host, the 6-benzylthioinosine analogs described in these references have limited efficacy *in vitro* or *in vivo*, and there appears to be little opportunity for further optimization without a better understanding of how they are transported into or metabolized by the parasite.

In contrast with the poor outlook for purine nucleotide metabolism as a practical target for intervention, the pyrimidine biosynthetic pathway shows considerably more promise. Although Toxoplasma salvages uracil, de novo pyrimidine biosythesis does appear to be essential. Knockout mutations in T. gondii carbamoyl phosphate synthetase II, which catalyzes the committed first step of pyrimidine biosythesis, are uracil auxotrophs and are completely avirulent in vivo (Fox and Bzik, 2002). The situation is potentially more favorable still in malaria, which lacks pyrimidine salvage enzymes altogether and is dependent on the de novo pathway for pyrimidine nucleotides (Gutteridge and Trigg, 1970; Reyes et al., 1982; Seymour et al., 1994). Consistent with its atypical nature, Cryptosporidium lacks a de novo pathway entirely and instead seems to have compensated for this by acquisition through horizontal gene transfer of three salvage enzymes unique to the Apicomplexa, including a fused eukaryotic like uridine kinase-uracil phosphoribosyltransferase and a protobacterial thymidine kinase (Striepen *et al.*, 2004).

19.3.2 Lipid metabolism

Apicomplexan apicoplast pathways implicated in lipid metabolism have attracted considerable attention as potential therapeutic targets, and have been a subject of previous reviews (McFadden and Roos, 1999; Roos et al., 1999c; Roberts et al., 2003; Seeber, 2003; Ralph et al., 2004; Lu et al., 2005). The existence of an apicoplast-associated bacterial type II fatty-acid pathway in Toxoplasma and Plasmodium was first discovered during searches for likely plastid-targeted protein genes in publicly available genomic and EST sequence databases. A bipartite leader peptide was identified as a signature for nuclear encoded genes imported into the apicoplast, and was shown to be sufficient for the targeting of a heterologous GFP fusion protein or nuclear encoded acyl-carrier and ribosomal S9 proteins to this organelle in Toxoplasma (Waller et al., 1998). As apicoplast-targeting sequences are highly degenerate and hard to predict with certainty, this import assay has subsequently been used to confirm the organellular compartmentation of other imported proteins. In this way, components of an apicomplexan mevalonate pathway, responsible for isoprenoid biosynthesis and malarial fosmidomycin senstivity, were also found to be localized in the plastid (Jomaa et al., 1999). Similarly, a Toxoplasma plastid acetyl CoA carboxylase (ACCase) was identified, which catalyzes the key committed step in FAS biosythesis, and which is sensitive to clodinafop and related herbicides (Jelenska et al., 2001; Waller et al., 2003). The sensitivity of Toxoplasma and Plasmodium to selective inhibitors of the bacterial II FAS pathway, including thiolactomycin and triclosan, has provided some additional pharmacological validation of this pathway (Waller et al., 1998, 2003; McLeod et al., 2001; Surolia and Surolia, 2001).

Although the use of herbicides or antibiotics to probe lipid biosythetic pathways is suggestive, it is far from definitive evidence of their indispensibility. Some of these reagents, including thiolactomycin derivatives (Waller et al., 2003) and fop herbicides (Zuther et al., 1999), show poor antiparastic efficacy both in vitro and against their hypothetical fabH and ACCase enzyme targets (10–50 μ M IC₅₀). Aureobasidin A, a well-characterized inhibitor of fungal inositol phosphporylceramide biosynthesis (Endo et al., 1997; Takesaiko et al., 1993), shows reasonable potency against tachzoites (~1 μ M IC₅₀), but its effects on parasite sphingolipid pools appear to be relatively minor and other antiparasitic targets cannot yet be ruled out (Sonda et al., 2005). Another well-known inhibitor is the antibacterial disinfectant triclosan, used commonly in toothpastes and soaps. In bacteria it shows selectivity for fatty-acid enoyl reductase (fabI). Triclosan shows good efficacy against Toxoplasma and malaria in vitro (sub-µM IC₅₀), and some activity in mouse models (McLeod et al., 2001; Surolia and Surolia, 2001). However, it has poor solubility and well-documented non-specific effects on membranes (Villalain et al., 2001; Lygre et al., 2003; Guillen et al., 2004; Paul et al., 2004). Much has been made of the observation that, unlike inhibitors of apicoplast DNA or protein synthesis, triclosan appears to act quickly (Surolia et al., 2004). However, it is not clear to what extent the efficacy is due to the inhibition of the fabI target. Knowledge of triclosan-reistant enoyl-reductase alleles in bacteria should allow the construction of transgenic Toxoplasma or Plasmodium parasites harboring orthologous resistance mutations (Heath et al., 2000; Brenwald and Fraise, 2003), to better evaluate the extent to which triclosan efficacy is dependent on this enzyme. Strategies to improve the cellular uptake of triclosan have been attempted, including the creation of prodrug forms by coupling to a peptide carrier for use against T. gondii tachyzoites (Samuel et al., 2003) or to a beta-lactam core for use as an antibiotic (Li et al., 2002; Stone et al., 2004). In both cases improved cellular uptake and improved efficacy was convincingly demonstrated, associated with intracellular release by cellular esterases and beta-lactamases respectively. However, neither of these studies showed encouraging data on the antimicrobial effects of these conjugates in vivo. To overcome the poor solubility of triclosan

for use as an antimalarial, new colloidal formulations using nano-technological devices have also been examined (Maestrelli *et al.*, 2004). Alternatively, the synthesis of a new structural class of broadspectrum fabI-directed antibiotics with more suitable pharmacokinetic properties may be worth exploring (Payne *et al.*, 2002).

A significant complicating factor in the use of Toxoplasma as a model for understanding apicomplexan lipid biosynthetic pathways and for purposes of target validation is the emerging evidence for significant differences among the species. While malaria has a single apicoplast bacterial (type II) FAS pathway, the Toxoplasma genome has additional genes encoding cytosolic (type I) FAS and ACCase enzymes (Jelenska et al., 2001; Ralph et al., 2004). Cryptosporidium lacks an apicoplast and has a cytosolic type I fatty-acid synthase (Zhu et al., 2000; Zhu, 2004). Theileria appears to harbor a highly reduced apicoplast that lacks a type II fattyacid biosythetic pathway but which retains the ability to produce isoprenoids (Gardner et al., 2005). A cytosolic type I fatty-acid biosynthetic pathway also appears to be absent.

Despite the promise of a plethora of potential therapeutic targets associated with the plastid lipid metabolism, a number of important questions remain about their druggability. Toxoplasma and malaria scavenge most of their lipid from infected host cells, including fatty acids, phospholipids, and cholesterol (Rock, 1971a, 1971b; Vial et al., 1999; Coppens et al., 2000; Charron and Sibley, 2002; Jackson et al., 2004). If the parasite does not make the bulk of its lipid de novo, what is the function of apicoplast lipid biosythesis? Labeling experiments and biochemical analysis have yet to identify specific products of this pathway. Will inhibition of these parasite pathways cause cytocidal, cytostatic, or delayed effects on growth? For the treatment of acute toxoplasmosis or cerebral P. falciparum malaria, cytocidal compounds that act quickly on essential targets are clearly desirable. On the other hand, cytostatic compounds, but probably not slow-acting ones, may be adequate for prophylactic use. Fortunately, with the availability of molecular tools for Toxoplasma and Plasmodium, forthcoming genetic dissection of the enzymatic components of parasite lipid biosynthetic pathways should provide some answers. A first step in this direction has been the construction of a *T. gondii* acyl carrier protein (ACP) conditional null mutant (Mazumdar *et al.*, 2006). Under non-permissive conditions, growth of this parasite cell line was reduced *in vitro*, with the severity of inhibition increasing with additional rounds of growth in HFF cell monolayers. These results demonstrate the importance of the FAS II pathway for tachyzoite growth, but also confirm the impression that chemotherapeutic targeting of this pathway may be limited by delayed cytostatic efficacy.

19.3.3 The cytoskeleton

Toxoplasma has proven to be an excellent model system for understanding mechanisms of apicomplexan motility as well as the underlying dynamics and architecture of the parasite cytoskeleton (Carruthers, 2002; Soldati et al., 2004; Soldati and Meissner, 2004; Wetzel, 2004). Cytoskeletal structural proteins are targets of a variety of non-selective natural product toxins, such as cytochalasin D (against actin), and antimitotic colchicines, vinblastines, and taxols (against tubulin). The high degree of conservation observed across eukaryotic species for these proteins would appear to make them far from ideal targets for the selective inhibition of eukaryotic pathogens. However, commercially successful synthetic inhibitors of tubulin polymerization have been developed for the eradication of fungal crop pathogens (benomyl) and parasitic helminths (benzimidazoles), and the selective control of weeds in arable crops (dinitroaniline herbicides). The dintroaniline herbicide trifluralin is also selectively toxic to protozoans, including apicomplexan parasites (Stokkermans et al., 1996; Bell et al., 1988; Armson et al., 1999; Dow et al., 2002) and kinetoplastid parasites (Traub Cseko et al., 2001). In cell culture, the apicomplexan parasites Toxoplasma, Plasmodium, and Cryptosporidium are significantly more sensitive to these agents (low µM to sub-µM range) than are kinetoplastid Trypanosoma and Leishmania parasites. Unfortunately their insolubility, a desirable property for a herbicide, precludes their use

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systemically as a drug in animals (Benbow *et al.*, 1998; Armson *et al.*, 1999; Dow *et al.*, 2002). Nevertheless, this structural class of compounds has served as a useful reagent – and *Toxoplasma* as a convenient model – for investigating tubulin as a therapeutic target.

Toxoplasma tachyzoites are highly sensitive to dinitroaniline herbicides ethafluralin, oryzalin (IC₅₀ 100 nM), and trifluralin (IC₅₀ 300 nM) (Stokkermans et al., 1996). Human foreskin fibroblasts (HFFs) used to cultivate the parasites are insensitive to these compounds, even at levels greater than 100-fold higher than those inhibiting parasite growth. Electron-microscope ultrastructural examination has revealed that all of the compounds disrupt mitotic spindle-pole formation (Stokkermans et al., 1996). At the same time, the compounds have little effect on cellular metabolism as measured by radiolabeled uracil incorporation. Oryzalin and ethafluralin have somewhat different effects on other aspects of the tubulin cytoskeleton. Oryzalin-treatment causes a loss of subpellicular microtubules, whereas ethafluralin has more of an effect on the endoplasmic reticulum and nuclear envelope. In this study (Stokkermans et al., 1996), chemically induced mutant parasites resistant to oryzalin, ethafluralin or trifluralin were selected exhibiting a variety of cross-resistance phenotypes, suggesting that the mechanism for resistance might be controlled by multiple loci.

A subsequent, more comprehensive, investigation of the mechanism of oryzalin resistance among a large collection of mutants has fulfilled this earlier prediction. A total of 21 different amino-acid substitutions at 16 positions in α -tubulin were identified that mapped to two distinct structural domains, based on a model of the bovine (Bos taurus) α-tubulin crystal structure (Morrissette et al., 2004). When introduced into parasites, either as transgenes or as allelic replacements, the mutations were sufficient to confer oryzalin resistance. Some of the mutations map to regions (M or N loops) that are thought to mediate lateral adhesion of protofilaments and probably counter dintroaniline action by hyperstabilizing microtubules. Most were found to be in the core of α -tubulin, where they have the potential to affect the conformation of the dintroaniline binding site. Docking simulations based on a similarity model of the T. gondii sequence were used to identify the location of a high-affinity oryzalin binding site. Results of the analysis suggest that oryzalin does not interact with equivalent residues in the bovine sequence. Two T. gondii α-tubulin substitutions in binding site residues, Thr239Ileu and Ser165Ala, are predicted to block oryzalin binding directly. The Thr239Ileu allele is also associated with resistance to dinitroanilines in the goosegrass weed (Anthony et al., 1998). As residues that are predicted to make contact with oryzalin in the T. gondii protein are not restricted to plant and protozoan α-tubulin, other amino acids (including a number of core domain drug resistance alleles implicated in this study) must indirectly stabilize the three-dimensional conformation of this binding site. Unfortunately, the implications of these findings - especially in the context of the limited resolution of the T. gondii α-tubulin similarity model - do not favor structure-based approaches for identifying alternative pharmacophores.

19.4 EMPIRIC SCREENING FOR SMALL-MOLECULE INHIBITORS

Although huge investments in high-throughput screening (HTS) and combinatorial chemistry technologies were made by the pharmaceutical industry during the 1990s, development of new antibacterial and antifungal drugs has fallen far short of expectations (Youngman, 2002; Projan, 2003; Mullin, 2004; Projan and Shlaes, 2004; Spellberg *et al.*, 2004). While enzyme-based HTS focused on well-validated targets has yielded some examples of selective inhibitors (Hackbarth *et al.*, 2002; Seefeld *et al.*, 2003), not a single new drug has emerged despite considerable effort at lead refinement through medicinal chemistry and structure-based design.

Fundamentally, the HTS screening paradigm applied to microbial pathogens has failed as a result of the limited structural diversity inherent in synthetic compound libraries combined with an intrinsic lack of favorable cell-permeability properties. Building the requisite pharmacokinetic properties into an HTS lead through medicinal chemistry is difficult enough for any development program, but converting a molecule into a drug that can also freely traverse the microbial cell wall has so far proven extraordinarily difficult. In the arena of natural products, where permeability has often been selected for by nature, the problem is somewhat different. Here, the challenge is to identify novel antimicrobial compounds from crude fermentation broths from which a broad range of common toxins and antibiotics have already been mined over several decades of intensive bio-prospecting.

Like fungal and bacterial pathogens, apicomplexans present additional permeability barriers for therapeutic compounds compared with the mammalian cells. To block the proliferation of the intracellular parasite, a compound must traverse the host-cell plasma membrane, the parasitophorous vacuole membrane, and the parasite plasma membrane. Access to targets in mitochondrial or plastid compartments requires transport across additional membrane barriers. Although the vacuolar membrane is permeable to small molecules, the parasite surface is not (Schwab et al., 1994). As a result, effective antimalarial and antitoxoplasmal drugs rely on a relatively narrow range of physical properties to promote access to intracellular targets. For example, effective antiparasitic antifolates such as pyrimethamine are typically more lipophilic than analogous drugs targeting mammalian cells. In addition to lipophilicity, drugs such as aminoquinolines or artemisinins that target the malarial food vacuole have a requisite weak-base property that promotes their trapping inside this acidic compartment (Egan et al., 2000; Hindley et al., 2002). As a result of such constraints, enzymebased HTS approaches for finding new leads to combat apicomplexan parasites are likely to be as unfruitful as they have been for antimicrobial targets.

An appreciation of the limitations of targetbased HTS in antimicrobial drug discovery has led to a renewed emphasis on a more empirical

approach to the screening of compound libraries, in which cell-based screens are used as the primary filter for identifying novel antimicrobial compounds. Using this strategy, compounds that effectively kill the pathogen in vitro are quickly identified. Secondary cell-based assays are used to eliminate toxic compounds and to prioritize hits based on spectrum of activity toward related pathogens. The challenge then becomes one of identifying the mechanism of killing so that targetbased assays can be established to promote lead refinement efforts. Examples of recent successes include the discovery of the first-line oxazolidinone antibacterial drug linezolid (Slee et al., 1987), and the identification of a promising compound for the treatment of tuberculosis (Andries et al., 2005). In both cases, the mechanism of action at respective ribosomal and ATPase targets was determined subsequently through a combination of biochemical and genetic approaches.

For apicomplexan parasites, empiric cell-based screening has long been the paradigm for commercial anti-coccidial drug discovery efforts. The target organisms in this case are a variety of avian Eimeria species, which afflict factory-farmed poultry with major agronomic impact. Broadspectrum coccidiostat compounds must be used prophylactically in these settings to prevent periodic outbreaks. The difficulty in cultivating Eimeria in vitro has led to the use of related but more amenable coccidians as experimental surrogates. Toxoplasma has played a useful role in investigating the biochemical mechanism of commercial coccidiostats, and in evaluating their susceptibility to drug resistance (Pfefferkorn et al., 1988; Hupe et al., 1991; Ricketts and Pfefferkorn, 1993). For screening compound libraries, an in vitro antiparasitic assay using the murine apicomplexan Besnoitia jellisoni has been commonly employed (Lunde and Jacobs, 1965; Ernst et al., 1968; Fayer et al., 1969). The assay is an adaptation of the a 96-well host-cell monolayer clearing assay that has also been described for Toxoplasma (Roos et al., 1994). Hits in compound libraries identified in this manner are then evaluated for activity against Eimeria tenella in a secondary radiometric uracil-uptake assay. In this VALIDATION OF cGMP-DEPENDENT PROTEIN KINASE (PKG) - A CASE STUDY

case sporozoites, freshly prepared from sporulated oocysts (Dulski and Turner, 1988), are inoculated onto Madin-Darby bovine kidney cell monolayers and parasite-specific uracil incorporation is quantitated (Schmatz et al., 1986). Examples of lead coccidiostat compounds identified through empiric screening include the identification of the natural product histone deacetylase inhibitor apicidin (Darkin Rattray et al., 1996; Meinke and Liberator, 2001) and a synthetic cGMP-dependent protein kinase inhibitor (Gurnett et al., 2002; Biftu et al., 2005). More recently, T. gondii has become the organism of choice for screening due to its adaptability to higher throughput formats and the ease with which it can be cultivated in vitro. Simple colorimeteric or fluorogenic assays for Toxoplasma have been developed, based on transgenic β -galactosidase and GFP reporter enzymes, which facilitate the rapid identification of active compounds (Seeber and Boothroyd, 1996; McFadden et al., 1997; Gubbels et al., 2003).

An example of a 384-well *T. gondii* β-galactosidase screening plate is shown in Figure 19.1. HFF cells are first seeded in 384-well microtiter plates. Once monolayers are confluent, parasites are inoculated into wells and compound delivered with a pin-tool (V and P scientific) to a final concentration of 2-5 µM. Control compounds (C1, compound 1; C2, compound 2; Figure 19.2) are included for internal reference. Following 4-5 days of growth, the colorimetric substrate CPRG is added and wells showing parasite growth inhibition are scored visually. Wells where parasite growth inhibition is observed appear yellow, due to a lack of parasite-specific β-galactosidase activity. Microscopic examination of 'positive' wells is used to eliminate compounds where inactivity is due to host-cell toxicity. The potency of promising activities is then quantified in subsequent dose-response titrations. In this example of a plate from a G-protein coupled receptor (GPCR) compound library screen, one potent and four weakly active compounds were identified. For three other 'hits', the lack of parasite growth was attributed to host-cell toxicity. In these cases, the HFF cells appeared vacuolated, shrunken, or lysed. Compounds showing promising levels of potency are evaluated for activity



FIGURE 19.1 Antiparasitic screening of compound libraries using transgenic parasites expressing β -galactosidase (Seeber and Boothroyd, 1996). Compounds are delivered from source plates by pin tool to a final concentration of 5 μ M. Example of a 384-well screening plate showing wells with active compound (yellow). Empty columns (1 and 24) are not shown. Reference compounds are included as internal controls (C1 and C2). Wells showing signs of toxicity to the HFF host-cell monolayer are highlighted in red.

This figure is reproduced in color in the color plate section.

against *E. tenella* sporozoites. When antiparasitic activity is confirmed against *E. tenella in vitro* and where availability permits, compounds are tested for *in vivo* efficacy in a chick *Eimeria* model.

19.5 VALIDATION OF cGMP-DEPENDENT PROTEIN KINASE (PKG) – A CASE STUDY

19.5.1 Characterization of the coccidian PKG target

A useful illustration of how the *Toxoplasma* model system has provided effective support for anti-coccidial drug discovery is Merck's cGMP-dependent protein kinase inhibitor program. An empiric screening hit was initially identified that showed promising broad-spectrum *in vitro* activity against a variety of coccidian parasites, including *B. jellisoni, E. tenella, T. gondii,* and *N. caninum*

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FIGURE 19.2 Reference compounds: SB203580 and SB202190, inhibitors of p38 MAP kinase; compound 1 and compound 2, inhibitors of parasite PKG; aminopurvalanol A and purvalanol B, inhibitors of mammalian CDK and parasite CK1.

(Gurnett et al., 2002; Wiersma et al., 2004). The lead, a 2,3-diarylpyrrole referred to as compound 1 (Figure 19.2), was active against several key pathogenic species of Eimeria in the chicken and against T. gondii in the murine model (Nare et al., 2002; Biftu et al., 2005). The compound also has in vitro and in vivo activity against S. neurona (Powles, Lindsay and Liberator, in preparation). It appears to be selectively toxic to coccidian parasites, showing little activity against mammalian cells in cell culture $(at < 10 \mu M)$ or toxicity to chickens or mice (at < 50 mg/kg doses). A ligand binding assay with tritiated compound 1 was used to track a single peak of binding activity in Eimeria oocyst lysates through multiple column chromatographic purification steps (Gurnett et al., 2002). Amino-acid sequencing of a highly enriched 120-kDa protein followed by degenerate PCR cloning led to the unambiguous identification of a parasite ortholog of vertebrate cGMP-dependent protein kinase (PKG). Unlike the animal enzyme, the purified Eimeria PKG is highly cooperative with respect to activation by cGMP and sensitive to sub-nM levels of compound 1. Like most protein kinase inhibitors, inhibition by compound 1 is ATP-competitive, and noncompetitive with respect to the peptide substrate.

Toxoplasma has been a highly effective system for the expression of active recombinant PKG enzyme for functional studies. Initial attempts to obtain active enzyme in a variety of heterologous systems, including E. coli, yeast, and bacculovirus, failed to produce enzyme with properties resembling those of the native enzyme. For example, although an adequate level of soluble recombinant enzyme was recovered using the baculovirus system, it exhibited poor specific activity and uncooperative cGMP-activation kinetics. In contrast, purification of FLAG epitope-tagged recombinant Eimeria tenella PKG (EtPKG) from transgenic Toxoplasma parasites yielded an enzyme that was indistinguishable in kinetic properties from the purified native enzyme (Donald and Liberator, 2002; Gurnett et al., 2002). The T. gondii PKG enzyme (TgPKG) was similarly expressed in active form from transgenic parasites (Donald et al., 2002). This technical feat facilitated a detailed molecular analysis of the coccidian PKG enzyme, explaining many of its unique properties. The novel third cyclic GMP allosteric site, absent from the animal PKGs, was shown to form the basis for the enzyme's stringent cGMP-regulatory properties and cGMP binding-site cooperativity (Salowe et al., 2002). This third site was also shown to be essential for enzyme activity, making it a potential inhibitor target in its own right. The membrane association of a larger isoform of EtPKG and TgPKG was shown to be attributable to dual N-terminal myristoylation and palmitoylation of a polypeptide initiated at an upstream methionine codon (Donald and Liberator, 2002). Indirect evidence from two distinct gene targeting strategies yielded strong evidence that the PKG gene is essential for tachyzoite proliferation, providing genetic validation of its importance as a therapeutic target. Using the hit-and-run gene targeting approach (Donald and Roos, 1998), PKG locus pseudodiploids only resolved to wild-type but not to PKG null segregants following counterselection to remove vector sequences. In the second approach, exhaustive gene-targeting experiments with PKG gene knockout vectors in wild-type parasites failed to generate PKG null mutants that prevent native PKG expression. However, such mutants could be readily recovered from transgenic lines expressing functional recombinant PKG in trans. Transgenes capable of complementing PKG knockout mutations include those expressing acylated or non-acylated forms of EtPKG, demonstrating that the Eimeria enzyme is fully functional and that PKG acylation per se is not essential in Toxoplasma tachyzoites.

As described in earlier examples, the mechanism by which a drug interacts selectively with its target is often revealed through an understanding of resistance determinants. The existence of alleles capable of conferring resistance to compound 1 was initially predicted from a similarity model of the 2,3-diarylpyrrole-enzyme complex based on the crystal structure of mammalian p38 MAP kinase (alpha isoform). The trajectory of the PKG enzyme backbone superimposed on the p38 MAP kinase structure predicts that the two enzymes display related catalytic site architecture.

The p38 enzyme is a major drug target for rheumatoid arthritis, as inhibitors block proinflammatory cytokine production in animal models

(Kumar et al., 2003). Compound 1 itself, originally created as part of a glucagon receptor antagonist program, is a known inhibitor of p38 MAP kinase (IC₅₀ 20 nM) (de Laszlo, 1999). Conversely, several commercially available p38 MAP kinase inhibitors also inhibit PKG, including trisubstituted imidazoles SB202190 (IC $_{50}$ 6 nM) and SB203580 (IC $_{50}$ 10 nM) (Figure 19.2) (Donald, unpublished results). Interestingly, these reference compounds also show some activity against Toxoplasma in vitro (Wei et al., 2002), although at levels that are tenfold higher than those observed for inhibition by compound 1. A putative Toxoplasma ortholog of p38 MAP kinase has been cloned and characterized, but it appears to be relatively insensitive to these inhibitors (Brumlik et al., 2004) (SB203580 IC₅₀ 135 nM). Therefore, it seems more likely that the limited antiparasitic activity of these reference p38 compounds is due to inhibition of parasite PKG.

The vulnerability of host-cell p38 MAP kinase and parasite PKG to structurally related inhibitors appears to be due to the presence of a common hydrophobic binding pocket that overlaps the ATP binding site. Previous modeling studies of the p38 MAP kinase suggested selective interaction between residues in this pocket and the inhibitor ligand (SB203580, Figure 19.2), a prediction that was subsequently confirmed by mutational analysis (Gum et al., 1998; Lisnock et al., 1998). In particular, when p38 MAP kinase residue threonine106 is substituted by bulky residues such as methionine or glutamine, inhibitor binding is prevented. Modeling of the EtPKG or TgPKG enzymes resulted in analogous predictions that identified a related accommodating interaction between the parasite enzyme and the inhibitor ligand. Apicomplexan PKG enzymes also have a threonine residue at this key position, while vertebrate PKGs harbor a methionine (cGK isoform I) or leucine residue (cGK isoform II) that would be predicted to interfere with compound binding. According to the model, this threonine residue makes close contact with the fluorophenyl group of the inhibitor. As with the p38 MAP kinase, substitution of a definitive PKG catalytic site threonine residue by methionine or glutamine (T770M/Q for EtPKG; T761M/Q for TgPKG) would be expected to
Gene	Hydrophobic binding pocket	Cmpd 1 IC ₅₀ (nM)	
Bt-cGKI	RTFKDSKYLYMLMEACLGGELWT (437)	>2000	
Pf-PKG	RTFKDSKYFYFLTELVTGGELYD (620)	3	
Cp-PKG	RTFKDSENIYLLTELIPGGELYD (699)	nd	
Et-PKG	RTFRDKEFLYFLTELVTGGELYD (770)	1	
- <i>T770M</i>	M	>2000	
- <i>T770Q</i>	Q	>2000	
Tg-PKG	RTFRDKEFLYFLTELVTGGELYD (761)	1	
-T761M	M	>2000	
-T761Q	QQ	>2000	
-T761G	GG	0.3	
-T761A	AA	0.4	

FIGURE 19.3 Sensitivity of apicomplexan parasite PKG to compound 1 is mediated by a catalytic site threonine residue. Methionine or glutamine substitutions render the enzyme refractory to inhibitor. Glycine or alanine substitutions confer hypersensitivity. Et-PKG, Tg-PKG, Cp-PKG, Pf-PKG correspond to PKG enzymes of *E. tenella, T. gondii, C. parvum,* and *P. falciparum,* respectively. Bt-cGKI is a bovine ortholog, cGMP-dependent protein kinase isoform I. Identical and related amino acids are shaded light and dark gray respectively. Numbers in parentheses reflect position relative to the N-terminus. nd, not done.

interfere with inhibitor binding. Figure 19.3 compares compound 1 IC₅₀ values obtained with recombinant parasite PKGs, various mutant derivatives, and a bovine cGKI control. Whereas wildtype parasite PKG enzymes are highly sensitive to compound 1 (IC₅₀ 1–3 nM), methionine or glutamine substitutions of the definitive active site threonine residue in the T. gondii or E. tenella enzymes are as refractory to inhibition as the bovine cGK1 control (IC₅₀ > 2μ M) (see also Donald et al., 2002). Substitution of TgPKG threonine 761 by smaller residues such as alanine or glycine actually hypersensitizes the enzyme to compound 1 (IC₅₀ <1 nM). These results suggest that steric effects play an important role in permitting access to this hydrophobic binding pocket by the inhibitor ligand. The data also explain the high degree of selectivity of compound 1 for the parasite PKG target compared with PKGs of the host. This is important, as inhibition of orthologous host-cell enzymes might be expected cause toxicity in vivo. The two isoforms of human PKG play important roles in gastrointestinal smooth-muscle physiology (cGKI) and fluid homeostasis (cGKII), and knockouts in these genes in mice display severe phenotypes, including gastrointestinal tract impairment and dwarfism (Pfeifer *et al.*, 1996, 1998).

It is important to note that although p38 inhibitors and PKG inhibitors exploit similar vulnerabilities in the catalytic site hydrophobic binding pocket, selectivity for host-cell p38 MAP kinase and parasite PKG clearly involves other catalytic site contacts. Some of the most highly selective p38 MAP kinase inhibitors exploit additional structural features of the p38 MAP kinase catalytic site that are not shared by PKG (Fitzgerald et al., 2003; Natarajan et al., 2003; Stelmach et al., 2003). Extensive structure-activity relationship (SAR) data from p38 MAP kinase and PKG inhibitor medicinal chemistry programs also support the concept that potency toward each enzyme is not necessarily linked. For example, the ratio of inhibitor selectivity of p38 MAP kinase compared with parasite PKG can vary by as much as two orders of magnitude (100×) for compounds of the 2,3-diarylpyrrole class (e.g. compound 1, Figure 19.2). For imidazopyridine compounds (e.g. compound 2, Figure 19.2), where lead optimization has focused separately on both PKG and on p38 MAP kinase targets, this ratio can be even higher (> 1000×).

The use of Toxoplasma knockout strains expressing PKG mutants that are refractory to compound 1 inhibition has provided unambiguous pharmacological validation of the coccidian PKG enzyme as a therapeutic target. Parasites expressing these mutant PKG alleles are highly resistant to compound 1 in vitro and in vivo (Donald et al., 2002). Such drug-resistant strains can also serve as tools, and compound 1 as a companion reagent, for investigating the role that the PKG enzyme plays in parasite biology. According to this strategy, PKG involvement can be inferred where sensitivity of a process to compound 1 is observed in wildtype parasites but not in an engineered drugresistant strain. In this way it was shown that PKG activity, and by inference cGMP signaling, is essential for the motility of extracellular tachyzoites and their capacity to attach to and invade host cells (Wiersma et al., 2004). Perhaps surprisingly, the intracellular growth of the Eimeria tenella sporozoites and Toxoplasma tachyzoites is relatively unaffected by the sub-µM levels of compound 1 that block parasite invasion.

Available evidence suggests that microneme secretion is integral to processes associated with invasion, such as parasite motility and host-cell attachment (Kappe et al., 1999; Brossier et al., 2003). Indeed, compound 1 inhibits the secretion of micronemal adhesins in Eimeria sporozoites (EtMIC1 and EtMIC2) and in tachyzoites (TgMIC2). In contrast, micronemal discharge, extracellular motility, and host-cell invasion remain unaffected by compound 1 in transgenic tachyzoites expressing compound-refractory EtPKG or TgPKG mutants, implicating PKG in these processes. However, PKG is certainly not the only protein kinase to regulate parasite motility and invasion. A calciumresponsive calmodulin-domain like protein kinase (CDPK1) has also been implicated (Kieschnick et al., 2001). The involvement of calcium signaling in invasion has been demonstrated in a number of studies with calcium ionophores and chelators which respectively stimulate and block microneme secretion, affecting motility, host-cell attachment,

and invasion (Carruthers and Sibley, 1999; Carruthers *et al.*, 1999a, 1999b; Lovett *et al.*, 2002; Lovett and Sibley, 2003). Interestingly, inhibition of TgMIC2 secretion by compound 1 is unaffected by such calcium modulators, suggesting that PKG acts downstream or perhaps independently of calcium signaling pathways.

The discovery that compound 1 acts primarily to block host-cell invasion is consistent with its cytostatic effects on parasite growth observed both in vitro and in vivo. The ability to control the proliferation of parasites without eliminating them entirely also explains the contribution of the host immune system to the efficacy of compound 1 observed in the toxoplasmosis mouse model (Nare et al., 2002). Compound 1 can control a lethal RH strain infection in mice, conferring long-term survival. In contrast, compound-treated gamma interferon (IFN-y) knockout mice succumb relatively rapidly to infection. The additional protection afforded by the immune system can be traced to a transient asymptomatic parasite recrudescence that occurs following withdrawal of drug therapy. In IFN- γ knockout mice, this recrudescence is not self-limiting and leads to unchecked parasite proliferation and death. These results allow us to infer that PKG inhibitors also act cytostatically against Eimeria in chickens and further emphasize the utility of Toxoplasma as a model system for coccidian parasites.

19.5.2 Other parasite PKG enzymes

Compound 1 has also been evaluated for activity against malaria both *in vitro* and *in vivo* (Diaz *et al.*, 2006). PKG is expressed throughout erythrocytic development, with highest levels of expression detected in ring and gamete stages. *P. falciparum* metabolic labeling experiments measuring hypoxanthine or adenosine uptake in infected red blood cell (RBC) cultures showed sensitivity in the 0.5–2 μ M range, with the variability depending on the strain used. This represents a 2.5–10-fold lower sensitivity than observed for cultured *Toxoplasma* or *Eimeria* parasites. In a *P. berghei* mouse model, using the same dosing regimen as successfully applied in the toxoplasmosis model, only marginal compound 1 efficacy was observed. Only 20-30 percent of the mice survived beyond the untreated controls, and these succumbed within 2 weeks of the initial infection. In order to determine whether the lower efficacy can be attributed to reduced potency at the level of the PKG target, a recombinant P. falciparum PKG was expressed in Toxoplasma and purified by FLAG-affinity chromatography as described for *Eimeria* and *Toxoplasma* PKG enzymes (Donald and Liberator, 2002). Previous attempts to express PfPKG in E. coli have yielded active enzyme, but the recombinant enzyme obtained is a truncated version that is less active and less responsive to cGMP than coccidian PKGs (Deng and Baker, 2002; Deng et al., 2003). In this case, a full-length fully synthetic P. falciparum gene was expressed incorporating T. gondii codon preference. A highly active recombinant PfPKG was recovered showing kinetic properties characteristic of coccidian PKGs, including cGMP cooperativity (Hill coefficient of 2), substrate preference, and sensitivity to compound 1 (Diaz et al., 2006). These results suggest that differences in parasite biology most likely account for the reduced susceptibility of blood-stage malaria to compound 1. Although microneme secretion is integral to the invasion of malarial erythrocytic stages and coccidian parasites alike, it is possible that underlying control mechanisms might differ. As malarial sporozoites show a greater physiological resemblance to their coccidian counterparts than do erythrocytic stages, it will be interesting to see if compound 1 shows greater potency against hepatocyte invasion.

Attempts have also been made to express the PKG from *Cryptosporidium parvum* in *Toxoplasma* (Donald, unpublished), but these have not been successful. Although some *C. parvum* proteins have been successfully expressed in *Toxoplasma* (Striepen *et al.*, 2002; O'Connor *et al.*, 2003; Slapeta and Keithly, 2004), the lower GC content of *C. parvum* genes (37 percent vs 54 percent coding sequence average) and related codon bias may hamper the heterologous expression of larger proteins like PKG (~ 100 kDa). In such cases the construction of codon optimized minigenes may be required, as is the case for most malaria genes.

19.5.3 Secondary targets of PKG inhibitors in *Toxoplasma*

Compound 1 and related 2,3-diarylpyrroles are not sufficiently potent against Eimeria parasites to be developed as economically viable coccidiostats, despite their broad-spectrum activity. A new structural class of PKG inhibitor was subsequently found through a combination of enzyme targetbased screening and antiparasitic counterscreening. A redirected medicinal chemistry effort, focused on an imidazopyridine heterocyclic core, was able to identify a new lead (compound 2, Figure 19.2) (Biftu et al., 2006) with enhanced PKG potency and antiparasitic activity. Compound 2 is two- to five-5 fold more potent than compound 1 against the coccidian PKG target, and five- to tenfold more active against Eimeria and Toxoplasma parasites in vitro. PKG mutants that are refractory to compound 1 in in vitro enzyme assays are also completely insensitive to compound 2 (EtPKG T770Q/M or Tg T761Q/M; IC₅₀ > 10 μ M), confirming a common mechanism of inhibition (Donald, unpublished). A Toxoplasma PKG knockout strain expressing such a PKG mutant can serve as a useful tool to measure the extent to which compound sensitivity is dependent on the PKG target (Donald et al., 2002). A comparison of the efficacy of the two compounds against cultured Toxoplasma is shown in Figure 19.4. For compound 1 (panel A), the refractory strain (PKG T761Q-KO, open triangles) is 15-fold more resistant than wild-type parasites (or against parasites expressing non-mutant recombinant PKG, not shown). As levels of compound approach 10 µM, proliferation of the drug-resistant strain also becomes inhibited, implying titration of other intracellular targets. In contrast, the refractory strain is only five-fold more resistant to compound 2 than wild-type parasites (panel B, compare open with closed circles). In addition, the resistant strain is more sensitive to compound 2 than wild-type parasites are to compound 1 (panel A, closed triangles), indicating that in this case non-PKG targets are being inhibited at therapeutically significant (sub-µM) levels.

The involvement of potential secondary targets with the newer compound is even more



FIGURE 19.4 Activity of coccidiosis leads against *Toxoplasma in vitro*. Dose response for inhibition of wildtype (RH) and compound refractory (TgPKG T761Q-KO) *T. gondii* strains by PKG compounds 1 and 2 (panels A, B; see also Figure 19.2). The metabolic activity of parasites growing in HFF cell monolayers was determined by measuring ³H-uracil uptake after 48 h growth in Cytostar-T scintillation tissue culture plates (GE Biosciences). IC₅₀ values generated in this representative experiment were calculated by using curve fitting software (GraphPad Prism v4).

pronounced in analogous experiments using the toxoplasmosis mouse model (Figure 19.5). Approximately 90 percent of mice infected with a lethal dose of wild-type strain RH parasites survive long-term when treated with compound 1 or compound 2 at equivalent efficacious doses. Whereas the PKG mutant *Toxoplasma* strain (T761Q-KO) is completely refractory to compound 1 treatment (panel A, closed circles), it remains partially sensitive to compound 2 (panel B, closed circles). In this case the infected mice treated with compound 2 survive 20 days longer than the



FIGURE 19.5 Activity of coccidiosis leads against *Toxoplasma in vivo*. Transgenic *T. gondii* expressing a compound refractory PKG mutant (T761Q-KO) are insensitive to compound 1 (panel A) but partially sensitive to compound 2 (panel B) in a toxoplasmosis mouse model. Mice were dosed with compound by twice daily IP injection (IP bid) for 10 days at levels (50 mg/kg for compound 1 and 25 mg/kg for compound 2) that yield ~90 percent long-term survival of wild-type parasites (RH).

analogous compound 1 treated group, which succumbs to infection at the same time as untreated controls. Taken together, the results of the in vitro and in vivo experiments suggest that although PKG is clearly a primary target of compound 2, inhibition of other targets also contributes to its efficacy. In other words, compound 2 is less selective a PKG inhibitor than is compound 1 - a property that has distinct advantages when considering the potential for drug resistance. The ability of compound 2 to inhibit other secondary kinases that are important for parasite proliferation has the potential to mitigate drug-resistance concerns. Certainly the ability to generate resistance to compound 1 in tachyzoites through a single substitution mutation - without apparent fitness cost to the enzyme - raises questions about the development of structurally related inhibitors as commercial coccidiostats. Perhaps surprisingly, though, preliminary results from an Eimeria tenella drug-resistance model in chickens suggest a relatively favorable drug-resistance profile for compound 1. After multiple cycles of application to Eimeria-infected chickens at a sub-efficacious dose, resistance to compound 1 emerges at a rate slower than for amprolium, a commercial coccidiostat that survived for more than a decade of use before the onset of resistance (Tamas Tamas, Merck and Co, unpublished data). The possible involvement of additional targets of compound 1 in Eimeria might explain the difference between theoretical predictions and these preliminary results, although a more definitive assessment awaits confirmatory drug resistance studies in floor pen trials.

The PKG knockout strain expressing the complementing inhibitor-refractory PKG mutant (T761Q-KO) was also used as tool for biochemical analysis to identify secondary targets that potentially contribute to the superior antiparasitic efficacy of compound 2 (Donald *et al.*, 2006). The absence of native PKG expression in this strain provides an ideal background for detecting the presence of other enzymes with strong affinity for compound 2. With tritiated compound 2 as ligand, a filter binding assay was employed to track activity in fractionated crude lysates of tachyzoites, in the same manner used originally to identify PKG as the primary intracellular target of compound 1 in *Eimeria* and in *Toxoplasma* (Donald *et al.*, 2002;

Gurnett et al., 2002). A single peak of compound 2 binding activity was identified following fractionation on ion-exchange and hydrophobic interaction chromatography columns. Biochemical characterization of the partially purified compound 2 binding activity showed that it corresponded to a nucleotideindependent protein kinase with preference for milk casein as a substrate and properties bearing superficial resemblance to casein kinase 1 (CK1). This result provided impetus for the cloning and characterization of two isoforms (alpha and beta) of Toxoplasma casein kinase I, one of which showed some sensitivity to PKG inhibitors (Donald et al., 2005). However, subsequent peptide antisera serology revealed that the compound 2 binding activity followed in fractionated tachyzoite extracts was not a classic casein kinase (type 1 or 2) enzyme, but rather a previously characterized calciumdependent calmodulin-domain like protein kinase 1 (CDPK1) (Kieschnick et al., 2001). The calcium dependence of its activity became apparent when peptide substrates were evaluated in in vitro assays instead of milk casein, itself a calcium source. Remarkably, partially purified or recombinant CDPK1 was at least as sensitive to compound 2 as PKG (sub-nM). The sensitivities of Toxoplasma PKG, CDPK1, and CK1 enzymes to compound 1 and compound 2 are summarized in Table 19.1. Compound 2 is a significantly more potent inhibitor of CDPK1 and CK1 α enzymes than compound 1, exhibiting much less selectivity for the PKG target than compound 2. The likely significance of the CK1 secondary target has been illuminated by the discovery that the closest ortholog of coccidian CK1α in Leishmania major promastigotes (LmCK1-2) is responsible for their susceptibility to compound 2 and other imidazopyridine inhibitors (Allocco et al., 2006). Further studies aim to investigate the extent to which coccidian CDPK and CK1 enzymes contribute to the antiparasitic efficacy of these compounds, and to understand the structural basis for inhibitor potency.

19.5.4 Apicomplexan protein kinases as therapeutic targets

As exemplified by studies of the PKG target, *Toxoplasma* has proven to be an invaluable genetic

protein kinase targets. Antiparasitic efficacy (uracil uptake) and recombinant protein kinase activitie	S
were assayed as described (Donald and Liberator, 2002; Donald et al., 2005). Average IC_{50} values (nM)	
from several experiments are shown, and were calculated from dose–response curves. Structures of	
compounds are shown in Figure 19.2. Values for compounds other than compound 2 were taken from	ļ
<i>Donald</i> et al. (2005).	

Inhibitor	³ H-uracil uptake	TgPKG	TgCDPK1	TgCK1β	TgCK1α	Rat CK1δ
Compound 1	200	1	63	4200	110	2230
Compound 2	20	0.7	0.5	500	18	100
Purvalanol B	> 5000	1900	550	>5000	123	3600
Aminopurvalanol A	360	302	586	3760	42	4000

and biochemical system for investigating and validating potential protein kinase targets of coccidian parasites. From the mining of genome databases of clinically important apicomplexans, it is clear that the evolutionary distance between essential protein kinases of the pathogen and orthologs of the host organism presents additional opportunities for selective chemotherapeutic intervention (Kappes et al., 1999; Doerig et al., 2002a; Doerig, 2004). Evidence from affinity chromatography experiments with an immobilized inhibitor ligand suggest that parasite cyclin-dependent kinases (CDKs) and CK1 enzymes contain catalytic site structural features that distinguish them from orthologous host enzymes. Based on the knowledge of CDK catalytic site architecture, mammalian CDK inhibitor purvalanol B (Figure 19.2) was strategically coupled to a chromatography matrix via its carboxyl moiety and used to probe lysates from sources including mammalian cells, invertebrates and a variety of protozoan parasites (Knockaert et al., 2000). Purvalanol B, and structurally related compounds purvalanol A and aminopurvalanol A, are well-characterized, highly selective inhibitors of mammalian CDK enzymes (Gray et al., 1998; Chang et al., 1999). Affinity purified enzymes were subjected to micro-sequencing analysis, and matches identified by comparisons with gene databases. While this affinity purification strategy succeeded in capturing CDK enzymes from most animal tissues, orthologous parasite CDKs failed to be recovered from L. mexicana, T. cruzi, P. falciparum, or T. gondii. Instead, a single band

corresponding to a CK1 enzyme was unexpectedly purified from all of these parasite species. The sensitivity of parasite CK1 for purvalanol inhibitors of mammalian CDK was subsequently confirmed by the cloning and characterization of an isoform of Toxoplasma CK1 (Donald et al., 2005). Aminopurvalanol A (Figure 19.2), the most potent antiparasitic compound of this inhibitor class, showed sub-µM efficacy (IC50 350 nM) and selectively inhibited parasite CK1 (IC $_{50}$ 42 nM) but not host-cell CK1 (IC₅₀ 4 µM). Taken together, these results provide impetus for further investigation of parasite CDK and CK1 enzymes as chemotherapeutic targets. With the cloning and characterization of CK1 and CDK enzymes from both Toxoplasma and Plasmodium (Barik et al., 1997; Doerig et al., 2002b; Khan et al., 2002; Donald et al., 2005), structure-based as well as target-based screening approaches are now theoretically feasible.

A similar affinity ligand approach has also been used to identify alternative targets of p38 MAP kinase inhibitor SB203580 (Figure 19.2; Godl *et al.*, 2003). The results of this study challenge early assumptions about the specificity of this compound for p38 MAP kinase, and serve as a useful lesson about potential pitfalls associated with protein kinase inhibitor development in general. As with the purvalanol B affinity ligand, structural information from kinase/inhibitor co-crystals was used to devise a successful p38 ligand coupling strategy, based on a determination of solvent accessible sites for the coupling of the inhibitor to the linker molecules on the sepharose bead matrix. Using HeLa cell lysates, at least eight protein kinases bound to the SB203580-derived matrix, including the expected p38 α target. The specificity of the putative protein kinase–ligand interactions was confirmed in subsequent *in vitro* kinase assays, and revealed that one of the kinases, RICK, was two-fold more sensitive to SB203580 than the p38 α target itself (IC₅₀ of 16 nM and 38 nM for RICK and p38 α , respectively). Two other kinases GAK and CK1 (α , δ , and ϵ isoforms) were in the same range of sensitivity as p38 α . These results raise questions about the selectivity of this compound and its widely described biological effects, including failure in clinical trials (Kumar *et al.*, 2003).

Unexpected additional protein kinase targets have also been identified with the purvalanol B CDK inhibitor ligand in mammalian cells (Knockaert et al., 2000). In screening various cell types and tissues for purvalanol interacting proteins, CDKs were identified in most cases, but a few other kinases were also found in some cell lines, including p42/p44 MAPK (Erk1/2). Although the sensitivity of p42/p44 MAPK to aminopurvalanol A in vitro is 10-100× lower than that of CDK1/cyclinB, indirect target-specific whole-cell assays confirm that at >5 μ M levels the antiproliferative properties of aminopurvalanol A are due to inhibition of both p42/p44 MAPK and CDKs (Knockaert et al., 2002). Clearly, a high degree of selectivity is hard to achieve with protein kinase inhibitors, even for compounds like the purvalanols that have been refined by extensive medicinal chemistry (Gray et al., 1998). As a result, it is safe to assume that most compounds will have a range of activities against multiple protein kinase targets. This conclusion has implications for the lead refinement process, where multiple protein kinase activities may need to be monitored in order to optimize efficay. This may be necessary not only for protein kinases in the target pathogen, but also for those in the host cell where off-target activities may contribute to host-cell toxicity.

The conventional industrial approach for the pharmacological validation of protein kinase targets is dependent on access to large, structurally diverse, small-molecule libraries. An elegant

alternative 'chemical genetics' method that bypasses this requirement and that might be applied to apicomplexan parasite kinases has been developed by Kevan Shokat and his laboratory (Shokat and Velleca, 2002; Specht and Shokat, 2002; Shah and Shokat, 2003). The strategy is based on the construction of a kinase allele that renders the target enzyme uniquely distinguishable from all other kinases in the genome through its sensitivity to a complementary inhibitor. This mutation is a substitution of a universally conserved bulky amino acid in the ATP-binding pocket, known as the 'gatekeeper' residue. When this residue is substituted for a smaller amino acid in a variety of target kinases, access to a deep hydrophobic 'specificity' pocket is created that has strong affinity for a modified ATP analog or inhibitor. In principle, this pocket is analogous to that exploited naturally by PKG compounds or structurally similar inhibitors of p38 MAP kinase (Figure 19.2). Significantly, the engineered mutation is usually silent in terms of enzyme function (Witucki et al., 2002), permitting specific labeling of the altered kinase or protein substrate by a radiolabeled derivative of a matching ATP analog. A small proprietary set of functional ATP analogs and potent inhibitors that fit only the engineered kinases have been synthesized and are available by license from CGI Pharmaceuticals, Inc., Branford, CT (CGI). Some of these compounds are highly potent, cell-permeable, and have excellent bioavailability combined with low toxicity in mice. As a result, they can be used to evaluate the physiological consequence of protein kinase inhibition both in vitro and in vivo. Naturally the technology requires time for the construction of the appropriate gene replacement strains, in which the transgene expressing the modified sensitized kinase target functionally replaces the endogenous wildtype gene copy. Nevertheless, the method has been used with great success for the characterization of a number of yeast and mammalian protein kinases and their cellular substrates (Holt et al., 2002; Shah and Shokat, 2002; Eblen et al., 2003; Sekiya Kawasaki et al., 2003; Sreenivasan et al., 2003; Ubersax et al., 2003; Hindley et al., 2004; Wan et al., 2004; Jones et al., 2005).

19.5.5 Use of small-molecule modulators to study the host-parasite interaction

Small-molecule modulators with well-characterized cellular targets have often been used as reagents to study biological processes. In Toxoplasma, for example, studies with cytochalasin D implicated actin as a key player in host-cell invasion (Dobrowolski and Sibley, 1996). Dinitroaniline inhibitors of tubulin polymerization have been employed to study cell division (Morrissette and Sibley, 2002). Brefeldin A, an inhibitor that blocks ADP-ribosylation factor recycling in the Golgi apparatus, has been used to investigate protein trafficking (Hager et al., 1999). Inhibitors have also been useful tools to help understand mechanisms of nutrient acquisition from the host (Coppens et al., 2000) and for probing immune-system signaling pathways that are induced in response to parasite invasion (Kim et al., 2004, 2005). Where the mechanism of action is well-validated, inhibitors can offer a simple alternative to genetic approaches. Because small molecules act quickly, the consequences of inhibition of a desired target can be studied in real-time.

Even when the mechanism of a small-molecule effect is not well understood at the outset, subsequent analysis can prove insightful when the associated phenotype is sufficiently compelling. A good example is the use of compound 1 (Figure 19.2) to investigate the development of the latent bradyzoite stage. Compound 1 was initially observed to be a strong inducer of bradyzoite differentiation in vitro (Nare et al., 2002). The mechanism responsible was traced to the host cell, since pretreatment with compound 1 is sufficient to induce developmental switching in subsequently invading parasites (Radke et al., 2006). The effect can be abrogated by 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB), a recognized inhibitor of gene transcription by RNA polymerase polymerase II, suggesting that host-cell gene expression mediates the effect. As commercially available p38 MAP kinase inhibitors are not strong inducers (e.g. SB203580), or even antagonize the compound 1-induced bradyzoite induction (e.g. SB202190),

MAP kinase signaling is probably not responsible. Genes that are selectively upregulated in response to compound 1 treatment were identified through DNA microarray analysis. Subsequent siRNA gene knockdown experiments confirmed that inhibition of transcription of individual host genes can independently block bradyzoite differentiation. These results have provided new direction to researchers investigating bradyzoite differentiation by emphasizing the role of the host-cell microenvironment in modulating stage differentiation.

In a bold new approach pioneered by Gary Ward and his colleagues, high-throughput screening (HTS) of compound libraries has been used to identify small molecules that interfere with a specific aspect of the host-pathogen interaction in order to provide insight into the underlying biology (Ward et al., 2002). In a pioneering evaluation of this strategy, a sophisticated microscope-based screen for modulators that influence host-cell invasion by Toxoplasma was developed (Carey et al., 2004). Using an automated 384-well imagecapturing system, the investigators were able to identify 30 non-cytotoxic compounds with effects on invasion from a library of 12160 structurallydiverse small molecules. Invasion of YFP-tagged parasites into compound-treated micro-well monolayers was quantified by measuring the ratio of extracellular parasites (labeled by indirect immunofluorescence) to intracellular ones (inferred from YFP-signal). Detection was sufficiently sensitive to discriminate compounds that enhance invasion (6 molecules) from those that inhibit it (24 molecules). In a series of secondary assays, different subsets of the inhibitors showed a range of activity profiles on invasion-associated processes - including gliding motility, calciuminduced microneme secretion, and conoid extrusion. Several of the inhibitors/enhancers of T. gondii invasion showed similar properties on the invasion of erythrocytes by Plasmodium merozoites, suggesting that they may target conserved components of the apicomplexan invasion machinery. Intriguingly, several of the compounds that blocked invasion and constitutive microneme secretion had no effect on secretion induced artificially by calcium ionophores, and none of the

invasion enhancers showed any effect on intracellular calcium levels. In the absence of any knowledge of the molecular targets of these compounds, the exercise has revealed an unexpected degree of complexity regarding the mechanisms of parasite invasion, particularly with respect to the role of calcium in these processes. Although none of the molecules identified in this screen shows sufficient antiparasitic potency to be considered drug development leads, they have potential as research reagents for identifying mechanisms underlying host-cell invasion.

In theory, compounds identified in such a parasite phenotypic screen can be used to identify the relevant cellular determinants through molecular labeling and proteomic techniques. In practice, target identification is the most challenging part of the overall strategy. Synthesizing a radio-labeled derivative with sufficiently high specific activity for use in ligand-binding assays requires access to a dedicated radiochemistry facility. For the construction of non-radioactive 'tags', such as biotin, fluorescent chromophores, or photo-affinity reagents, considerable empirical tinkering may be required in order to find modifications that do not compromise activity. In the absence of highresolution structural information, the design of affinity ligands for coupling to a chromatography support is a hit-or-miss endeavor. Once a biologically active tagged derivative is identified, access to a first-rate proteomics facility is needed to enable the identification of the labeled target from extracts of parasites fractionated by electrophoretic or chromatographic methods. In cases where the compound is sufficiently potent and when genetically dominant compound-resistant mutants can be selected, complementation cloning of the target protein can also be considered (Striepen et al., 2002).

Limitations aside, this new chemistry-based paradigm for biological investigation has become increasingly feasible with improved access to a variety of public and commercial small-molecule collections. Compound libraries available to qualified investigators include those supported by the National Cancer Institute and the National Institute of Neurological Disorders and Stroke. Several well-endowed academic institutions, such as the University of Wisconsin, Stanford University, Harvard, and MIT, have developed well-equipped core facilities that provide access to a number of commercial libraries (Chembridge, Microsource, and others), as well as automation support. Although the focus of this small-molecule approach is to provide a greater understanding of biological systems, it has the added benefit of potentially validating new chemotherapeutic strategies. With some luck, there is hope that it may even identify lead compounds for further development.

19.6 FUTURE OUTLOOK

The implementation of an ever-increasing collection of molecular tools has helped to solidify the position of Toxoplasma as a model system for the apicomplexa. The development of a genetic complementation system has facilitated the cloning of genes for which a selectable or screenable phenotype exists (Striepen et al., 2002). The technique employs a high-frequency insertion library and an efficient recombination rescue strategy to identify cDNA clones capable of complementing a parasite mutant. It has been successfully used to clone the Cryptosporidium IMPDH (Striepen et al., 2002) and to rescue a conditional cell-cycle mutant through the capture of a mitotic suppressor protein (White et al., 2005). When combined with flow cytometry and fluorescence microscopy, the method has also been used to clone a sporozoite stage-specific surface antigen (Radke et al., 2004), and to recover YFP-tagged genes whose proteins are targeted to a variety of subcellular locations (Gubbels et al., 2004). This system should prove particularly useful for understanding mechanisms of drug resistance, by facilitating the identification of protein determinants following chemical mutagenesis and in vitro selection of complementing clones.

Perhaps the most powerful genetic tool for use in target validation is a regulatable gene expression system that enables the construction of conditional gene knockout mutants. Until recently, the demonstration that a gene is essential in the

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haploid tachyzoite could only be inferred indirectly - for example, by showing that a gene can only be inactivated in the presence of a complementing copy or through the inability to recover a null mutant from an engineered gene locus pseudodiploid (Donald and Roos, 1994, 1998). The ability to turn off gene expression with a smallmolecule modulator has become possible in mammalian cells with the adaptation of a tetracycline-regulated trans-activating system from E. coli (Gossen and Bujard, 1992). This so-called 'Tet-Off' switch permits a direct assessment of gene function with the construction of the appropriate transgenic background, one in which the targeted gene is constitutively expressed until a tetracycline modulator is added that turns off gene expression. The successful implementation of this technology for Toxoplasma required the selection of a functional chimeric tet-transactivator capable of constitutive activation of a tet-operator (tetO) containing responsive promoter in the absence of tetracycline. This was accomplished through the insertional trapping of an activating domain in frame with a proximal E. coli tetR DNA-binding domain, using downstream HXGPRT and lacZ genes as selectable and screenable markers respectively (Meissner et al., 2002). Once captured and validated, the transactivator was used to construct a conditional knockout of the MyoA gene, in which the endogenous gene was disrupted in the presence of a second regulatable copy. Attenuation of myosin-A expression with tetracycline addition caused severe impairment in hostcell invasion, unambiguously establishing the essential nature of this motor both in vitro and in the mouse model (Meissner et al., 2002). The general utility of this methodology in Toxoplasma has been demonstrated with the construction of a conditional knockout in the acyl carrier protein (alluded to earlier), which confirms that the T. gondii FAS II pathway is necessary for parasite proliferation (Mazumdar et al., 2006). Significantly, Toxoplasma-specific tet-transactivators also function in P. falciparum, and so should be adaptable for the generation of conditional knockouts for malarial bloodstream-stage genes (Meissner et al., 2005).

The full potential of this conditional 'Tet-Off' gene knockout approach has been applied on a large scale to the fungal pathogen Candida albicans for use as a drug discovery tool (Haselbeck et al., 2002; Roemer et al., 2003). The implementation of this technology on a comprehensive scale for antifungal drug development illustrates a possible future path for apicomplexan drug discovery, assuming that sufficient resources can be focused on such an initiative. For the diploid C. albicans, conditional knockouts are built in two successive steps involving targeted homologous recombination in a strain expressing a chimeric transactivator protein containing the yeast GAL4 activation domain. In the first step, a heterozygous mutation in the target gene is made by gene replacement. In the second, expression of the remaining allele is placed under the control of the regulatable Tet promoter. This approach has been used in a systematic fashion to assess the relative significance of 823 gene orthologs of essential genes from S. cerevisiae (Roemer et al., 2003). The analysis permitted a rank ordering of genes deemed the most attractive drug targets based on phenotypic information from both in vitro and in vivo gene dial-down experiments. Such an assessment involves the consideration of whether gene knockdown is accompanied by cytocidal or cytostatic kinetics, or how dramatically virulence in a mouse model was affected. Heterozygotes or conditional knockouts corresponding to high-priority genes can subsequently be used as screening tools in cell-based assays to identify compounds with selectivity for a target of interest. The underlying principle of such assays is the concept of haploinsufficiency, where lowering the dosage of a gene results in a strain that is sensitized to any drug that acts on the product of this gene (Giaever et al., 1999). The selectivity of a compound for a specific gene product is demonstrated when a strain attenuated in expression of that gene appears hypersensitive in comparison with a parental strain control. For the C. albicans 'Tet-Off' system, the levels of tetracycline required to achieve optimal hypersensitivity without excessive growth suppression are somewhat strain-dependent and are determined empirically. For conditional knockout strains

corresponding to drug targets, such as Erg11p, Alg7p, or His3p, it has been possible to establish conditions that yield 30–100-fold greater sensitivity to known inhibitors (Haselbeck *et al.*, 2002). With the availability of analogous regulatable systems for *T. gondii* and *P. falciparum*, this approach is similarly feasible for apicomplexan parasites.

Related chemical genomics platforms have also been developed for profiling compounds in order to elucidate mechanism of action (Haselbeck et al., 2002; Lum et al., 2004). Strains sensitized in a target-specific manner can be assembled into a comprehensive panel for evaluating the fitness of individual strains in response to drug exposure. The mode of action of selective inhibitors may be inferred in such a fitness test by quantifying the depletion of sensitized strains growing competitively in the presence of drug. Strains are grown in pools, and are detected by DNA microarray hybridization or by multiplex PCR. When applied to collections of heterozygous S. cerevisiae or C. albicans, this approach has been used to confirm the mechanism of antifungal drugs (Giaever et al., 1999; Haselbeck et al., 2002; Lum et al., 2004). A similar chemical genomics strategy has also been applied to Staphylococcus aureus, only using regulatable antisense RNA expression as a means to dial down gene expression (Forsyth et al., 2002). Such fitness test profiling platforms serve as powerful tools to identify target-specific inhibitors among hits identified in empiric whole-cell screens, and to prioritize leads according to target biology.

Unfortunately, resources for the genome-level application of available molecular tools do not yet extend to the antiparasitic therapeutic area. In the coccidiosis arena, regulatory concerns and economic pressures have resulted in a renewed interest in vaccine development, with investment in drug discovery programs seen as increasingly risky. Due to the relatively small volume of cases, there is little commercial interest in developing new drugs to treat opportunistic cryptosporidiosis or toxoplasmosis. For malaria, where there is considerable publicity and concern over the devastating loss of life associated with *P. falciparum* in sub-Saharan Africa, the problem for commercial development is clearly one not of volume but

of margin. With pressure to produce drugs on an 'at-cost' basis for developing countries, and with heightened regulatory and product liability concerns in the face of increasing development costs, the potential returns (public relations payoff or profit) are simply inadequate to offset the inherent risk. With the arrival of the new millennium, increasing pressures on the pharmaceutical industry have impacted other areas of infectious disease drug discovery as well. Many of the major companies have either totally eliminated or severely cut back antibacterial research and development (Projan and Youngman, 2002; Projan, 2003; Projan and Shlaes, 2004). Under the current climate, where are the future drugs to treat diseases caused by apicomplexan parasites likely to come from?

One possible source of new drugs to treat orphan-cause infectious diseases is the animal health arena. A precedent-setting example is the successful use of ivermectin (Mectizan) to treat onchocerciasis, or African river blindness, in sub-Saharan Africa. Used primarily as an animal health drug to treat worm infections in livestock and in companion animals, it later achieved considerable clinical success, largely as part of a well-publicized donation program by the manufacturer (Merck and Co.). For apicomplexan parasites, commercial coccidiosis drug discovery programs are a potential conduit for clinical drugs. In this case, the record so far has been somewhat mixed. Sub-optimal pharmacokinetic properties of coccidiostat compounds applied to a clinical setting are likely to be a common limitation, as they are optimized for use in chicken feed, where dosing is continuous rather than on a once- or twice-daily basis. Perhaps the most widely used class of coccidiostat compounds in poultry are the polyether ionophores, such as monensin and salinomycin (Greif et al., 2001). Unfortunately, they are too non-selective and toxic for clinical use despite their broad-spectrum anti-coccidial activity (Novilla, 1992; Mead et al., 1995; Oehme and Pickrell, 1999). In contrast, the successful triazine coccidiostats diclazuril and toltrazuril - the mechanism of which is unknown - have found use beyond the poultry houses. Diclazuril has been used with some success in small uncontrolled

studies to treat Isospora and Cryptosporidium infections in AIDS patients (Menichetti et al., 1991; Limson Pobre et al., 1995). Unfortunately, available data suggest that diclazuril is absorbed slowly and reaches relatively low levels in the blood (Soave et al., 1990). Nevertheless, ponazuril, a metabolite of toltrazuril, has been approved by the FDA for the treatment of equine protozoal myeloencephalitis (EPM) caused by Sarcocystis neurona infections. Ponazuril also shows good oral efficacy against Toxoplasma in a mouse model (Mitchell et al., 2004), which suggests potential clinical application against a broad range of opportunistic coccidian parasite infections. An obvious advantage of exploring animal health drugs for clinical use is the availability of extensive pharmacokinetic, efficacy, and safety data that can help hedge the chances of success in clinical trials.

Unexpectedly, antiparasitic compounds have also been discovered among drugs already approved for clinical use by the FDA. Bisphosphonates that are used to treat bone resorption diseases have been shown to inhibit the in vitro growth of kinetoplastid and apicomplexan parasites (T. gondii and P. falciparum) (Martin et al., 2001). The likely target of these drugs is the enzyme farnesyl pyrophosphate synthase (FPPS), which marks the branching point of the mevalonate pathway leading to sterol and dolichol biosynthesis as well as the farnesyl and geranylgeranyl modification of proteins (Keller and Fliesler, 1999; van Beek et al., 1999; Bergstrom et al., 2000; Grove et al., 2000). Two of the most potent antiparasitic compounds from among a collection of 60 structurally diverse bisphosphonates showed excellent protective activity in a toxoplasmosis mouse model (Ling et al., 2005). Both compounds show a good therapeutic window, displaying at least 1000× greater potency against Toxoplasma than against a human cell line in a control proliferation assay. Since a transgenic Toxoplasma strain overexpressing recombinant TgFPPS required considerably higher levels of these bisphosphonates to achieve 50 percent growth inhibition, FPPS is a likely intracellular target. Although these more active compounds are not the more common bisphosphonates used in bone resorption therapy, they constitute leads for further optimization to improve bioavailability and potency. Lest this example of finding novel activities from existing drugs be dismissed as improbable, a β -lactam antibiotic was recently identified with an unexpected clinical application. The antibacterial agent was found to induce glutamate transporter expression and help prevent neurodegeneration associated with elevated glutamate neurotransmitter levels in a variety of neurological disorders, including stroke, spinal cord injury, ALS, AIDS neuropathy, and Alzheimer's disease (Rothstein *et al.*, 2005). Collections of approved drugs are now commercially available to facilitate screening for new biological activities (MicroSource Inc.; http://www.msdiscovery.com/index.html).

With the limited participation of the pharmaceutical industry in finding new drugs to treat protozoal diseases, is it possible for academic research groups to fill the void? The success of the drug nitazoxanide demonstrates that early research and development can indeed be accomplished in academia. This benzamido-5-nitrothiazole compound was first synthesized by a research group at the Paris Pasteur Institute, led by Jean-Francois Rossignol in the early 1970s (Rossignol and Cavier, 1975). However, clinical development did not occur until much later, after Rossignol himself founded Romark Labs in 1993. With the requisite industrial sponsorship and success in clinical trials to treat protozoal diarrhea, it has since become an important broad-spectrum agent for the treatment of Giardia lamblia and Cryptosporidium parvum (Rossignol et al., 2001; White, 2004; Fox and Saravolatz, 2005; Ochoa and White, 2005). Amazingly, the drug is also effective against intestinal nematodes, tapeworms, and trematodes (Rossignol and Maisonneuve, 1984; Favennec et al., 2003). Its activity against anaerobic bacterial strains susceptible to metronidazole (Dubreuil et al., 1996; Yamamoto et al., 1999) suggests a common mechanism through reductive activation of the drugs' nitro group via an oxygensensitive pyruvate-NADP oxidoreductase (Dubreuil et al., 1996; Coombs and Muller, 2002).

Perhaps the most encouraging development in the fight to find cures for orphan-cause diseases is the foraging of joint industry-public consortium-based initiatives, such as the Medicines for Malaria venture (MMV; http://www. mmv.org/), or One World Health (OWH; http:// www.oneworldhealth.org/). According to this new drug discovery paradigm, early stage development may involve pharmaceutical-based resources, but the brunt of the costs and liability risks associated with clinical development and beyond have migrated to the public domain with the backing of private endowments and grants (for example, from the Bill and Melinda Gates foundation). The partnership is typically facilitated by the transfer of intellectual property rights associated with early drug development from commercial as well as academic sectors. So far, compounds in the MMV and OWH development pipeline have been limited primarily to artemisinin compounds with which there are cost issues and production concerns, derivatives of older compounds (diamines), or combinations of both (aminoacridines, proguanil, fosmidomycin, and atovaquone). Clearly, the advent of novel antiparasitic compounds will require a greater participation by the industrial component of this partnership in order to leverage access to critical resources such as compound libraries and medicinal chemistry expertise. Fortunately, there is evidence of greater collaboration, as demonstrated by the commitment of GlaxoSmithKline to the MMV partnership. Nevertheless, the emphasis of global health consortia will always be on the major killers (malaria, HIV, TB), where there is likely to be greater impact, rather than on diseases caused by lesser pathogens. As a result, new therapies for opportunistic coccidian parasites such as Toxoplasma and Cryptosporidium are most likely to emerge as a byproduct of commercial antimicrobial or perhaps antimalarial drug development programs. On the other hand, as illustrated by the example of nitazoxanide, good luck and persistence can also occasionally lead to success following early research and development in an entirely academic setting.

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Proteomics of Toxoplasma gondii

J.M. Wastling

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20.1 INTRODUCTION

Identifying and characterizing the function of proteins in *Toxoplasma gondii* has been the focus of extensive research into this parasite since the availability of techniques which made possible the study of individual genes and molecules. The mechanism of host-cell invasion, the structure and composition of the apical organelles, stage conversion, and evasion of host immune defenses have, among others, been important and recurring themes. Our understanding of gene expression in the context of these fundamental problems has been transformed by the sequencing of the *T. gondii* genome and the subsequent development of sophisticated genetic tools to study this parasite.

Proteins are the end product of genes, and it is ultimately the actions of proteins, not genes, that are central to the fundamental processes of parasitism that we wish to understand. Until the advent of proteomics, the large-scale study of proteins had fallen significantly behind that of genes. The recent development of proteomic methods for *T. gondii* and the availability of genome sequence to underpin a proteomic database now represent an unprecedented opportunity to characterize protein composition and function in this parasite.

To understand the evolution of proteomic studies in *Toxoplasma*, it is helpful to look briefly at the development of transcriptional analysis for this parasite. Microarrays provide the exciting possibility of studying the transcriptional patterns of not a handful but thousands of genes at any one time in different biological contexts. A number of largescale transcriptional gene expression studies using microarrays have already been undertaken for T. gondii (Blader et al., 2001; Cleary et al., 2002; Matrajt et al., 2002; Sibley et al., 2002; Singh et al., 2002). However, transcriptional studies have several disadvantages. There is a less than predictable relationship between gene transcription and protein expression, although recent work using biosynthetic labeling of RNA with uracil phosphoribosyltransferase to allow microarray analysis of mRNA synthesis and decay in T. gondii may go some way to address this problem (Cleary et al., 2005). Moreover, the activity of a protein is dependent not just on its abundance but also on its state of activation, often mediated through a post-translational event such as phosphorylation. Finally, the activity of a protein is also dependent on interactions with other proteins, so that identifying and understanding the nature of these interactions is vital in studying protein function. Unfortunately, microarray-based transcriptional studies provide little help with addressing these questions. The proteome, by definition, contains all the expressed proteins in all their various states of activation and modification. Proteomics or, perhaps more appropriately, functional proteomics thus has the potential to bridge the gap between our knowledge of the genome and the functional biological processes intrinsic to an organism.

The progression from genome to transcriptome and then to proteome is one not only of increasing complexity, but also of increasing instability. Proteomes are highly dynamic entities. Even if it were technically possible to 'map' a truly complete proteome for Toxoplasma (and at present it is not), this proteome would be specific only for the precise biological conditions in which the parasites were prepared for analysis. This would include their life stage, the point in their cell cycle, whether the parasites were derived in vitro or in vivo, etc. Thus the complexity of proteomes, whilst technically challenging, provides an indication of the potential power of proteomics analysis. Since protozoa like Toxoplasma are complex microorganisms, any proteome analysis which is able to reflect this complexity therefore has the potential to provide the key to understanding how this parasite functions and interacts with its environment.

Although large-scale microarray and proteomics studies present exciting new opportunities, they have also attracted some criticism. Many early problems of both microarrays and proteomics were due to inadequate bioinformatics tools for handling the unprecedented amount of complex data that these type of analyses produce. Although much remains to be done, in recent years significant advances have been made in the development of powerful bioinformatics tools to handle these data (Hancock et al., 2002; Haley-Vicente and Edwards, 2003; Taylor et al., 2003; Jones et al., 2004; Hamady et al., 2005; Kremer et al., 2005; Souchelnytskyi, 2005). Other criticisms have a more philosophical basis. Before the so-called 'post-genomic era', a typical conventional molecular study of Toxoplasma might have begun with the identification of a single gene or protein associated with a particular biology event such as invasion or stage-conversion. This gene would usually be sequenced, cloned, and re-expressed to provide localization and other functional data; its gene structure may have been mapped by Southern blotting, its transcriptional expression by Northern blotting, and the expression of the protein for which it codes by Western blotting. Homologous genes might have then been sought using degenerate PCR primers, and the process repeated. Consequently, a large amount of detailed information was gathered about a very small number of genes, often based around a single hypothesis. Many excellent studies founded on the above 'hypothesis-driven' pattern have provided the grounding for our present understanding of T. gondii; however, the process is slow and does not exploit the present wealth of genome sequence data in the most efficient way. By contrast, microarray and proteomics experiments provide initially lower-grade information, not on a few but rather on hundreds or even thousands of genes. Moreover, the types of genes and proteins to be investigated before an experiment are not pre-selected (hence sometimes attracting the pejorative description of 'fishing experiments').

In fact, large-scale microarray and proteomics experiments are specifically designed *not* to prejudge the outcome of the analysis by identifying in an unbiased way genes and proteins that are associated with specific biological events. These experiments are thus instrumental in hypothesis formation rather than necessarily always being hypothesis-testing. Large-scale microarray and proteomics experiments are not therefore in conflict with so-called hypothesis-driven experiments, but should be partners in an iterative hypothesis development and testing process.

In this chapter we review the present state of proteomics analysis of Toxoplasma and discuss how these data have been used to enhance our understanding of the biology of this parasite. We look at specific examples of how data from proteomics have both confirmed previous hypotheses and revealed unexpected results that would have been difficult to predict without adopting a proteomics approach. Developed in the late 1990s, proteomics is a relatively young field, although almost since its inception proteomics has been exploited by those interested in the biology of Toxoplasma. In such a rapidly developing subject, it is inevitable that the detail of any review will become outdated almost immediately. However, the aim of this chapter is not just to provide an update regarding the present state of research, but also to act as an introduction to some of the principles that guide proteomics studies and are thus likely to hold true in what is an exciting and fast-moving field in Toxoplasma research.

20.2 FUNDAMENTALS OF PROTEOMICS

There are many excellent generic reviews on proteomics in the literature (Aebersold and Mann, 2003; Morrison *et al.*, 2003; Gorg *et al.*, 2004; Johnson *et al.*, 2004; Yates, 2004; Bradshaw and Burlingame, 2005; Dhingra *et al.*, 2005; Lane, 2005; Phillips and Bogyo, 2005); these and others should be referred to for a more thorough introduction to the subject. However, before dealing specifically with proteomics studies of *Toxoplasma* we will

briefly review some of the basic principles of this relatively new area of technology.

Mass spectrometry analysis (MS) of proteins has essentially replaced classical methods for protein micro-sequencing, such as Edman degradation. MS enables the mass measurement of ions derived from molecules, and is capable of forming, separating, and detecting molecular ions based on their mass-to-charge ration (m/z). The development of two ionization methods in the later 1980s made MS routinely available to biological researchers. These were Matrix-Assisted Laser Desorption/Ionization mass spectrometry (MALDI-MS), and Electro-Spray Ionization tandem mass spectrometry (ESI-MS/MS). Proteomics essentially entails the matching of two datasets, one experimental and one virtual, to enable rapid identification of proteins. A general scheme for a proteomics experiment is shown in Figure 20.1. The experimental dataset consists of MS data, which in the simplest form contain the precise masses of peptides obtained from proteins digested with an enzyme such as trypsin. Measured using MALDI-MS, the individual masses of each peptide give rise to a unique peptide mass fingerprint (PMF). Alternatively, peptides can be fragmented by ESI-MS/MS to produce tandem mass spectrometry, or MS/MS, data. Once again a unique fingerprint is obtained, this time for each individual peptide. In both cases, the experimental MS data can then be matched using algorithms to a corresponding virtual peptide mass database. Virtual mass databases are dependent on the availability of a substantial quantity of genome sequence, or EST data, and are derived from translations of all possible open reading frames into protein sequences, or from protein prediction models. Since the completion of the genome sequencing project for Toxoplasma, these virtual or hypothetical protein databases can be readily constructed, thus enabling precise matches with experimental MS data.

20.2.1 Soluble and insoluble proteomes

The successful application of proteomics is crucially dependent on the enrichment and separation of the



FIGURE 20.1 A general scheme for a proteomics experiment. In proteomics, the identification of proteins is facilitated by the matching of two data sets, one experimental (bottom) and one virtual (top). Parasite proteins can be separated by a variety of methods, such as two-dimensional electrophoresis, and the individual proteins are then analyzed by mass spectrometry (MS) to produce an experimental data set consisting of either peptide mass fingerprints (MALDI-MS), or peptide fragmentation data (ESI-MS/MS). The experimental MS data are then matched using appropriate algorithms to a corresponding virtual peptide mass database derived from genome sequence, or EST data and containing translations of all possible open reading frames into protein sequences. Since the completion of the genome sequencing project for *Toxoplasma* these hypothetical protein databases can be readily constructed from ToxoDB, thus enabling precise matches with experimental MS data and the rapid identification of *T. gondii* proteins. This figure is reproduced in color in the color plate section.

proteins under investigation before MS analysis. The method of choice for protein separation depends on the nature of the proteins: typically either two-dimensional electrophoresis (2-DE) or non-gel based methods, such as liquid chromatography (LC) are used. 2-DE is particularly suitable for analysis of soluble proteomes, and produces high-resolution two-dimensional maps of protein constituents which enable the relative level of steady-state protein expression to be estimated as well as the potential identification of post-translational modifications. Individual protein spots can then be picked from the gel, and the samples containing the protein digested and analyzed by MS to identify each protein spot. Unfortunately, some proteins, notably hydrophobic proteins and those at the extremes of molecular weight, are notoriously difficult to resolve using 2-DE. Very low abundance proteins are also difficult to detect by 2-DE.

An alternative to 2-DE protein separation is to undertake a two-dimensional protein separation using a combination of either one-dimensional SDS-PAGE followed by nano-liquid chromatography and MS, or two serial liquid chromatographic separations followed by MS. In this latter approach,

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also know as multidimensional protein identification technology (MudPIT), proteins are digested using trypsin, and the peptides separated first using a strong cation exchanger and second by reverse phase LC. The peptides which are eluted from the second LC column are fed directly into an ESI ion-trap mass spectrometer, where they are ionized, mass selected, and fragmented. Fragmented peptides are then matched to proteins in a database using appropriate algorithms. MudPIT has been used partially to resolve the proteomes of a number of microbes, including yeast, where 1484 proteins and 5540 peptides were resolved and identified from Saccharomyces cerevisiae (Washburn et al., 2001). Significantly these proteins contained low-abundance proteins such as transcription factors and kinases, as well as proteins with extremes of PI and molecular mass, and membrane proteins not normally resolved by 2-DE. Both MudPIT and one-dimensional SDS-PAGE followed by nano-LC have been used to analyze the proteomes of protozoan parasites, including those of Plasmodium falciparum (Florens et al., 2002; Lasonder et al., 2002) and Trypanosoma cruzi (Paba et al., 2004). Analysis of the tachyzoites of T. gondii by MudPIT is currently underway (Wastling, unpublished).

At first, MudPIT analysis might seem like the obvious replacement to 2-DE based proteomics as it circumvents many of its inherent limitations and offers a relatively 'hands-off' procedure which can quickly yield large amounts of data. However, the simplicity of MudPIT is at the cost of the loss of information about the proteins which are being analyzed. One of the advantageous features of 2-DE protein separation is that it reveals the complexity of the proteome in a way that MudPIT currently cannot. For example, 2-DE proteomic analysis of T. gondii (Cohen et al., 2002) and other parasites (Jones et al., 2006) reveals that single proteins are often represented by more than one gel spot. These additional spots are not artefacts but represent potentially important functional features of proteins, such as an alternative splicing event, or a post-translational modification like phosphorylation. Moreover, whilst the relative abundance of proteins represented on 2-DE gel

can be reproducibly compared using comparative gel approaches (i.e. DIGE), MudPIT is notoriously non-quantitative. This latter feature may present particular difficulties during the analysis of enriched sub-proteomes, since distinguishing between the sub-proteome under investigation and minor contaminants can be highly problematic (Sam-Yellowe *et al.*, 2004).

20.3 WHICH PROTEOME? PROTEOMES AND SUB-PROTEOMES OF *T. GONDII*

The success of proteomic analysis depends on careful experimental design, of which sample preparation is the first and arguably the most important step. If the aim of an experiment is to map a complete or sub-proteome, then it is essential that the proteins which are being mapped represent a pure, or at least highly enriched, fraction to prevent costly analysis of many proteins not belonging to the proteome under investigation. Whilst this might appear obvious it is not trivial, and in the case of obligate intracellular parasites like T. gondii necessitates the successful separation of parasites from host cells. Whilst there are reliable methods for the separation of tachyzoites from host cells grown in vitro (we have found filtration of tachyzoites through 3-µM pore filters to be highly efficient for subsequent proteome analysis) (Cohen et al., 2002), the requirement for separation from host tissues makes the analysis of in vivo-derived tissue cysts problematic. Even the best methods for enriching for tissue cysts using density gradient centrifugation result in preparations of cysts that contain very high proportions of host tissue. Moreover, the number of mice required to produce sufficient material can be prohibitively high. Proteomic analysis of other in vivo stages of T. gondii, such as the sexual stages in the cat, are an attractive prospect, but unfortunately it is difficult at present to imagine how sufficient material could be obtained, first to develop an efficient enrichment of the material and second to perform preparative analysis of these stages.

The proteome of any cell consists of a heterogeneous mixture of soluble and hydrophobic proteins, some of which may be present in large, stable quantities whilst others are in transitory and almost undetectable amounts. An obvious way to overcome the challenge of complexity is to simplify the mixture of proteins before analysis. This principle has led to development of 'sub-proteomics'. Sub-proteomics relies on the preparation or enrichment of a smaller fraction of the total proteome, a strategy employed, for example, by Zhou and co-workers to analyze the protein repertoire of the excretory-secretory products of T. gondii (Zhou et al., 2005). This approach is highly useful when the study is restricted to the proteins of a particular organelle in which a method for purifying the organelle has been established (Yates et al., 2005). There are, however, several important caveats to a sub-proteomics approach. First, interpretation of the resulting data must take regard of the confidence placed on the preparation method and the degree of enrichment obtained for the organelle or sub-proteome under investigation. Second, the protein composition of organelles may be in a continual flux depending on the biological status of the cell. Third, many molecules in the cytoplasm interact with organellar proteomes and may be associated with these proteins, although not strictly derived from the organelle itself. Thus, with sub-proteomics and organellar proteomics experiments, reproducible sample preparation, immunolocalization to verify the origin of newly discovered proteins, and careful bioinformatics interpretation of proteomics data are important adjuncts to successful studies.

20.4 MASS-SPECTROMETRY ANALYSIS OF T. GONDII PROTEINS

For the purposes of most proteomics analyses of *T. gondii*, the present choice is whether to use MALDI-MS or ESI-MS/MS for protein sequence determination. MALDI-MS and ESI-MS/MS generate different types of data, and the choice of analysis depends on whether PMF or peptide fragmentation

data is required. The first protein identifications by MS for *Toxoplasma* were performed before there was substantial genome information for the parasite, and were forced to depend on identification of proteins using the then handful of genes in NCBI and a limited EST database (Nischik et al., 2001; Cohen et al., 2002). Since EST sequences are by nature short, they rarely encode sufficient peptides for unambiguous PMF identification of proteins. Only in the case of long, overlapping consensus EST sequences is MALDI-MS identification of proteins using PMF more efficient. However, with the development of a sophisticated $10 \times$ coverage genome database for *T. gondii*, it is now possible to search efficiently MALDI-MS data against predicted open reading frames and, in particular, against thousands of hypothetical predicted proteins derived from protein prediction programs such as TgTwinScan, TigrScan and GlimmerHMM (www.toxodb.org).

The advantage of ESI is that it allows for tandem MS, or MS/MS capability. The key event in MS/MS is that it involves the energetic dissociation of anions to cause peptide fragmentation, and thereby derives structural information relating to the sequence of a peptide. Thus, in contrast to PMF, where only the masses of individual peptides are known, MS/MS peptide fragmentation data give rise to peptide sequence information. For organisms of limited genome data, or where genome data have not been completely annotated, this provides an immediate advantage.

It is important to recognize some of the other characteristics of both MALDI-MS and ESI-MS/MS. Easy to use and less expensive, MALDI-MS is generally of a lower resolution than ESI-MS/MS. By contrast, the cost of purchasing and maintaining an ESI-MS/MS instrument can be prohibitively high for many laboratories, and the degree of skill required for successful operation is considerably greater than for MALDI. However, as well as MS/MS capabilities, ESI-MS/MS is excellent for determining peptide modifications, whilst MALDI only has limited capabilities in this area. The ability to determine peptide modifications is critical in what will undoubtedly become one of the major areas of proteomics – the characterization

of post-translational modifications of proteins. Developments in MS instrumentation for biological applications are fast-moving, and will undoubtedly have implications for *Toxoplasma* proteomics research in the future. For example, MALDI instruments have recently become available with MS/MS capability (MALDI-tof/tof), which should combine the flexibility of MALDI technology with the ability to acquire MS/MS peptide fragmentation data.

20.4.1 *Toxoplasma* protein databases for MS searching

As previously discussed, the availability of a sophisticated genome database for T. gondii is essential to underpin analysis of Toxoplasma MS data, since the experimental MS data need to be matched against a suitable protein database. At present, MS data can be searched against theoretical protein sequences contained within the most current version of ToxoDB (www.toxodb.org), a database of Toxoplasma genomic, cDNA sequences, and protein prediction models compiled from sequences representing a total of ~ $10 \times$ the size of the Toxoplasma genome and thus likely to contain virtually the entire genome. For example, Bradley and co-workers searched Toxoplasma rhoptry protein MS data against a locally constructed database of a total of 881411 sequences downloaded from ToxoDB, including 790796 genomic open reading frames greater than 50 amino acids, ESTs from ~69000 cDNA clones, 4954 Glimmer HMM protein predictions, 8336 TigrScan protein predictions, and 7588 TwinScan protein predictions for Toxoplasma (Bradley et al., 2005). For each identified protein, the exact position of each peptide was checked against alignments of the genome with ESTs and protein prediction models using a generic gene model organism database construction set (GBrowse, www.gmod.org/) available for T. gondii at www.toxodb.org.

ToxoDB also supports the direct searching of MS/MS data in a variety of common outputs, such as those from MASCOT (http://www.matrix-science.com/), using the Global Proteome Machine (GPM) (http://www.toxodb.org/tandem/) made

available by the Global Proteome Machine Organization (www.thegpm.org). MALDI PMF data searches are also supported by ToxoDB by EMOWSE (http://www.toxodb.org/restricted/emowse.shtml). For increased flexibility and for using other MS search tools it is necessary to consider constructing a local database for MS analysis, although this database will obviously need to be continually updated by ftp download in line with changes in ToxoDB.

20.5 CAN PROTEOMICS BE QUANTITATIVE?

20.5.1 Gel-based analysis

In common with all proteomes, Toxoplasma proteomes are highly dynamic and reflect the biological state of the parasites at the time of sampling. Comparing proteomes at different life-stages and under different conditions thus has the potential for revealing something of the nature of the biological response to specific stimuli. For example, we may wish to understand what happens to the proteome when tachyzoites transform to bradyzoites, or what happens when we knock out a gene coding for a protein in a specific biochemical pathway. Such comparisons are useful for understanding not only the basic biology of the parasite, but also how chemotherapeutic compounds may act on the parasite, the basis of drug-resistance, and the host response to infection. For these types of analyses, the proteomic method of choice must be capable of some form of quantitative measurement.

It is important to make the distinction between absolute quantitation and relative quantitation in proteomics. Absolute quantitation in proteomics is an extremely challenging area which is very much still in its infancy. Whilst some advances are being made in absolute quantitation (Godovac-Zimmermann *et al.*, 2005; Ishihama *et al.*, 2005), most 'quantitative proteomics' studies refer to the relative quantitation of proteins – that is, determining the extent to which a protein or group of proteins is up- or downregulated in different conditions. Early gel-based and non-gel-based proteomics methods were criticized for being notoriously non-quantitative. For example, the separation of proteins by 2-DE can result in unacceptable gelto-gel variation if not performed expertly. Conventional gel analysis thus required multiple gels to be run to eliminate experimental variation. Recent advances have, however, transformed the quantitative nature of 2-DE comparisons. First, the availability of sensitive fluorescent protein stains with a high dynamic range, such as Sypro Ruby (Steinberg et al., 1996), have eliminated the problems of the non-linearity and poor dynamic range of silver-staining. Second, advances in gelbased technology, such as the introduction of immobilized pH gradient strips for first-dimension separation, the availability of pre-cast plasticbacked second dimension gels, the introduction of robotics for spot detection, spot picking, and protein digestion prior to MS, and the development of sophisticated gel analysis software, have minimized the problem of experimental variation in 2-DE, leaving the researcher free to concentrate on the real challenge of biological variation.

The latest development in comparative 2-DE technology is Difference Gel Electrophoresis, commonly known as DIGE. DIGE effectively eliminates gel-to-gel variation, and circumvents the problems inherent with gel-to-gel comparisons. The premise of DIGE is that two samples of interest are labeled with different fluorescent dyes, mixed together and run on the same 2-DE gel. A third sample, the internal standard, allows for accurate gel-to-gel matching and detection of minor differences in protein expression with statistical confidence. Specialized software is then used for the detection, quantitation, positional matching, and differential protein expression analysis. The statistical analysis is based on (1) Differential In-gel Analysis (DIA), involving protein spot detection and quantitation on a pair of images from the same gel; and (2) Biological Variation Analysis (BVA), involving the matching of multiple images from different gels to provide statistical data on differential expression levels between multiple groups. A general scheme showing DIGE analysis of Toxoplasma proteins is shown in Figure 20.2.

With the assistance of powerful imaging software, DIGE enables the sensitive and reproducible mapping of hundreds of potential protein changes between two samples, which can then be picked using a robotic spot-cutter and identified by mass spectrometry.

20.5.2 MS quantitation of proteins

Proteins that are not resolvable by 2-DE are of course not amenable to DIGE analysis, and non-gel based methods need to be considered for relative protein quantitation. Stable isotope-labeling methods have been developed that show considerable potential for MS-based quantitation of proteins. One such method involves the use of Isotope-coded Affinity Tagging (ICAT) reagents (Smolka et al., 2001). This entails a cysteine-reactive moiety, a biotin affinity tag for isolation of tagged proteins, and a linker region consisting of eight hydrogens (light tag) or eight deuteriums (heavy tag). Each proteome is then labeled with the light or heavy ICAT reagent, and the samples are mixed and analyzed simultaneously by LC-MS/MS. For quantitation, the relative signals of identical peptides labeled with either the heavy or light tag analyzed in the MS mode can then be used to measure the relative quantity of one protein against another. However, ICAT has several limitations, the primary one of which is the requirement for a free cysteine on the protein being labeled. It is estimated that as much as 20 percent of any given proteome lacks a free cysteine residue, hence eliminating these from the analysis. ICAT has, however, been used successfully with microorganisms such as Mycobacterium tuberculosis to compare virulent and attenuated forms of the bacterium (Schmidt et al., 2004), and the trypomastigote and amastigote stages of Trypanosoma cruzi (Paba et al., 2004). Future advances in non-gel-based quantitation probably lie in the development of technologies for in vivo isotopic labeling of proteins. In this approach, stable isotopes are incorporated into metabolic products and the relative differences between these products from parasites grown in normal and isotope-labeled media are compared.



FIGURE 20.2 DIGE analysis of *Toxoplasma gondii*. In a typical experiment, two parasite samples are labeled each with a different CyDye (Cy3, Cy5 respectively), and an internal standard consisting of a mixture of the samples labeled with Cy2. The three labeled samples are then mixed together and separated by 2-DE. The gel is scanned at the three different excitation and emission wavelengths unique to each CyDye. Specialized software matches the CyDye-labeled samples on each gel, and also matches spots across gel sets to enable detection of differentially expressed proteins. DIGE Fluor minimal dyes have an NHS-ester group which can form a covalent bond with the epsilon amino group of lysine residues in proteins via an amide linkage. All three CyDyes are size and charge matched ensuring equal migration on a 2-DE gel. CyDyes are very sensitive and capable of detecting around 125 pg of a single protein with a linear response to protein concentration up to five orders of magnitude.

This figure is reproduced in color in the color plate section.

Although not yet developed for *Toxoplasma, in vivo* isotopic labeling exploiting parasite uptake of heavy isotope-containing isoleucine has been used successfully as a quantitative proteomic approach for *Plasmodium* (Nirmalan *et al.*, 2004).

20.6 APPLICATION OF PROTEOMICS TO THE STUDY OF *T. GONDII*

There is presently no complete proteome map of *T. gondii*, although a number of large-scale wholeproteomic analyses of tachyzoites are currently underway (Wastling, unpublished). However, it is now generally recognized that no single separation technique is sufficient to resolve the complete proteome of even relatively simple eukaryotic organisms, including protozoan parasites such as *Toxoplasma*.

One of the first two-dimensional separations of Toxoplasma proteins used 2-DE to compare protein patterns from the RH and BK strains of T. gondii; however, with proteomic technology still developing, verification and identification of the differentially expressed proteins was not achieved using MS (Geissler et al., 1999). Instead, two-dimensional Western blotting was used to characterize antibody reactivity to seven different marker antigens of T. gondii which proved useful in differentiating between acute and latent toxoplasmosis. Similarly, Dlugonska and co-workers used 2-DE to characterize a subset of excretory antigens in T. gondii (Dlugonska et al., 2001). Again, protein identification was by Western blotting and hence limited by previously available immunological reagents against these antigens. The ability to sequence Toxoplasma proteins by mass spectrometry utilizing a limited genome and EST database for T. gondii was first undertaken in studies by Nischik and co-workers, and Reichmann and co-workers (Nischik et al., 2001; Reichmann et al., 2001). Nischik and co-workers were interested in determining virulence factors in T. gondii, and used comparative 2-DE separations to identify differentially expressed proteins in attenuated isolates of T. gondii. In this study, the expression of some excretory proteins (which were identified by immunoblotting) appeared to be modulated in attenuated parasites. In addition, a handful of other differentially expressed proteins was detected and, significantly, for the first time identified using ESI-MS/MS of tryptic peptides eluted from 2-DE protein spots. At the same time, Reichmann and co-workers were able to use a combination of Edman degradation, MALDI-MS, and ESI-MS/MS to characterize a tachyzoite-specific isoform of lactate dehydrogenase (Reichmann *et al.*, 2001).

The first significant proteomic mapping of T. gondii tachyzoites was undertaken by Cohen and co-workers, who used a range of immobilized pH gradients and high-resolution 2-DE to separate over 1000 Toxoplasma proteins. Mass spectrometry was used to analyze 71 of these proteins by MALDI-MS and MALDI post-source decay analysis (PSD) (Cohen et al., 2002). Notably, this study showed that in many instances several protein spots appeared to be encoded by the same gene, indicating that post-translational modification and/or alternative splicing events may be a common feature of functional gene expression in T. gondii. Peptide mass fingerprinting by MALDI-MS enabled unambiguous protein identifications to be made where full gene sequence information was available. However, interpretation of peptide mass fingerprint data using T. gondii EST sequences was less reliable. Peptide fragmentation data, acquired by PSD MALDI-MS, proved a more successful strategy for the identification of Toxoplasma proteins using the EST database. Post-source decay analyses, like ESI-MS/MS (which has now largely replaced it) yields peptide sequence information and does not rely on matching mass fingerprints to near complete predicted protein sequences. This early experiment demonstrated the viability of proteomic analyses for T. gondii, even in the absence of complete genome sequence, as well as the potential value of proteomic data as an aid to annotation of sequence databases in T. gondii.

20.7 SUB-PROTEOMES OF T. GONDII

Although around 30 unique *T. gondii* tachyzoite proteins were identified by Cohen and co-workers,

the most readily identified proteins being the more highly expressed rhoptry, dense-granule, and structural proteins (Cohen *et al.*, 2002). This reflects the inherent difficulties of analyzing a total proteome dominated by a sub-set of highly expressed proteins. In order to mine deeper into the proteome, an alternative approach is required in which the sub-proteome of interest is enriched before analysis. The potential for analyzing sub-proteomes of *T. gondii* has been recognized as a powerful tool to study sub-sets of proteins involved in important biological processes, including perhaps the most defining feature of the Apicomplexa – the ability to invade cells.

20.8 PROTEOMICS ANALYSIS OF THE RHOPTRY ORGANELLES OF *T. GONDII*

The rhoptry organelles are specialized secretory organelles, and have been the focus of considerable attention in an effort to understand the mechanisms by which Toxoplasma is able to invade cells. The availability of proteomics methods for T. gondii has facilitated the identification of a large number of novel rhoptry proteins and the discovery of a completely new class of proteins which localize to the neck of the organelle (Bradley et al., 2005). This improved understanding of the composition of the rhoptries has led to new insights into their structure and function, and set the groundwork for a more complete understanding of how they contribute to the process of invasion and intracellular survival (Wastling and Bradley, 2007).

Until recently, there were eight known rhoptry proteins of *Toxoplasma* designated ROP proteins: ROP1, ROP2, ROP4, ROP8, and ROP9 (Sadak *et al.*, 1988; Ossorio *et al.*, 1992; Beckers *et al.*, 1994, 1997; Reichmann *et al.*, 2002), and two rhoptry proteases (a subtilisin-like protein TgSUB2 and a cathepsin B-like protein, Toxopain-1) as well as a sodium–hydrogen exchanger named TgNHE2 (Que *et al.*, 2002; Miller *et al.*, 2003; Karasov *et al.*, 2005). It was anticipated that this handful of proteins did not represent the full complement of

rhoptry protein constituents, and with the completion of the genome sequence for Toxoplasma it was hoped that bioinformatics analysis might reveal many additional members of the 'rhoptry family' of proteins. This approach unfortunately proved far less successful than was originally anticipated because of an apparent lack of homologous proteins and/or conservation among apicomplexan rhoptry proteins. Furthermore, the paucity of rhoptry sequences in the database meant that it was impossible to determine a rhoptry targeting signal that could be used to identify additional proteins associated with the compartment. However, although bioinformatics approaches proved less successful in the search for additional rhoptry proteins, the development of proteomic methods for T. gondii (Cohen et al., 2002) and the availability of genome sequence to underpin a proteomic database presented a unique opportunity to characterize rhoptry protein composition.

20.8.1 Preparation of the rhoptry sub-proteome

As with all sub-proteome experiments, the success of the rhoptry proteome project required the development of a reliable method for obtaining a highly purified sub-proteome in quantities sufficient for MS analysis. This was achieved with considerable success by the adaptation of a Percoll gradient method originally devised by Dubremetz et al. (Leriche and Dubremetz, 1991) and used to isolate a fraction enriched for rhoptries. Analysis of the fraction obtained by the original protocol using 2-DE revealed that it contained substantial proportions of contaminants, mainly dense granules, mitochondria, and plastids. To improve on the initial enrichment, this fraction was further subjected to sucrose gradient floatation (Bradley et al., 2005). Subsequent analysis of 1-D SDS PAGE gels of this fraction by immunoblotting using antibodies to known proteins from the rhoptries, dense granules, and mitochondria showed that this new sucrose floatation step was able to enrich for the rhoptries whilst effectively removing the vast majority of dense granules and contaminating mitochondria.

20.8.2 The hydrophobic nature of the rhoptry contents

The largely hydrophobic nature of rhoptry proteins was determined using sodium carbonate extraction (Bradley et al., 2005) - a procedure that releases proteins that are soluble or associated with membranes through ionic interactions, leaving behind proteins that are anchored in the membranes by transmembrane domains or other strong interactions such as glycosylphosphatidylinositol anchors. Sodium-carbonate extraction of partially enriched rhoptry fractions showed the predicted partitioning of known soluble (NTPase) and membrane-associated proteins (ROP2/3/4), but also indicated that the majority of novel rhoptry proteins were likely to be found within the membrane fraction and thus not amenable to analysis by 2-DE (Bradley et al., 2005). Thus, when this enriched rhoptry fraction was subjected to 2-DE the representation of proteins on the gel was poor, with membrane-associated rhoptry proteins such as the ROP2-8 family being poorly resolved because of their hydrophobic nature making them less amenable to solubilization in the isoelectric focusing step of 2-DE.

20.8.3 Proteomic identification of rhoptry proteins

Due to the hydrophobic nature of the rhoptry proteins, an alternative to 2-DE proteome analysis was employed to separate the proteins, using conventional one-dimensional SDS-PAGE followed by the excision of 51 contiguous gel slices, each of which was subjected to in-gel trypsin digestion and then tandem MS (MS/MS) to obtain peptide fragmentation data suitable for proteomic database searching (a schematic of the complete approach is shown in Figure 20.3). As expected, known rhoptry proteins were readily identified, including members of the ROP2/4/8 family and ROP9. In addition, 38 previously unidentified candidate novel rhoptry proteins were detected in the fraction. A combination of approaches was used to determine the true localization of the novel proteins identified, including epitope tagging and the production of antibodies against peptides and recombinant proteins. Of 13 randomly chosen proteins that were tested, 12 were indeed found to be localized to the rhoptries. These results indicate that a large percentage of the remaining proteins will also be rhoptry-localized and validate the purification and analysis scheme. Table 20.1 summarizes all confirmed rhoptry proteins identified by proteomic analysis (a full list of all proteins in the analysis can be found in Bradley *et al.*, 2005).

One of the most exciting findings regarding proteins in the rhoptry proteome was the subgroup of proteins that localize exclusively to the duct-like rhoptry necks. To distinguish these from rhoptry body proteins, called ROPs, rhoptry neck proteins were named RONs (Bradley et al., 2005). As previously noted, four RON (RON1-4) proteins were confirmed by antibody production and localized by IFA to the necks of the organelle. To exclude the possibility that these proteins were staining a compartment other than rhoptry necks, localization was confirmed for one of these, RON4, by immunoelectron microscopy (Figure 20.4). Upon rhoptry release during invasion, the rhoptry neck protein RON4 is localized to the moving junction - a structure that forms the interface between the surface of the parasite and the hostcell plasma membrane. RON4 is part of a complex of rhoptry neck proteins that includes RON2, and a protein identified in the rhoptry proteome as TgTwinscan_4705, which has recently been shown to localize to the rhoptry necks and named RON5 (Bradley et al., 2005; Lebrun et al., 2005; Wastling and Bradley, 2007). All three proteins are believed to be present at the moving junction, although localization has only directly been shown for RON4 (Alexander et al., 2005). Proteomic analysis has thus helped to reveal for the first time the presence and participation of rhoptry neck proteins in moving junction formation, and strongly suggests the conservation of this structure at the molecular level among Apicomplexa.

Unexpectedly, proteomic analysis also identified the known *Toxoplasma* proteins Rab11 and toxofilin as being present in the rhoptry fraction



PROTEOMICS ANALYSIS OF THE RHOPTRY ORGANELLES OF T. GONDII

FIGURE 20.3 Schematic for the proteomic analysis of the rhoptry organelles of *T. gondii*.

(A) Rhoptry organelles were prepared from tachyzoites lysed in isotonic sucrose to maintain intact organelles and the organelles fractionated by Percoll and sucrose gradients (only a single gradient is shown here for simplicity).

- (B) The purified organelles were then separated by one-dimensional SDS-PAGE and 51 individual bands were excised from the gel and digested.
- (C) The bands were subjected to LC followed by ESI-MS/MS. MS data were searched against a database containing 881 411 protein sequences downloaded from ToxoDB to identify the proteins in each gel slice.(D) 38 novel rhoptry proteins were identified.
- (D) 38 novel moptry proteins were identified.
- (E) A sub-set of these were selected and expressed as His6-tag fusion proteins and purified by nickelagarose chromatography.
- (F) The purified proteins were injected into mice for polyclonal antibody production.
- (G) Co-localization of these novel rhoptry proteins was verified by immunofluorescence (Bradley *et al.*, 2005).

This figure is reproduced in color in the color plate section.

(Bradley *et al.*, 2005). These findings were confirmed by immunolocalization using antibodies raised against purified recombinant proteins and, in the case of toxofilin, parasites engineered to express a C-terminal HA-tagged version of the protein. Finding toxofilin in the rhoptries was

surprising, because this protein had previously been localized largely to the parasite apical cytoplasm and shown to interact with parasite actin and a protein phosphatase 2C (Poupel *et al.*, 2000; Delorme *et al.*, 2003). The possible biological significance of these findings is discussed elsewhere

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TABLE 20.1 Rhoptry (ROP) and rhoptry neck(RON) proteins of Toxoplasma gondii detectedby proteomic analysis and confirmed byimmunolocalization

Name	MW (kDa)	pI	Function	
ROP1	43	5.8	Penetration enhancing factor?	
ROP2*	64	7.8	PVM-Host mitochondria association	
ROP4*	64	8.5	Unknown	
ROP5*	61	9.8	Unknown	
ROP8*	65	9.2	Unknown	
ROP9	35	7.1	Unknown	
ROP10	61	4.2	Unknown	
ROP11*	58	7.7	Unknown	
ROP12	25	4.5	Unknown	
ROP13	45	9.4	Unknown	
ROP14	122	9.0	Unknown	
ROP15	34	8.6	Unknown	
ROP16	76	9.0	Putative kinase	
RON1	127	4.9	Unknown	
RON2	156	9.7	Moving junction complex	
RON3	223	9.3	Unknown	
RON4	107	6.3	Moving junction complex	
Toxofilir	n 27	9.6	Actin/PP2C binding?	
Rab11	25	10.6	Membrane trafficking of GTPase	

The calculated molecular weight (MW), isoelectric point (pI) and putative function (where known) of rhoptry and rhoptry neck proteins as detected by proteomic analysis and confirmed by immunolocalization (Bradley *et al.*, 2005). The MW and pI are calculated from the primary translation product including signal peptide when present; * = ROP2 family proteins.

(Bradley *et al.*, 2005; Wastling and Bradley, 2007); however, the somewhat surprising nature of these observations underlines the advantage of a proteomic study which assumes nothing of the expected outcome and is not dependent on the availability of specific reagents that may limit the breadth of experimental investigation.



Bradley et al, JBC 2006, vol 80, with permission

FIGURE 20.4 Immunolocalization of RON 4 to the rhoptry necks of *Toxoplasma gondii*. Immunoelectron microscopy with anti-RON4 antibodies demonstrates that RON4 is localized to the neck portion (arrows) of the rhoptries (R) and is not present in the bulbous bodies of the organelle. Lines also point to the conoid (C) and the micronemes (M). RON4 appears to be most prominent at the junction of the body and neck portion of the organelle, and is present in samples from both tachyzoites and bradyzoites.

All *T. gondii* rhoptry proteins studied to date appear to be synthesized as pro-proteins that are then processed to their mature forms. To understand the role of the *pro*-region in rhoptry protein function, MS analysis has been used to define the processing site of the pro-region of the rhoptry protein ROP1 (Bradley and Boothroyd, 1999). Efforts to determine such processing sites had previously been prevented by blocked N-termini of mature proteins isolated from *T. gondii*, thus preventing analysis by conventional N-terminal amino-acid sequencing. To overcome the problem, an engineered form of ROP1 was designed and MS used to demonstrate that pro-ROP1 is processed to its mature form between the glutamic acid at position 83 and alanine at position 84.

20.9 PROTEOMICS ANALYSIS OF EXCRETORY/ SECRETORY PROTEINS OF T. GONDII

The apical complex of Toxoplasma is involved in the secretion of compounds that play a key role in host-cell invasion and survival during infection. These compounds are derived not just from the rhoptries but also from the dense granules and the micronemes. Bioinformatics analysis of the T. gondii genome reveals more than 800 genes encoding proteins with putative secretory signal peptides (Zhou et al., 2005). Although many of these putative secretory proteins are likely to be associated only with internal organelles, a substantial proportion might be exported externally for interaction with the host. Until recently only a limited number of such secretory proteins had been discovered, and the wider spectrum of these molecules thought to be involved in parasite invasion and survival remained unknown. These proteins, known as excretory/secretory antigen (ESA) proteins, are by nature soluble, and thus ideally suited to mapping and identification by 2-DE. Zhou and co-workers have recently applied proteomics technology to analyze a large cohort of freely released Toxoplasma secretory proteins by using the complementary methodologies of 2-DE and liquid chromatography ESI-MS/MS (MudPIT) (Zhou et al., 2005).

A sub-proteome of ESA proteins can be readily prepared from tachyzoites by separating the parasites from host cells and incubating them in medium containing 1% ethanol before concentration and proteomics analysis. Mapping of these ESA secretory products by 2-DE revealed approximately 100 spots, most of which were successfully identified by protein micro-sequencing or MALDI-MS. Separation of the ESA proteins on 2-DE gels showed clearly that many proteins were present in multiple species, suggesting they are subjected to substantial post-translational modification (PTM), although the nature and significance of these PTMs remains to be elucidated (Zhou *et al.*, 2005).

MudPIT proteomics analysis of the same secretory fraction revealed several additional proteins, including novel putative adhesive proteins, proteases, and hypothetical secretory proteins similar to products expressed by other related parasites, including Plasmodium (Zhou et al., 2005). As with the analysis of rhoptry proteins (Bradley et al., 2005), Zhou and co-workers characterized a subset of these novel ESA proteins by localization experiments, this time by re-expressing them as fusions to yellow fluorescent protein. This screen revealed shared and distinct localizations within the secretory compartments of T. gondii tachyzoites, confirming the hypothesis that these proteins were indeed derived from these secretory organelles. This proteomic study has significantly broadened our understanding of the ESA proteins of Toxoplasma in a way that would have been difficult to achieve with classical techniques. Interestingly, only 1 of the 38 novel rhoptry proteins identified by Bradley and co-workers was found in the ESA fraction of Zhou and co-workers. This extremely small overlap between the two subproteomes is a demonstration of the purity of the respective sample preparations, and a testament to the importance of good sample preparation when undertaking sub-proteomic studies.

20.10 OTHER SUB-PROTEOME STUDIES OF T. GONDII

Essentially, any compartment or sub-proteome of *Toxoplasma* can be studied, providing material of sufficient quantity and quality can be generated and some method of verification, such as immunolocalization, can be employed. Unfortunately, some of the life stages of *T. gondii* for which proteomics analysis would prove most biologically interesting, such as the sexual stages, which occur exclusively in the cat gut, are some of the most difficult to obtain and to prepare suitable material from. Proteomics studies of the cytoskeleton and the

inner-membrane complex of T. gondii tachyzoites are currently underway (Wastling, unpublished), and many other similar studies are certain to follow in the coming years. 2-DE mapping, in particular, reveals the enormous complexity of gene expression in T. gondii, indicating the extent to which isoforms of proteins are a common feature of protein expression and also the extent to which post-translational modifications feature in the proteome. For example, 2-DE analysis of the cytoskeleton shows large and complex families of alpha and beta tubulin, myosin, and membrane skeletal proteins among others (Wastling, unpublished). The nature of the post-translational modifications which give rise to this complexity is at present completely unknown. Such modifications are likely to be functionally important, and determining when and why they occur will be essential to understanding the biology of the parasite.

20.11 THE DYNAMIC PROTEOME OF *T. GONDII*

So far we have referred to the proteome of Toxoplasma as if were a relatively stable entity, rather like its genome. All proteomes are in fact highly dynamic, and reflect the biological context of the cell at the time of analysis. Rather than proving a hindrance, this phenomenon can be turned to our advantage so that comparative proteomics analysis can be exploited as a tool to characterize the biological activity of the parasite. Thus proteomics analysis can be used to help understand important facets of the biology of T. gondii, such as the changes that occur from one life-stage to another and the analysis of gene knockout phenotypes and virulence factors, as well as for the study of the action of chemotherapeutic agents and the basis of drug resistance.

20.11.1 Proteomics analysis of *Toxoplasma* virulence factors

Neudeck and co-workers used comparative proteome analysis to demonstrate that the level of GRA 7 expression correlates with virulence in the BK strain of T. gondii, although it is not known whether the loss of virulence is a direct consequence of the reduced GRA7 expression (Neudeck et al., 2002). Cohen also demonstrated changes in the proteome of S48 strain T. gondii tachyzoites (Cohen, 2002). S48 strain tachyzoites are thought not to form tissue cysts and are the basis of Toxovax[™], the only commercial vaccine against toxoplasmosis, used to protect sheep against abortion (Buxton and Innes, 1995). Nischik and co-workers used comparative 2-DE to try to identify determinants of Toxoplasma virulence, and noted modulation of a range of proteins, including actin, catalase, nucleoside triphosphate hydrolase 1, microneme, dense-granule and rhoptry proteins in attenuated forms. Gastens and Fischer also used comparative proteome analysis to compare mouse virulent and attenuated Toxoplasma. Gel-to-gel analysis revealed a 53-kDa protein which was markedly reduced in attenuated parasites. Subsequent analysis by ESI-MS/MS detected three peptides, one of which matched a T. gondii EST sequence in attenuated parasites which appeared to be a homolog of eukaryotic translation initiation factor eIF4A. The identification of this protein was confirmed by immunoblotting using antibodies against the predicted N-terminus of T. gondii eIF4A, and differential expression between virulent and avirulent parasites reconfirmed. The downregulation of this protein in attenuated parasites and also in bradyzoites suggests that it is involved in the homeostasis of protein synthesis in Toxoplasma (Gastens and Fischer, 2002). However, the proteins identified in these studies perhaps seem unlikely candidates as primary determinants of virulence, appearing rather to reflect proteins associated with faster growth, intracellular replication, and invasion. It remains a challenge of proteomics to improve experimental design to focus on primary rather than secondary mechanisms of action. This can only be achieved by placing greater emphasis on sub-proteome studies which enable deeper mining of the proteome, and by regarding proteomics not in isolation but as one of several complementary tools to study gene function in T. gondii.

Proteomics represents an ideal tool to characterize the phenotypic response of a parasite to a gene knockout event, particularly in cases where there is no obvious morphological or biological change in the organism. The term 'no phenotype' for a gene knockout experiment is often misleading, as often it incorrectly refers to phenotypes that can be immediately observed - such as loss of virulence. In fact, the deletion of a gene and the subsequent survival of the parasite can reveal considerable information as to the complexity of its biology and the presence of alternative metabolic pathways. Proteomics is an excellent way to detect unseen compensatory biochemical responses to gene knockout events. Bhatti and co-workers used comparative 2-DE gels and MS identification of proteins to identify gene products affected by the loss of the histone acetyltransferase GCN5 in T. gondii (Bhatti et al., 2006). GCN5 is a histone acetyltransferase that is essential for development in mammals and critical to stress responses in yeast. Loss of GCN5 in T. gondii yields viable parasites and does not attenuate virulence in an in vivo mouse model, but does cause downregulation of proteins involved in metabolism and cell signaling, as well as those associated with invasion. Together with other data, these studies helped to drive the discovery of a second GCN5 (TgGCN5-B) which has the potential to compensate for TgGCN5-A and reveal the unexpected complexity in the GCN5 machinery of Toxoplasma.

20.11.2 Proteomics analysis of protein processing in *T. gondii*

In an elegant experiment by Zhou and co-workers, a variety of proteomics techniques were used to study the proteolytic processing of the *T. gondii* transmembrane micronemal protein MIC2 and its partner M2AP, which form an adhesive complex required for host-cell invasion (Zhou *et al.*, 2004). The MIC2/M2AP complex undergoes proteolytic processing on the parasite surface during invasion. In a three-stage proteomics experiment, MALDI intact mass measurements followed by enzymatic digestion and inhibitor profiling were used to demonstrate that the protein M2AP is processed by two proteases: MPP2 and MPP3. Second, differential protein expression patterns detected by 2-DE DIGE were used to define the substrate repertoire of MMP2. Finally, mass spectrometry was used to show that MIC2 is shed by membrane cleavage within its anchor domain (Zhou *et al.*, 2004). This study demonstrates the power and versatility of mass spectrometry analysis to help determine the function of proteins. Notable is the high degree of mass accuracy achievable by MS, which enables intact mass measurements to be made and interpreted in a meaningful way.

20.12 PROTEOMICS AS A TOOL TO DISSECT THE HOST IMMUNE RESPONSE TO INFECTION

20.12.1 Immunopathological responses to infection with *T. gondii*

Proteomic analysis of the host response to parasite invasion and the resulting immunological response to infection can be equally important in trying to understand the nature of the host– parasite relationship.

Proteins separated by 2-DE can be transferred to immobilized membranes for immunoblotting in the same way that one-dimensional SDS-PAGE gels can be used to investigate antibody responses to infection. In theory, this should enable the precise identification of the proteins which elicit specific antibody responses during infection. If immunological reagents are available against specific antigens, then these antibodies can be used to probe 2-DE blots to determine their pattern of expression. However, 2-DE immunoblotting can also be adapted to enable detection of novel antigens by probing 2-DE blots of whole parasite antigen with sera from animals infected with T. gondii. Unfortunately it is not possible to determine protein sequence by MS analysis directly from membranes to which proteins that have been transferred and subsequently immunoblotted, due to interference with the blocking reagents and chemicals used in Western blotting.

Nonetheless, it is possible to identify proteins from replicate preparative gels or immobilized membrane blots that have not undergone immunological detection. The availability of immobilized plastic-backed 2-DE gels and excellent imaging software to match analytical and preparative gels makes this task possible, although still something of an art.

Since the effective immune response to T. gondii infection is dependent on cell-mediated immunity, high-resolution analysis by 2-DE blotting to determine antibody responses has only limited value in helping elucidate the mechanisms of immunity to this parasite. To help characterize T-cell stimulatory antigens, the challenging but potentially powerful technique of T-cell blot analysis of electrophoretically separated proteins may be employed followed by MS analysis to identify specific antigens (Reichmann et al., 1997). Reichmann and co-workers separated whole tachyzoite proteins by 2-DE, and electro-eluted individual soluble fractions using an electroelution device (Gulle et al., 1990) to identify protein spots that stimulated a specific T-cell clone. One of the proteins able to stimulate this clone was LDH, for which the T-cell epitope was mapped to a nine-amino-acid partial sequence. Unfortunately it was not possible to demonstrate that the LDH molecule was immunogenic during infection in vivo, since neither sera nor T cells from infected mice reacted with recombinant LDH, or with the eluate from the gel. Nonetheless, the separation of proteins by 2-DE or other methods combined with T-cell analysis and proteomic detection of T-cell stimulatory antigens remains a potentially powerful and relatively unexploited tool to help understand the immunogenic profile of T. gondii proteins.

Comparative 2-DE analysis can also be used on whole tissues to determine pathological changes resulting from infection with *T. gondii*. As part of a study to understand the development of toxoplasmic encephalitis (TE) in the brain, we analyzed murine brain tissue before and after infection with various strains of *T. gondii*. Tissue was processed and analyzed by 2-DE, which revealed significant changes in the expression of key proteins involved in the pathology of TE (Wastling, unpublished). Proteins including glial fibrillary acidic protein (GFAP) were shown to be significantly upregulated in brain tissue following infection. This result was confirmed by immunohistochemistry using antibodies against GFAP to provide strong evidence of the importance of GFAP in the development of TE (Fischer *et al.*, 1997; Stenzel *et al.*, 2004).

20.11.2 Modulation of the host-cell proteome by *T. gondii*

To understand the way in which the T. gondii proteome interacts with host cells, it is essential to know something of how the host responds to the parasite during and after invasion of the cell. Changes in gene and protein expression might be anticipated during intracellular invasion and establishment, but determining the exact nature of these modulations is important in understanding how and why Toxoplasma is such a ubiquitous and successful intracellular pathogen. Blader and co-workers have examined in vitro host-cell gene expression changes during invasion of T. gondii, using microarrays consisting of approximately 22 000 known genes and uncharacterized expressed sequence tags (Blader et al., 2001). Early during infection (1-2 h), < 1 percent of all genes showed a significant change in the abundance of their transcripts, with a significant upregulation of proinflammatory genes as well as genes involved in organelle redistribution and protection from apoptosis. The correlation between steady-state transcriptional data and protein synthesis in eukaryotes is not known. Moreover, for many proteins their functional activity is dependent not on abundance of their mRNA, but on posttranslational modifications such as phosphorylation, which are not tractable by transcriptional analysis. Consequently, we have recently attempted to model changes in the soluble host-cell proteome during and after in vitro invasion of cells by T. gondii using proteomics. In this experiment, conventional 2-DE and DIGE experiments were used to detect several hundred protein changes that occurred after invasion of host cells with Toxoplasma.

After DIGE analysis of parasite-infected cells, differentially expressed proteins were identified by MS analysis and protein functions assigned for each, using protein function assignation tools such as the Bioinformatic Harvester program (www.harvester.embl.de) and those at the Human Protein Resource Database (www.hprd.org), in order to model pathway changes following parasite invasion. All of the proteins modulated in the study were assigned a functional classification based on a schema put forward by the Munich Information Centre for Protein Sequences (http:// mips.gsf.de/projects/funcat). A scheme for the bioinformatic handling of DIGE data from parasiteinfected cells is shown in Figure 20.5. Classes of protein that were modulated included those associated with apoptosis, mitosis, glycolysis, lipid metabolism, nucleoside synthesis, and the cytoskeleton. Changes in the host-cell proteome were consistent with the arresting of both apoptosis and cell division. In addition, glycolysis was upregulated, as was the synthesis of cytoskeletal proteins, presumably due to hostcell remodeling. Of considerable note was the large number of host mitochondrial proteins that were changed in expression during infection, which accounted for almost one-third of all modulated proteins.

The design of the above proteomics experiment was deliberately intended to mirror that of the earlier transcriptional studies by Blader and coworkers in order to provide an opportunity to examine the correlation between transcriptional changes and the host proteome during the invasion process. Since only differentially expressed proteins were picked for MS analysis, only a comparison of this subset of proteins was possible. Nevertheless, sufficient data were available to show that around 60 percent of the differentially expressed transcriptional data agreed with the proteome data (although, perhaps more significantly, 40 percent did not agree). Interestingly, proteomics analysis of expression changes during invasion of the same cells with the non-apicomplexan trypanosomatid parasite Leishmania major showed a very small overlap with those proteins modulated during Toxoplasma

invasion. Combined with the data of Blader and co-workers, who also examined transcriptional changes during invasion with another trypanosomatid parasite, *T. cruzi*, these data suggest very strongly that the response to *Toxoplasma* invasion is highly specific, and more complex than a simple response to stress. These studies provide a wealth of information on which to build more detailed functional studies on the subversion of the host cell by this parasite.

20.13 CHEMICAL PROTEOMICS

One of the drawbacks of proteomics methods as they stand is that they mostly provide information only on the identity and abundance of proteins. Although much information is potentially obtainable by MS analysis, such as the nature and extent of PTMs, in practice efficient methods for the rapid identification of PTMs (such as phosphorylation) are largely underdeveloped and have not yet been implemented for Toxoplasma. The characterization of protein-protein or inhibitor-protein interactions has also been mostly overlooked by classical proteomics methods. As a response, the field of chemical proteomics has recently been established as a means to profile global patterns of enzyme activity through the use of synthetic small molecules known as activity-based probes (ABPs) (Cravatt and Sorensen, 2000; Greenbaum et al., 2000, 2002a). In combination with classical biochemistry and genetic studies, these chemical reagents are designed to target a distinct subset of enzymatic targets to help identification and activitybased profiling (ABPP). Activity-based probes can be used to profile the activity of several protein targets in a single experiment without the need to generate reagent quantities of recombinant enzymes. For example, a general cysteine protease probe, DCG-04, has been used to profile cysteine protease activity in Plasmodium falciparum (Greenbaum et al., 2002b). More recently, the first high-throughput screen for small molecules capable of disrupting biological processes in T. gondii was performed (Carey et al., 2004). Using a microscopybased assay, over 12000 structurally diverse small



FIGURE 20.5 Bioinformatics analysis of differential expression of host-cell proteins following infection

- with T. gondii.
- (A) Proteins that are differentially expressed between infected and non-infected cells are readily detected by specialized DIGE software including average volume change between samples and statistical analysis. The statistical analysis is based on Differential In-gel Analysis (DIA) involving protein spot detection and quantitation on a pair of images from the same gel and Biological Variation Analysis (BVA) involving the matching of multiple images from different gels to provide statistical data on differential expression levels between multiple groups.
- (B) Protein spots which are significantly different can then be picked and identified by MS analysis.
- (C) Protein functions are assigned for each, using protein function assignation tools such as the *Bioinformatic harvester* program (www.harvester.embl.de) and those at the Human Protein Resource Database (www.hprd.org) in order to model pathway changes following parasite invasion. In this example the protein peroxiredoxin 1 was shown to be significantly downregulated in infected cells. Peroxiredoxin 1 is functionally classified as being associated with cell-cycle/mitosis, and its modulation is consistent with the suppression of cell mitosis after *Toxoplasma* infection.
- (D) The pie chart shows the functional redistribution of 260 host proteins that change after *T. gondii* invasion.

This figure is reproduced in color in the color plate section.

molecules were screened for the ability to modulate the invasion of *T. gondii* into cells. The study demonstrated that different inhibitors perturb different aspects of invasion, including gliding motility, secretion of host-cell adhesins from apical organelles, and extension of the conoid. Unexpectedly, the screen also identified molecules that dramatically enhanced invasion, gliding motility, and microneme secretion. The small molecules identified using this approach reveal a previously unrecognized complexity in the control of parasite motility and microneme secretion, and they constitute a set of useful probes for dissecting the invasive mechanisms of *T. gondii* and related parasites (Carey *et al.*, 2004).

20.14 DATABASE MANAGEMENT OF *T. GONDII* PROTEOMICS DATA

With the development of robotics, high-throughput protein separation and MS analysis, even modest proteomics experiments have the capacity to generate vast quantities of data. Database management plays an important role in maximizing the accessibility and handling of proteomics data. Effective database management requires consideration of the following:

- 1. How to present the data in a summarized yet informative way
- 2. In what form to store raw data so that it can be re-queried at a later date and used by others who might wish to address a different set of biological questions
- 3. How to integrate proteomics data with other data, such as genomics and microarray data.

Before looking at how these points might be addressed for *T. gondii*, it is necessary to understand something of the wider context of the challenges facing proteomics data-handling in general. Those engaged in large-scale proteomics projects now have to address problems similar to those faced during the early years of microarray experiments, in which the necessity to integrate diverse types of data meant that completely new bioinformatics tools were required to be developed and implemented. For some time now, object models such as MAGE-OM (the Microarray Gene Expression Object Model) have been available for managing microarray experiments. MAGE-ML (Microarray Gene Expression Mark-up Language), an XML implementation of MAGE-OM, is a language designed to describe and communicate information about microarray-based experiments. MAGE-ML can describe microarray designs, microarray manufacturing information, microarray experiment setup and execution information, gene expression data, and data analysis results. A programming interface is already available for formatting microarray data into MAGE-ML (http://www.mged.org/ Workgroups/MAGE/magestk.html), and MAGE-ML acts as a format for sending data to publicly available databases.

Recently, considerable effort has been directed into developing a similar object model for proteomics experiments, thus enabling microarray and proteomics data to be handled in a similar format. Using 2-DE, MS data, and experimental protocols derived from 2-DE proteomics analysis of T. gondii, Jones and co-workers developed an object model capable of representing microarray and proteomics data, including separation techniques such as 2-DE and MS protein identification (Jones et al., 2004). The model stores experimental protocols, raw data, and data analysis, and is known as the Functional Genomics Experiment Object Model (FGE-OM). FGE-OM was developed from three main sources: the MAGE model for microarrays (Spellman et al., 2002), the PEDRo model developed at the University of Manchester (Taylor et al., 2003), and a model developed at the University of Glasgow, referred to as Gla-PSI.

The Human Proteome Organization Proteome Standards Initiative (HUPO-PSI) (http://www.hupo. org/research/psi/) has also developed standards for MS and protein–protein interaction data, as well as being engaged in developing an integrative format for the full representation of a proteomics experiment. PEDRo (the Proteome Experimental Data Repository) is one of a number of schemes which aims to make comprehensive proteomics datasets available for browsing, searching, and downloading, and is designed for storing, searching, and disseminating experimental proteomics data. The PEDRo database also provides access to a collection of comprehensive descriptions of experimental datasets in proteomics, which should serve as a starting point for the standardization of proteomics experiments.

20.14.1 *T. gondii* proteomics data management

Proteomics analysis of T. gondii was instrumental in providing some of the first data used in developing proteomics standards (Jones et al., 2004), and it remains important that large-scale Toxoplasma proteomics experiments and data should attempt to adhere to these developing common formats. Whilst a proteomics experiment in isolation can yield valuable results, there is considerable potential for extending the value of those data to the wider community with the careful use of common standards. Moreover, the principle that raw data should always be stored alongside processed data (as with sequence and microarray data) is also an important one. Whilst raw data are unlikely to be accessed widely by the user-community, its retention preserves the possibility of re-querying the original data at a later time. This has already proved important with the MS identification of T. gondii proteins, where a re-analysis of raw MS data during a rapid period of the evolution of the Toxoplasma genome database was required on more than one occasion (Bradley et al., 2005). Similarly, the algorithms for protein identification from MS data are constantly being modified and improved, making it sometimes desirable retrospectively to analyze MS data. In common with microarray experiments, the cost and effort involved in performing experiments justifies careful data storage and archiving, as it is rarely desirable (or possible) to repeat the same work. Large-scale proteomics data for T. gondii should thus be viewed as a community resource, with community-wide access, to encourage further exploitation of the data.

For proteomics data to be of benefit to the wider community, there must be an easy route of entry for researchers whose primary interest may not be proteomics but for whom proteomics data might be of value. As with genome and expression data, the most obvious portal for this is ToxoDB (http://www.toxodb.org/). Proteomics data for Toxoplasma have their greatest value in the context of other genome and expression data, where they still have considerable potential for informing gene finding and annotation. Efforts are currently underway with T. gondii and other apicomplexan parasites, including Cryptosporidium sp. and Plasmodium sp., to develop a common proteomics format for the display of proteomics data in their respective databases. Ideally these will include the alignment of peptide sequences against protein prediction models, such as those displayed in the Generic Gene Model Organism Database Construction set (GBrowse www.gmod.org/) available for T. gondii at ToxoDB. Moreover, the provision to 'drill-down' into the data to mine the wealth of additional information in proteomics experiments needs to be retained. Thus, maintaining a close link between ToxoDB and standardized proteomics data repositories will be essential.

20.15 CONCLUSION AND PERSPECTIVES

Toxoplasma has been increasingly at the forefront of proteomics, and this trend is set to continue with wider accessibility of this rapidly developing technology to researchers. However, major challenges lie ahead if proteomics studies are indeed to revolutionize our understanding of Toxoplasma biology. If we are really to use proteomics to establish the link between the genome and its function, then proteomics must move forward to provide more detailed and complete functional data on the proteins that it describes. The key to this lies partly in being better able to profile the activity of proteins, for example, through high-throughput analysis of PTMs and protein-protein interactions. These goals are not trivial. In the case of PTM analysis, for instance, it is not just the presence or absence of PTMs such as phosphorylation that needs to be identified; the degree of

phosphorylation and the location of phosphorylation sites for proteins must be determined - all this in the context of an exceptionally transient and labile system. Advances in both relative and absolute protein quantitation also need to be applied to the study of Toxoplasma if proteomics data are to be used more effectively with other postgenomic technologies, such as transcriptional analysis and metabolomics in a systems biology approach. Finally, just as mRNA analysis cannot tell us everything about the function of genes, proteins too cannot be understood entirely in isolation. Sugars and lipids are also important cell components, and their large-scale analysis through the emerging fields of glycomics and lipidomics is now a possibility. Proteomics for Toxoplasma must develop in the context of these nascent technologies. Perhaps the most certain aspect of the coming years is that the post-genomics picture is going to get more complex before it get simpler, thus placing bioinformatics at the forefront of all future developments.

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21

Cerebral Toxoplasmosis: Pathogenesis and Host Resistance

Y. Suzuki, S. Halonen, X. Wang and X. Wen

- 21.1 Introduction
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- 21.3 Producers of IFN-γ
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21.1 INTRODUCTION

Toxoplasma gondii is a ubiquitous, obligate intracellular protozoan parasite in humans and animals. Chronic (latent) infection with this parasite is likely one of the most common infections of humans, affecting 10–25 percent of the world's population. During the acute stage of the infection, tachyzoites quickly proliferate within a variety of nucleated cells and spread throughout host tissues. Following the acute stage, the parasite forms cysts (latent stage) in various organs, especially the brain, heart, and skeletal muscle, establishing chronic infection. Infection in immunocompetent individuals is usually unnoticed or a benign, self-limiting illness, and results in a latent chronic infection. Immunosuppression in chronically infected individuals may result in reactivation of a latent infection, which is initiated by disruption of cysts, followed by proliferation of tachyzoites. Such reactivation of *T. gondii* infection usually presents as toxoplasmic encephalitis (TE). TE has emerged as a major opportunistic infectious disease in the central nervous system in AIDS patients. Since immunocompetent individuals do not usually suffer apparent untoward effects, including development of encephalitis, it is clear that the immune response is critical for prevention of TE. The parasite can also be acquired congenitally, and in these cases the brain is the major organ affected by the infection.

Murine models have mainly been used to analyze the mechanisms of host resistance to acute infection and development of TE during the late stage of infection. IFN-γ-dependent cell-mediated immunity has been shown to play the major role in resistance to T. gondii, although humoral immunity is also involved. A number of different types of cells are involved in the resistance as producers of IFN- γ . Microglia that reside in the brain have been revealed to be a cell population that produces IFN-y following infection with T. gondii (Suzuki et al., 2005). Multiple types of cells also participate as effector cells that become activated by IFN-y to control the parasite. Additionally, multiple cell types are also important as producers of IL-12, which is required for the induction of IFN-γ.

21.2 PRODUCERS OF INTERLEUKIN (IL)-12 REQUIRED FOR IFN-7 PRODUCTION

21.2.1 Dendritic cells

IL-12 is the most important inducer of IFN- γ synthesis during the acute stage of infection. Neutralization of IL-12 with anti-IL-12 antibodies results in 100 percent mortality in mice infected with an avirulent strain of T. gondii, and the mortality is associated with decreased IFN- γ production (Gazzinelli et al., 1994). Dendritic cells were identified to be the source of IL-12 in the spleen in response to T. gondii (Reis e Sousa et al., 1997). All of the IL-12-positive cells in the spleens of T. gondii-stimulated mice were found in T-cell areas and were CD8α+, CD11c+, DEC205+ dendritic cells. CCR5 expressed on the surface of dendritic cells is responsible for their migration into splenic T-cell areas following stimulation (Reis e Sousa et al., 1997). CCR5 signaling also plays an important role in the activation of

dendritic cells to produce IL-12 (Aliberti *et al.*, 2000). CCR5-deficient mice have impaired IL-12 production by dendritic cells, and are highly susceptible to *T. gondii* infection. Cyclophilin-18 has been identified as the principal molecule derived from the parasite that triggers IL-12 production through CCR5 (Aliberti *et al.*, 2003). Recently, a profilin-like protein of the parasite was also found to bind to Toll-like receptor 11 and stimulate IL-12 production by dendritic cells (Yarovinsky *et al.*, 2005).

During the chronic stage of infection, cells bearing dendritic cell markers such as CD11c and 33D1 are located at inflammatory sites in the brains of mice (Fischer et al., 2000). These brain dendritic cells are mature as indicated by high-level expression of MHC class II molecules, CD40, CD54, CD80, and CD86, and are able to trigger antigen-specific T-cell responses in vitro. These dendritic cells have been shown to be the major producers of IL-12 in mononuclear cells isolated from the brains of infected animals (Fischer et al., 2000). GM-CSF appears to be important for the induction of these dendritic cells in primary brain cell cultures with T. gondii (Fischer et al., 2000). Since IL-12 is necessary for the maintenance of IFN-7 production in T cells mediating resistance to chronic infection (Yap et al., 2000), dendritic cells in the brains of the infected mice probably play a role in maintaining IFN- γ production by T cells in this organ.

21.2.2 Macrophages

Macrophages produce IL-12 in response to tachyzoites or tachyzoite antigens *in vitro* (Gazzinelli *et al.*, 1994, 1996). Since macrophages infiltrate the brains of mice following infection with *T. gondii* (Suzuki *et al.*, 2005; Wilson *et al.*, 2005), these cells, in addition to dendritic cells, may be an important source of IL-12 in resistance against the parasite in the brain.

21.2.3 Neutrophils

Neutrophils rapidly infiltrate the peritoneal cavity of mice following intraperitoneal infection with *T. gondii*. Approximately 85 percent of the neutrophils displayed intracellular storage of IL-12 (Bliss *et al.*, 2000). Depletion of neutrophils during

mortality in mice in association with decreased production of IL-12 and IFN- γ by splenocytes (Bliss *et al.*, 2001). Rapid infiltration of neutrophils into the site of infection appears to play an important role in induction of the protective Th1-type immune responses against *T. gondii* during the early stage of infection. However, it is unknown whether neutrophils are involved in the resistance in the brain to control *T. gondii* during the chronic stage of infection.

21.3 PRODUCERS OF IFN-γ

21.3.1 Involvement of 'innate immunity'

21.3.1.1 Microglia and blood-derived macrophages

αβ T cells are essential to control T. gondii in both acute and chronic stages of infection (see section 21.3.2). We found, however, that in addition to T cells, IFN-γ production by cells other than T cells is required for the prevention of reactivation of T. gondii infection (TE) in the brain of chronically infected mice (Kang and Suzuki, 2001). Athymic nude, SCID, and IFN-y-deficient mice were infected with T. gondii and treated with sulfadiazine to establish chronic infection. After discontinuation of sulfadiazine, each of these animals developed severe TE due to reactivation of the chronic infection and died. When the animals received adoptive transfer of immune spleen or T cells before discontinuation of sulfadiazine, infected athymic nude and SCID mice did not develop TE and survived. However, the infected IFN-y-deficient mice still developed TE and died even after receiving the cell transfer (Kang and Suzuki, 2001). Before cell transfer, IFN-y mRNA was detected in brains of the nude and SCID mice, but not in brains of the IFN-y-deficient mice. IFN-y mRNA was also detected in brains of infected SCID mice depleted of NK cells, and such animals did not develop TE after receiving immune T cells (Kang and Suzuki, 2001). Thus, IFN-y production by non-T cells, in addition to T cells, is required for prevention of reactivation of T. gondii infection in the brain.

These IFN- γ -producing non-T cells do not appear to be NK cells.

Suzuki et al. (2005) recently identified these non-T, non-NK cells that produce IFN-y in the brains of nude and SCID mice chronically infected with T. gondii. Intracellular staining for IFN- γ followed by flow cytometry revealed that approximately 45–60 percent of the cells expressing IFN- γ in their brains were positive for CD11b or F4/80 (markers for microglia/macrophages) on their surfaces. Smaller portions of the cells were positive for pan-NK marker. Further smaller portions were positive for CD11c (a marker for dendritic cells), and these cells were less than 5 percent of the IFN- γ expressing cells in brains of infected SCID mice. In addition to IFN-y proteins, large amounts of mRNA for IFN-y were detected in CD11b+ cells purified from brains of infected mice, but this was not the case in the cells obtained from uninfected animals. In infected SCID mice depleted of NK cells (by treatment with anti-asialo-GM1 antibody), cells expressing IFN- γ in their brains were all positive for CD11b, and IFN- γ -producing cells were detected in both CD45^{low} and CD45^{high} populations. These results suggest that CD11b+ CD45low microglia and CD11b+ CD45high blood-derived macrophages are the major non-T, non-NK cells which express IFN- γ in the brains of mice infected with T. gondii. Therefore, it is possible that IFN- γ production by microglia and/or macrophages plays an important role in prevention of TE in collaboration with $\alpha\beta$ T cells.

21.3.1.2 γδ T cells

During the acute stage of infection with *T. gondii*, increased numbers of T cells expressing the $\gamma\delta$ T-cell receptor have been observed in the spleen and peritoneal cavity of mice and the peripheral blood of humans. $\gamma\delta$ T cells are cytotoxic to infected target cells and produce IFN- γ and TNF- α in response to the parasite *in vitro*. Involvement of $\gamma\delta$ T cells in resistance against acute infection with *T. gondii* has been shown in mice. Mice deficient in $\gamma\delta$ T cells due to treatment with anti-TCR $\gamma\delta$ mAb (Hisaeda *et al.*, 1995) or lack of the functional TCR δ gene (Kasper *et al.*, 1996) die earlier than control mice during the acute stage of infection. $\gamma\delta$ T cells may also be involved in prevention of TE during the late stage of infection, as $\gamma\delta$ T cells are detectable in brains of chronically infected mice and rats. Of interest, the relative percentages of $\gamma\delta$ T cells in lymphocyte preparations isolated from brains of infected mice are significantly higher than in their spleens (Suzuki *et al.*, 1997). This raises the possibility that $\gamma\delta$ T cells preferentially infiltrate into the brain of *T. gondii*-infected mice. Lepage *et al.* (1998) have suggested a possible role for $\gamma\delta$ T cells in enhancing the protective activity of CD8+ $\alpha\beta$ T cells in studies using adoptive transfer of the lymphocyte populations.

In a patient with CD40L defect (hyper-IgM syndrome) who had developed TE, a marked increase in $\gamma\delta$ T cells was observed in his peripheral blood (Leiva *et al.*, 1998). The patient responded well to anti-toxoplasmic chemotherapy and to high-dose immunoglobulin replacement therapy. $\gamma\delta$ T cells may have contributed to controlling the disease, but their protective activity is not sufficient by itself to prevent development of TE.

21.3.1.3 NK cells

NK cells are an important source of IFN- γ in resistance against *T. gondii* during the early stage of infection. Depletion of NK cells results in early or increased mortality in SCID and wild-type mice. In contrast to the early stage of infection, NK cells do not appear to be crucial for prevention of TE during the late stage of infection. Kang and Suzuki (2001) reported in SCID mice with adoptive transfer of immune T-cells that the depletion of NK cells (cells were undetectable by flow cytometry in their brains and spleens) did not abolish the resistance of these animals to the development of TE.

21.3.2 The importance of 'acquired immunity' involving CD4+ and CD8+ $\alpha\beta$ T cells

It is clear that $\alpha\beta$ T cells are essential for resistance against *T. gondii*, since athymic nude and SCID mice which lack T cells succumb to acute infection and their mortality is associated with proliferation of large numbers of tachyzoites in various organs including the brain. CD8+ T cells are the major efferent limb of the protective cellular immunity against acute infection, although CD4+ T cells are also involved. The protective activity of CD8+ T cells is predominantly mediated by interferon gamma (IFN- γ), and these cells appear to be a major source of IFN- γ during the acute stage of infection (Suzuki and Remington, 1988). However, both CD8+ and CD4+ T cells obtained from the spleen of infected mice are able to produce this cytokine *in vitro* following stimulation with tachyzoite antigens.

IFN-γ also plays a critical role in prevention of TE during the late stage of infection in mice (Suzuki et al., 1989a; Gazzinelli et al., 1992). Neutralization of the activity of IFN- γ in chronically infected mice by treatment with anti-IFN-y monoclonal antibody (mAb) resulted in severe acute inflammation and development of large areas of necrosis in their brains (Suzuki et al., 1989a). In the areas of acute inflammation and necrosis, tachyzoites and T. gondii antigens were detected, indicating that such inflammatory responses were caused by proliferation of tachyzoites. A marked increase in numbers of tachyzoites in brains of mice following treatment with anti-IFN-y mAb was also demonstrated by detecting increased amounts of tachyzoitespecific SAG1 and SAG2 mRNA using the reverse transcriptase-polymerase chain reaction (RT-PCR) (Gazzinelli *et al.*, 1993). Thus, it is clear that IFN- γ is critical for prevention of proliferation of tachyzoites in the brains of mice. The same appears to be true in humans, since AIDS patients have an impaired ability to produce IFN- γ and they frequently develop TE.

Both CD4+ and CD8+ T cells infiltrate the brain of mice following infection. Gazzinelli *et al.* (1992) reported that depletion of both CD4+ and CD8+ T cells is required to induce severe TE in chronically infected mice. Brown and McLeod (1990) reported that CD8+ T cells are involved in resistance by regulating the numbers of *T. gondii* cysts in the brains of mice. By using adoptive transfer of immune T cell populations into infected nude mice, Kang and Suzuki (2001) demonstrated that both CD4+ and CD8+ T cells are able to prevent TE in recipient animals. The protective activity of the T cells is through their production of IFN- γ (Wang *et al.*, 2004).

When T-cell receptor VB chain usage was examined in the T cells that produce IFN-y in the brains of infected BALB/c mice (genetically resistant to TE), the cells bearing T-cell receptor Vβ8 chain were found to be most abundant (Wang et al., 2005). Also in their spleens, Vβ8+ T cells produced markedly greater amounts of IFN- γ than did the Vβ8⁻ population after stimulation with tachyzoite antigens in vitro (Kang et al., 2003). Adoptive transfer of splenic immune V_{β8+} T cells into infected nude mice prevented TE in the recipients (Kang et al., 2003). Therefore, T cells bearing TCR Vβ8 appear to play an important role in genetic resistance of BALB/c mice against development of TE. When immune VB8+ T cells were divided into CD4+ and CD8+ T-cell populations, the CD8+ population conferred much greater resistance to development of TE in infected nude mice than did the CD4+ population. However, the protective activity of total Vβ8+ T cells was greater than that of CD8+ Vβ8+T cells (Wang et al., 2005). Therefore, the CD8+ population plays a major role in the activity of Vβ8+ immune T cells against reactivation of infection in the brain, although the CD4⁺ population works additively or synergistically with the CD8+ population.

Bcl-3 oncoprotein, a distinct member of the I-κB family which functions as a positive regulator of nuclear factor (NF)-kB activity, has recently been shown to play a critical role in mounting a protective Th1 immune response to T. gondii (Franzoso et al., 1997). Bcl-3-deficient mice survive the early acute stage of the infection; however, most of them die between 3 and 5 weeks after infection. The ability of spleen cells to produce IFN-y in response to T. gondii antigens is normal in the early stage (7 days post-infection) but impaired in the later stage (12-31 days post-infection) of infection. The cytotoxic activity of T cells, but not NK cells, is also defective in these mice. These results suggest a critical role for Bcl-3 in antigen-specific priming of the long-term, protective Th1-type T cells following infection with T. gondii.

With regard to NF- κ B family transcription factors, Caamano *et al.* (2000) demonstrated the

importance of NF-kB(2) for T-cell responses in resistance to TE. NF- κ B(2)-deficient mice have no defect in their ability to produce IL-12 and IFN-y during the acute stage of infection; however, during the chronic stage of infection these deficient mice succumbed to TE in association with a reduced capacity for IFN-y production. Apoptosis of T cells appears to be involved in the reduced production of this cytokine. c-Rel is also involved in resistance against T. gondii in the brain (Mason et al., 2004). c-Rel-deficient mice survive the acute phase of infection, but develop severe TE associated with decreased numbers of CD4+ T cells and reduced production of IFN-y in their brains. Therefore, c-Rel plays an important role in the optimization and maintenance of Th1-type immune responses during T. gondii infection (Mason et al., 2004).

Subauste *et al.* (1999) reported that CD40-CD40L signaling is required for optimal T-cell production of IFN- γ in response to *T. gondii* in humans. However, the role of CD40–CD40L interaction in the T-cell response appears to differ in mice. CD40L-deficient mice produce comparable levels of IFN- γ to control animals following infection, although CD40L is important for resistance to TE (Reichmann *et al.*, 2000).

In addition to production of IFN-y, both human and mouse CD4+ and CD8+ T cells are capable of killing T. gondii-infected target cells in vitro in a major histocompatibility complex (MHC)-restricted manner; however, the cytotoxic activity of T cells has not been shown to be the critical factor in the prevention of TE in mice. By using C57BL/6 (genetically susceptible to TE)-background mice, Denkers et al. (1997) reported that approximately half of the perforin-deficient animals survived until 150 days after infection whereas CD8+T-celldeficient mice all died by 50 days after infection (Denkers et al., 1997), suggesting that perforinmediated cytolysis by T cells appears to play a limited role in resistance against T. gondii. More recently, Wang et al. (2004) demonstrated, by using TE-resistant BALB/c mice, that adoptive transfer of immune T cells from perforin-deficient mice into infected nude mice prevented the reactivation of infection and TE in the recipient animals (Wang et al., 2004). Therefore, perforin-mediated

cytotoxic activity of T cells is not required for genetic resistance of BALB/c mice to development of the disease.

21.4 THE INVOLVEMENT OF OTHER CYTOKINES AND REGULATORY MOLECULES IN RESISTANCE

21.4.1 TNF-α

Murine peritoneal macrophages become activated after treatment with a combination of IFN-y and TNF- α in vitro, and the activated cells inhibit intracellular replication of tachyzoites through the generation of NO by inducible NO synthase (iNOS) (Adams et al., 1990). It was recently demonstrated that TNF- α and iNOS are not essential, however, for the control of acute infection in vivo, since mice lacking TNF receptor type 1 (R1) and type 2 (R2) and those lacking iNOS control parasite growth in their peritoneal cavity following intraperitoneal infection (Scharton-Kersten et al., 1997; Deckert-Schluter et al., 1998; Yap et al., 1998). Thus, the protective mechanism(s) which require IFN- γ but do not require TNF- α or iNOS are sufficient in mice to control parasite growth during the acute stage of the infection. Recently, IGTP and LRG-47, members of a new family of IFN-y-inducible genes, have been demonstrated to be required for the IFN-y-mediated resistance against acute infection with T. gondii (see section 21.6.2).

In contrast to the acute stage of the infection, mice deficient in TNF R1/R2 or iNOS succumbed to necrotizing TE during the late stage of the infection (Scharton-Kersten *et al.*, 1997; Deckert-Schluter *et al.*, 1998; Yap *et al.*, 1998). These results are consistent with those of earlier studies; treatments of infected wild-type mice with either anti-TNF- α mAb or aminoguanidine (an iNOS inhibitor) resulted in development of TE (Gazzinelli *et al.*, 1993; Hayashi *et al.*, 1996). Thus, TNF- α and iNOS are critical for prevention of proliferation of tachyzoites in the brain. As mentioned earlier, IFN- γ plays the central role in resistance of the brain against this parasite (Suzuki *et al.*, 1989a; Gazzinelli *et al.*, 1992). Since neutralization of IFN- γ or TNF- α results in decreased iNOS expression and development of severe TE (Gazzinelli *et al.*, 1993), activation of iNOS mediated by IFN- γ and TNF- α appears to play a key role in prevention of TE. Microglia and astrocytes are likely the effector cells involved in this protective mechanism (see section 21.7). More recently, Collazo *et al.* (2002) reported an involvement of IGTP in the IFN- γ -mediated protection against TE.

With regard to induction of iNOS in the brain, Yap et al. (1998) reported that iNOS induction in the brain was unimpaired in infected TNF R1/R2deficient mice which are susceptible to TE, suggesting that TNF-dependent immune control of T. gondii expansion in the brain involves an effector function distinct from iNOS activation. More recently, Deckert-Schluter et al. (1998) reported that mice lacking TNF R1 but not those lacking TNF R2 developed necrotizing encephalitis following infection, and that a remarkable reduction of iNOS synthesis was observed in the brains of TNF R1-deficient animals as compared with TNF R2-deficient or control animals. They concluded that signaling through TNF R1, but not TNF R2, provides the stimulus required for the induction of iNOS activation in the brain following infection (Deckert-Schluter et al., 1998). It therefore appears that there are two pathways to activate iNOS in the brain of T. gondii-infected mice: one TNF-dependent and the other TNF-independent. Since different strains of T. gondii were employed in the studies mentioned above (Deckert-Schluter et al., 1998; Yap et al., 1998), the strain of the parasite could also be an important factor affecting the activation pathway for iNOS.

With regard to the role of TNF- α and iNOS in prevention of TE, Suzuki *et al.* (2000a) reported that IFN- γ -deficient mice infected and treated with sulfadiazine developed severe TE after discontinuation of sulfadiazine treatment, but these animals expressed equivalent amounts of mRNA for TNF- α and iNOS in their brains when compared to control animals. These results indicate that expression of TNF- α and iNOS in the absence of IFN- γ is insufficient to prevent TE in genetically resistant BALB/c mice.

21.4.2 Lymphotoxin

Lymphotoxin (LT), in addition to TNF- α , is the ligand of TNF R1. Schluter *et al.* (2003) reported that mice deficient in LT failed to control intracerebral *T. gondii* and succumbed to necrotizing TE following infection. IFN- γ expression in their brains was equivalent to that of control mice when the deficient animals had developed TE. Experiments with bone-marrow chimera mice showed that hematopoietic cells need to express both LT and TNF- α to control *T. gondii* in the brain.

21.4.3 IL-4

CD4+T cells are known to be heterogeneous (Th1 and Th2) with regard to cytokine secretion. Th1 cells preferentially secrete IL-2 and IFN-y, whereas Th2 cells preferentially produce IL-4, IL-5, IL-6, and IL-10. IL-4 has been reported to have a dominant effect on determining the pattern of cytokines (Th2-type) produced by CD4+ T cells upon subsequent antigen stimulation in vitro. Since the role of IFN-γ is critical for prevention of development of TE, the role of IL-4 in the immunopathogenesis of toxoplasmosis has been addressed in mice. Surprisingly, IL-4-deficient (IL-4-/-) mice demonstrate an increase in mortality compared to control animals (Roberts et al., 1996; Suzuki et al., 1996a; Alexander et al., 1998). Therefore, IL-4 plays a protective role in resistance against T. gondii. It should be appreciated, however, that the timing of the mortality and development of TE in IL-4^{-/-} is controversial.

Suzuki *et al.* (1996a) reported that $IL-4^{-/-}$ mice all died during the late stage (from 6 to 20 weeks) of infection, whereas control mice all survived. The mortality of $IL-4^{-/-}$ mice was associated with greater numbers of cysts and areas of acute focal inflammation with tachyzoites in their brains (Suzuki *et al.*, 1996a). These results suggest that IL-4 protects against the development of TE by preventing cyst formation and the proliferation of tachyzoites in the brain. In these studies, at 8 weeks after infection, spleen cells of control mice produced significantly greater amounts of IFN-y following stimulation in vitro with soluble T. gondii antigens than did those of IL-4^{-/-}mice (Suzuki et al., 1996a). Therefore, IL-4 appears to play a role by enhancing IFN- γ production during the late stage of infection, and the impaired ability of IL-4^{-/-} mice to produce IFN-γ likely contributes to their susceptibility for development of severe TE (Suzuki et al., 1996a). In relation to these findings, it was reported that IL-4 enhances IFN-y production by T cells which have already been primed (differentiated), whereas it suppresses differentiation of unprimed T cells to IFN-y-producing cells (Noble and Kemeny, 1995). During infection with T. gondii, IFN-y production occurs earlier than IL-4 production (Beaman et al., 1993). Thus, it may be that IL-4 does not affect the differentiation of unprimed T cells to IFN-γ-producing cells following T gondii infection because of the absence (or very low production) of IL-4 at the early stages of infection, whereas it enhances IFN-y production by differentiated T cells at the late stages of infection.

In contrast, Roberts et al. (1996) reported that an increased mortality occurs in IL-4^{-/-} mice during the acute stage of infection. However, in the late stage of infection, greater numbers of cysts and more severe pathology in the brain were observed in the control than in IL-4-/- mice. During the acute stage of infection, which was the time that IL-4^{-/-} mice died, increased IFN-y production was observed in spleen cells of IL-4-/- mice when compared to those of control animals. Therefore, the authors suggested that IL-4 plays a protective role in preventing mortality by downregulating proinflammatory cytokine production during the acute stage of infection. Reasons for the different outcomes in the studies described are unclear. However, since genetic backgrounds of mice and the strain of T. gondii differ between these two studies, these variables may have contributed to the different outcomes. In support of this possibility, the genetic background of mice has been demonstrated to affect the outcome of T. gondii infection in IL-4^{-/-} mice (Alexander *et al.*, 1998).

In addition to the regulatory effects of IL-4 on IFN- γ production, IL-4 has recently been shown to have an activity which modifies the intracellular replication of tachyzoites in murine macrophages (Swierczynski *et al.*, 2000) and human fibroblast cell lines (Chaves *et al.*, 2001). More studies are needed to elucidate the role of IL-4 in the immunopathogenesis of toxoplasmosis.

21.4.4 IL-5

IL-5 enhances expression of IL-2 receptors on B cells and promotes B-cell proliferation and differentiation (Swain et al., 1988; Takatsu et al., 1988). IL-5-deficient (IL-5-/-) mice had an increased mortality during the late stage of infection, and their mortality was associated with greater numbers of cysts and tachyzoites in their brains when compared to infected control mice (Zhang and Denkers, 1999). IL-12 production by spleen cells from infected mice in response to tachyzoite antigens in vitro was markedly lower in IL-5^{-/-} than in control animals, and this decrease correlated with a selective loss of B cells during the culture. Therefore, IL-5 plays a protective role against T. gondii and its protective role is related to the production of IL-12.

21.4.5 IL-6

IL-6 is a multifunctional cytokine that regulates various aspects of the immune response, acutephase reaction, and hematopoiesis (Akira et al., 1993), and acts in the nervous system (Hirota et al., 1996). IL-6 mRNA is expressed in brains of mice infected with T. gondii (Gazzinelli et al., 1992; Hunter et al., 1992; Deckert-Schluter et al., 1995), and is detected in the CSF of those mice (Schluter et al., 1993). IL-6-deficient (IL-6-/-) mice formed significantly greater numbers of T. gondii cysts and areas of inflammation associated with tachyzoites in their brains than did control mice (Suzuki et al., 1997; Jebbari et al., 1998). These results indicate that IL-6 is protective against development of TE by preventing formation of cysts and proliferation of tachyzoites in brains of infected mice. In brains of infected IL-6-/- mice, the amounts of mRNA for IFN- γ detected by RT-PCR were found to be significantly less when compared to control mice, whereas the amounts of IL-10 mRNA were greater than in control animals (Suzuki et al., 1997). In addition, lymphocyte preparations isolated from brains of infected IL-6^{-/-} mice had significantly lower ratios of $\gamma\delta$ T cells and CD4+ $\alpha\beta$ T cells, but higher ratios of CD8+ $\alpha\beta$ T cells than those of infected control mice (Suzuki et al., 1997). Of interest, no differences were detected in the ratios of these T-cell subsets in spleens between these animals (Suzuki *et al.*, 1997). In another study, serum IFN- γ levels were significantly lower in control than IL-6^{-/-} mice during the early stage of infection (Jebbari et al., 1998). Therefore, the protective activity of IL-6 against the development of TE appears to be due to its ability to stimulate IFN-y production (systemic in the early stage of infection and in the brain in the later stage) and to induce infiltration and accumulation of different T-cell subsets in the brains of infected mice. In relation to the protective role of IL-6 against TE, it was reported that human fetal microglia treated with IL-6 inhibits intracellular replication of tachyzoites in vitro (Chao et al., 1994), and that IL-6 acts synergistically with IFN- γ to inhibit proliferation of tachyzoites in murine astrocytes (Halonen et al., 1998).

21.4.6 IL-10

IL-10 is an important negative regulator of inflammatory responses (Kuhn *et al.*, 1993). IL-10-deficient (IL-10^{-/-}) mice die during the acute stage of infection with *T. gondii*, and their mortality is associated with development of severe immunopathology mediated by Th1 immune responses in the liver (Gazzinelli *et al.*, 1996) and intestine (Suzuki *et al.*, 2000b). Therefore, IL-10 is crucial for downregulating IFN- γ -mediated immune responses and preventing development of pathology caused by the immune responses. When IL-10^{-/-} mice were treated with sulfadiazine to control proliferation of *T. gondii* in the early stage of infection, the animals survived the acute stage but developed lethal inflammatory responses in their brains in

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the later stages of infection (Wilson *et al.*, 2005). Therefore, IL-10 plays an important downregulatory role to prevent immunopathology during the course of infection with *T. gondii*.

21.4.7 Lipoxin (LX) A4

LXA4 is an eicosanoid product generated from arachidonic acid. Following infection with T. gondii, wild-type mice produced high levels of serum LXA4, beginning at the onset of chronic infection. 5-lipoxygenase (5-LO) is an enzyme critical in the generation of LXA4, and mice deficient in 5-LO (5-LO^{-/-}) succumbed to infection during the chronic stage, displaying marked inflammation in their brains (Aliberti et al., 2002). However, numbers of cysts in the brain were significantly lower in 5-LO^{-/-} than in control mice, indicating that the mortality in 5-LO-/- mice is not due to defective control of the parasite, but due to enhanced inflammatory responses induced by the parasite. The increased mortality in the 5-LO-/animals was associated with elevations of IL-12 and IFN- γ , and was completely prevented by the administration of a stable LXA4 analog. Therefore, LXA4 is important for downregulation of proinflammatory responses during the chronic stage of T. gondii infection.

21.5 INVOLVEMENT OF HUMORAL IMMUNITY IN RESISTANCE

Antibodies are involved in resistance against *T. gondii*, although cell-mediated immunity plays the major role. Frenkel and Taylor (1982) examined the effect of depletion of B cells by treatment with anti- μ antibody on toxoplasmosis in mice infected with a virulent strain and treated with sulfadiazine. They observed mortality associated with pneumonia, myocarditis, and/or encephalitis in infected anti- μ -treated mice after discontinuation of sulfadiazine treatment. Administration of antisera to *T. gondii* reduces mortality in these animals. These results suggest that antibody

production by B cells may be important for controlling the latent persistent infection. However, these studies did not provide conclusive information because of the potential side effects of anti-µ antibody treatment on the immune system.

More recently, Kang et al. (2000) reported the role of B cells in resistance to T. gondii by using B-cell-deficient (µMT) mice generated by disruption of one of the membrane exons of the µ-chain gene. B-cell-deficient mice died between 3 and 4 weeks after infection, whereas no mortality was observed in the control mice until 8 weeks. At the stage during which µMT animals succumbed to the infection, large numbers of tachyzoites were detected only in their brains. Treatment of infected µMT mice with anti-T. gondii IgG antibody resulted in reduced mortality and a prolonged time to death. These results indicate that B cells play an important role, through production of specific antibodies, in prevention of TE in mice. In regard to the protective role of antibodies, Johnson and Sayles (2002) reported that treatment of CD4-deficient mice with anti-T. gondii sera prolonged their survival during the chronic stage of infection.

21.6 IFN-γ-INDUCED EFFECTOR MECHANISMS

Several mechanisms of IFN-y-induced anti-Toxoplasma activity in various host cells have been described. These anti-Toxoplasma effector mechanisms include nitric oxide (NO) production, tryptophan starvation, generation of reactive oxygen species, iron deprivation, and induction of the recently described p47 GTPases, including IGTP, IRG-47, and LRG-47. The actions of IFN-γ are initiated by the binding of IFN-y with its IFN-y receptor (IFN- γR) at the cell surface and the initiation of a signaling cascade, involving the JAK family of tyrosine kinases and STAT family of transcription factors. Upon IFN-y stimulation, STAT1 dimerizes and translocates to the nucleus, where it binds gammaactivated sequence elements in the promoter regions of IFN- γ -inducible genes. The actions of IFN- γ are mediated by the proteins encoded by these

IFN- γ inducible genes. IFN- γ -inducible genes, which are of particular importance for resistance to T. gondii, include genes involved in the generation of NO, tryptophan degradation, and generation of reactive oxygen intermediates, and genes encoding for the p47 GTPases, IGTP, LRG-47, and IRG-47, as mentioned above. Recent evidence found that STAT1 null mice (Stat1-/-) could still produce IFN- γ and develop some Th1 responses, but STAT1 null mice failed to upregulate many of the antimicrobial effector functions, such as production of nitric oxide synthetase, induction of some of the p47 GTPases, and the ability to control parasite replication (Gavrilescu et al., 2004; Lieberman et al., 2004). These results indicate that the major role of STAT1 is for the development of antimicrobial effector functions, and also highlight the importance of these IFN-y-induced antimicrobial mechanisms in the protection against T. gondii.

Studies indicate that the expression of these antimicrobial mechanisms vary among the tissues, though several of these antimicrobial effector mechanisms have been shown to be important for controlling *T. gondii* in the brain. Of particular importance for *T. gondii* in the brain are genes encoding for NO synthesis and IDO-induced tryptophan starvation, and the genes encoding for the p47 GTPases. A brief summary of each of these anti-*Toxoplasma* effector mechanisms and the mechanism of action of each is included below.

21.6.1 NO production

NO is one of the main IFN- γ -induced antitoxoplasmocidal mechanisms known to mediate resistance to *T. gondii* in mice (Gazzinelli *et al.*, 1993). IFN- γ induces synthesis of the enzymeinducible nitric oxide synthetase (iNOS), which produces NO from L-arginine (Adams *et al.*, 1993). The L-arginine-dependent production of NO, and subsequent conversion to nitrate and nitrites, is thought to have direct antimicrobiocidal activity and result in parasite killing. IFN- γ -induced NO production is a major anti-*Toxoplasma* effector mechanism operating in macrophages and microglia, (see section 21.7); however, mice treated with iNOS inhibitor or mice deficient in iNOS are relatively more resistant than mice deficient in IFN- γ , indicating that other effector mechanisms induced by IFN- γ are also important (Hayashi *et al.*, 1996; Scharton-Kersten *et al.*, 1997). Mice deficient in NO are, however, more susceptible to TE, indicating that NO is important in the latent stage of the infection. NO has also been found to induce bradyzoite differentiation, and thus NO may also have a role in maintaining the parasite in the chronic infection (Bohne *et al.*, 1994).

21.6.2 IGTP

The p47 GTPases are a family of proteins, 47-48 kDa in size, that are strongly induced by IFN- γ (Taylor et al., 1996, 2004). There are six members of the p47 GTPase family: IGTP, IRG-47, LRG-47, TGTP, IIGP, and GTPI. Three of these p47 GTPases, IGTP, IRG-47, and LRG-47, have been shown to be important in resistance to T. gondii. IGTP- and LRG-47-deficient mice both displayed a profound loss of resistance to acute infection with T. gondii, while IRG-47 deficient mice displayed only partial loss of resistance, which was not manifested until the chronic phase (Collazo et al., 2001). Additionally, IGTP and LRG-47 were found to be necessary for IFN-y-induced inhibition of T. gondii in macrophages, while IRG-47 was found not to be necessary (Butcher et al., 2005). Thus, these three p47 GTPases appear to have vital but distinct roles in immune defense against T. gondii. The function of IGTP, LRG-47, and IRG-47 is not known, but IGTP localizes to the endoplasmic reticulum, suggesting that IGTP and possibly other p47 GTPases may be involved in protein processing or trafficking (Taylor et al., 1997). In infection with Mycoplasma tuberculosis, LRG-47, and was found to influence the acidification of the phagosome, lending support to the idea that the p47 GTPases affect endocytic vesicular trafficking within host cells (MacMicking et al., 2003). Given that T. gondii resides in a unique intracellular compartment that is normally nonfusogenic with host-cell vesicular compartments, this is a very interesting idea that deserves further study. IFN-y-induced IGTP gene expression is dependent upon STAT1, and resistance to T. gondii was observed only when IGTP was present in both hematopoietic and non-hematopoietic compartments (Collazo et al., 2002). This suggests that IGTP is necessary in macrophages and microglia, as well as the non-professional effector cells like enterocytes, endothelial cells, and astrocytes. In contrast to the essential role of IGTP in resistance against acute acquired infection with T. gondii, this GTPase has been reported to play only a partial role in controlling the parasite during the chronic stage of infection in mice (Collazo et al., 2002). In relation to the involvement of IGTP in resistance against chronic infection, IFN-y-activated astrocytes have been demonstrated to inhibit intracellular replication of tachyzoites in vitro by a mechanism dependent on IGTP (Halonen et al., 2001).

21.6.3 Tryptophan starvation/ indoleamine-2,3-dioxygenase (IDO) pathway

IFN-\gamma-induced inhibition of T. gondii via tryptophan starvation was first described in fibroblasts (Pfefferkorn, 1984). Human fetal fibroblasts stimulated with IFN-y were found to significantly inhibit the growth of T. gondii. Treatment of the host cells for 24 hours prior to infection was required for inhibition of parasite growth. The mechanism of inhibition was found to be due to the induction of IDO, which results in the degradation of tryptophan to kynurenin (Pfefferkorn and Guyre, 1984; Pfefferkorn et al., 1986). Inhibition of parasite growth is due to tryptophan starvation, as tryptophan is apparently an essential amino acid for their growth, and as an addition of excess amounts of tryptophan in the culture antagonizes the toxoplasmostatic effect induced by IFN-y. This IFN-yinduced, IDO-mediated tryptophan starvation pathway has been described to inhibit growth of Toxoplasma in a variety of other host cell types, including epithelial cells, endothelial cells, tumor cell lines, and a variety of host cell species including human, rat, and mouse (Dimier et al., 1992; Nagineni et al., 1996; Daubener and MacKenzie, 1999). In vitro studies also found that IFN-y induction of the IDO pathway in epithelial and endothelial cells inhibits the growth of T. gondii, suggesting that this pathway is of importance in limiting parasite growth in the intestinal phase of the infection and passage to the fetus in congenital toxoplasmosis, respectively. The IFN- γ -induced IDO pathway has also been found to exert anti-*Toxoplasma* activity in some of the resident CNS cells, such as pigment epithelial cells and microvascular endothelial cells, and thus the IDO pathway may also be of importance in controlling the replication of the parasite in the CNS. The importance of IDO in resistance against *T. gondii in vivo* is supported by evidence of an induction of IDO expression and decreased levels of tryptophan in the lungs and brains of infected mice (Gazzinelli *et al.*, 1993; Silva *et al.*, 2002).

21.6.4 Reactive oxygen intermediates (ROI)

IFN-y-activated production of reactive oxygen intermediates (ROI) has been demonstrated to induce anti-toxoplasmocidal activity in human macrophages (Murray et al., 1985). The reactive oxygen intermediates generated include superoxide ion (O_2^{-}) and hydrogen peroxide (H_2O_2) . Reactive oxygen scavengers, or inhibition of these enzymes via superoxide dismutase, catalase, and mannitol, inhibit the anti-toxoplasmocidal effect in macrophages. ROI intermediates have also been found to mediate IFN-γ inhibition of T. gondii in dendritic cells but not in microglia (Jun et al., 1993; Aline et al., 2002). The physiological significance of the ROI pathway still remains unclear, especially in mice. It has been reported that the parasites are resistant to the oxygen metabolites produced in murine macrophages (Chang and Pechere, 1989), and p47phox-deficient mice (which lack an inducible oxidative burst) can control both the acute and chronic stages of T. gondii infection (Scharton-Kersten et al., 1997).

21.6.5 Iron deprivation

IFN- γ has been shown to inhibit growth of *T. gondii* via iron deprivation in a few cell types such as enterocytes (Dimier and Bout, 1998; Bout *et al.*, 1999). IFN- γ -induced iron deprivation was demonstrated

by experiments in which adding excess iron via a Fe²⁺ salt was able to overcome the IFN-γ-induced inhibition, and treating host cells with the iron chelator desferrioxamine (DFO) was able to induce inhibition (Dimier and Bout, 1997). Cells usually acquire iron via the transferrin receptor pathway, in which iron bound to transferrin binds to the transferrin receptor at the cell surface and the receptor-transferrin complex is internalized via endocytosis, with subsequent acidification of the endocytic vesicle causing the ferric ions to dissociate from transferrin. The iron ions are transported across the vesicle membrane into the cytoplasm and can then enter the iron pool of the cell. This intracellular pool of iron is available for metabolic processes and is available for chelation by DFO. T. gondii replicates within the parasitophorous vacuole, a membranous compartment within the host cell that does not fuse with the lysosomes and is not contiguous with irontransferrin as it passes through the endocytic pathway. Thus, rather than acquiring iron from transferrin, experiments with enterocytes indicate that IFN-γ can inhibit T. gondii replication by limiting the availability of intracellular iron for the parasite to scavenge from its host cell. The mechanism by which this occurs is not understood. Inhibition of *T. gondii* growth via IFN-γ-induced iron deprivation has only been shown for enterocytes, and may be an important anti-toxoplasmic mechanism on mucosal surfaces as a first line of defense against pathogen invasion. It is not clear, however, whether this IFN-y-induced irondependent mechanism also occurs in other cell types and thus has a broader role in the control of Toxoplasma in the latent stage of infection and congenital toxoplasmosis.

21.7 EFFECTOR CELLS IN THE BRAIN WITH ACTIVITY AGAINST T. GONDII

The establishment of a chronic asymptomatic *T. gondii* infection requires the cytokine IFN- γ , and resistance to *T. gondii* has been found to be

dependent upon IFN- γ -responsive cells in both the hematopoietic compartments and nonhematopoietic compartments (Yap and Sher, 1999). Multiple types of cells in the brains have been shown to respond to IFN- γ and inhibit intracellular replication of tachyzoites.

21.7.1 Microglia

Microglia are the resident macrophage population in the CNS, and are probably the major effector cells in the prevention of T. gondii tachyzoite proliferation in the brain. Microglia are activated to inhibit growth of *T. gondii in vitro* with IFN-γ plus lipopolysaccharide (Chao et al., 1993a, 1993b, 1994). TNF- α and IL-6 are also involved in the inhibition of T. gondii in human microglia (Chao et al., 1994). NO was shown to mediate the inhibitory effect of activated murine microglia on intracellular replication of tachyzoites, since treatment of these cells with NG-monomethyl-L-arginine (which blocks the generation of NO) ablates their inhibitory activity (Chao et al., 1993a). Recently, Freund et al. (2001) reported an involvement of both NO-dependent and -independent mechanisms in the resistance of murine microglia activated by a combination of IFN- γ and TNF- α . In contrast to these observations in murine microglia, it was reported that NO is not involved in the inhibitory effect of activated human microglia against T. gondii (Chao et al., 1994). In vivo, following T. gondii infection, microglia become activated to produce TNF- α , and IFN- γ mediates the activation (Deckert-Schluter et al., 1999). IFN-γ-mediated activation of microglia in collaboration with autocrine TNF- α is likely one of the resistance mechanisms of the brain against T. gondii.

Activated microglia produce IL-1 β , IL-12, and IL-15, and express MHC class I and II, LFA-1, and ICAM-1 (Schluter *et al.*, 2001). Therefore, in addition to their functions as effector cells, IFN- γ -activated microglia may act as antigen-presenting cells through MHC class I and II molecules on their surfaces, and function as regulatory cells on the immune responses through their IL-1 β , IL-12, and IL-15 production.

21.7.2 Astrocytes

In vitro studies have found that IFN-y-activated astrocytes can inhibit the growth of T. gondii. In human astrocytes, IFN-y plus IL-1ß stimulated astrocytes to inhibit the growth of T. gondii via an NO-mediated mechanism (Peterson et al., 1995). TNF- α and IFN- γ have been shown to inhibit growth of T. gondii in human glioblastomaderived cells and native astrocytes via induction of indoleamine 2,3-dioxygenase (Daubener et al., 1993). Co-activation of indoleamine 2,3-dioxygenase (IDO) activity and NO in human astrocytes resulted in an inhibition of IDO activity, indicating a cross-regulation between these two pathways in astrocytes (Oberdorfer et al., 2003). Murine astrocytes have also been shown to inhibit the growth of T. gondii in vitro (Halonen et al., 1998). In murine astrocytes IFN-y alone induced a significant inhibition in growth of T. gondii, while IFN-γ in combination with TNF- α , IL-1, or IL-6 acted synergistically to enhance this inhibition. None of TNF- α , IL-1, or IL-6 alone had any effect on growth of T. gondii in murine astrocytes. The inhibitory effect of IFN-y-activated astrocytes was found not to be mediated via NO or IDO (Halonen and Weiss, 2000), as was the case for human astrocytes or astrocytoma cells. The mechanism of IFN-yinduced inhibition in murine astrocytes was also found to be independent of reactive oxygen intermediates and iron deprivation. The mechanism of IFN-y-induced inhibition in murine astrocytes was, however, found to involve the IFN-\gamma-induced p47 GTPase, IGTP, as the IFN-γ inhibition was reversed in IGTP-/- astrocytes (Halonen et al., 2001). As discussed previously, this mechanism of IFN-y-induced-IGTP-dependence is not understood at this time, but since IGTP localizes to the endoplasmic reticulum it has been hypothesized that it may be involved in trafficking of molecules into the parasitophorous vacuole. Considering the fact that astrocytes are the dominant glial cells in the brain, the IGTP-mediated microbiostatic effects of astrocytes could have a significant effect on limiting the growth of the parasite in the brain and allowing the more potent antimicrobiocidal

activity of microglia to clear the parasites from the brain.

21.7.3 Endothelial cells

IFN-γ-stimulated human brain microvascular endothelial cells have been shown to induce inhibition of *T. gondii* in a dose-dependent manner (Daubener *et al.*, 2001). This antiparasitic effect was enhanced by TNF- α , although TNF- α alone had no effect on parasite growth. The effector mechanism in these cells has been reported to be mediated via tryptophan starvation due to the induction of IDO. Since one of the first steps in the development of cerebral toxoplasmosis is penetration of the blood–brain barrier, IFN- γ induced inhibition in these cells may prove to be important in limiting the replication of *T. gondii* in the brain.

21.7.4 Dendritic cells

T. gondii infection results in the development of mature dendritic cells, expressing MHC Class II, CD40, CD54, CD80, and CD86 in the brain of mice. Dendritic cells have been shown to inhibit intracellular replication of tachyzoites via reactive oxygen-mediated mechanisms in vitro when activated by IFN-y (Jun et al., 1993; Aline et al., 2002); therefore, these cells may function as effector cells to control the parasite in the brain. However, dendritic cells probably play a more important role as antigen-presenting cells and IL-12 producers. Since both microglia and astrocytes need IFN-y to upregulate their expression of MHC molecules on their surfaces, and since GM-CSF (a cytokine that astrocytes produce in response to T. gondii; Fischer and Bielinsky, 1999) induces dendritic cell maturation in primary brain-cell cultures with the parasite, dendritic cells may play a crucial role as antigen-presenting cells in the brain following infection. On the other hand, dendritic cells may also play a pathogenic role in the development of TE, because a recent study found that dendritic cells are responsible for transporting single tachyzoites into the brain (Courret et al., 2006).

21.8 THE ROLE OF CELLS HARBORING *T. GONDII* IN THE BRAIN

T. gondii persists for the lifetime of the host within cysts located predominantly in the brain. Tissue cysts are located intracellularly, encased by a thick cyst wall, and contain hundreds to thousands of bradyzoites. The host cell for the cyst stage in the brain has been addressed in a variety of in vivo and in vitro studies. Ultrastructural studies from the murine model of toxoplasmosis indicated that neurons were the major host cell for cysts in the brain (Frenkel and Escajadillo, 1987; Ferguson and Hutchinson 1987a, 1987b; Sims et al., 1988, 1989a, 1989b). The development of bradyzoites, however, has been described in other neuronal cells in vitro, including astrocytes and microglia (Jones et al., 1986; Fischer et al., 1997; Halonen et al., 1998). Studies conducted with organotypic brain-slice cultures, which preserve the three-dimensional architecture of intact brain tissue and thus may serve as a better model system, found that intracerebral replication and spontaneous conversion from the tachyzoite to bradyzoite form is supported by both neurons and astrocytes. Microglia cells, while capable of being infected, appear to function predominantly to inhibit parasite growth in the CNS (Luder et al., 1999). Collectively, these studies indicate that both neurons and astrocytes are host cells for the cyst stage in the brain. However, the predominant hostcell type in vivo, or the quantitative contribution of the different cell types of the CNS in the intracerebral development of T. gondii and the differentiation to persisting bradyzoites, still remains unclear.

Cysts are maintained in the brain for the lifetime of the host, but studies in mice indicate that cysts periodically rupture and re-encyst (Ferguson *et al.*, 1989). Thus, although the cysts persist for years without harming the host, the lifespan of an individual bradyzoite or cyst may be shorter. The requirement for the immune system to maintain the latency of chronic infection is evident from reactivation of infection in immunocompromised patients, such as those with AIDS (Israelski and Remington, 1993; Wong and Remington, 1994). Tissue cysts are the most likely source of reactivation toxoplasmosis in immunocompromised patients. In reactivated infection (i.e. TE) bradyzoite-to-tachyzoite conversion is thought to occur, and the resulting uncontrolled proliferation of the tachyzoites in the brain results in pathology. While the cysts appear to be located primarily in neurons and astrocytes, the tachyzoites may replicate in astrocytes, microglia, and possibly neurons. The mechanisms and dynamics of tachyzoite-tobradyzoite and bradyzoite-to-tachzyoite conversion in the brain are not fully understood, but are areas that are clearly important in understanding the persistence of *T. gondii* in the brain and for devising treatments for cerebral toxoplasmosis.

21.8.1 Growth of *T. gondii* in neuronal cells

A few studies on the growth of T. gondii in neurons in vitro have been performed using either singlecell cultures or organotypic cultures (Halonen et al., 1996; Fagard et al., 1999; Luder et al., 1999; Schluter et al., 2001). In all of these studies, the growth of the parasite in neurons was slower than that in astrocytes or other host cells such as fibroblasts. For example, vacuoles of parasites in neurons contained only 4-8 parasites 48 hours after invasion, as compared to astrocytes, which contained 16-32 parasites (Halonen et al., 1996). Interestingly, neither IFN-y- nor TNF-a-stimulated neurons were found to inhibit the invasion or growth of T. gondii (Schluter et al., 2001), although these cytokines inhibit proliferation of the parasite in microglia and astrocytes. Therefore, IFN-y and TNF- α play a role in controlling *T. gondii* in microglia and astrocytes, but are not sufficient to control replication of the parasite in neurons. However, the replication of the parasite in neurons is relatively slow, and this in itself is indicative of some intrinsic ability of neurons to limit parasite growth. It is not clear whether neurons spontaneously support tachyzoite-tobradyzoite conversion or whether IFN-y can induce or enhance this conversion. Given the biology of the latent/chronic infection, and the presumption that neurons are the dominant host

cell for the cyst stage of the parasite, this is an interesting and important question that remains to be answered.

Additionally, infected neurons secrete CC chemokines MIP-1 α and MIP-1 β , which attract inflammatory leukocytes, with MIP-1ß being strongly upregulated by the combination of IFN- γ and TNF-a. (Schluter et al., 2001). These results indicate that T. gondii-infected neurons may contribute to the recruitment of inflammatory cells to the site of infection. Intracellular parasites are commonly surrounded by an infiltrate of T cells and granulocytes while the bradyzoite-containing cysts are usually devoid of inflammatory cells, indicating that the neuronal production of chemokines may be dependent upon the stage of the parasite in neurons. This is an interesting area, and of importance to the understanding of both cerebral and neurological manifestations of congenital toxoplasmosis and TE.

21.8.2 Role of astrocytes in cerebral toxoplasmosis

Both in vivo and in vitro studies have found that astrocytes support the cyst stage. For example, cysts in astrocytes have been reported in human brain (Ghatak and Zimmerman, 1973; Powell et al., 1978), and T. gondii parasitophorous vacuoles in vitro in astrocytes exhibit bradyzoite characteristics and a cyst wall similar to cysts observed in vivo (Jones et al., 1986; Halonen et al., 1998). Cysts in astrocytes have been found to be surrounded by layers of glial filaments in both in vitro and in vivo studies (Ghatak and Zimmerman, 1973; Powell et al., 1978; Halonen et al., 1998; Luder et al., 1999). This type of cytoskeletal alteration might simply be cytoskeleton that is displaced from its original cytoplasmic localization toward the periphery of the growing cyst as it develops in the cytoplasm. Alternatively, it could be an important factor in mediating physical stability of the cysts (Halonen et al., 1998; Luder et al., 1999). A recent study with glial fibrillary acidic protein (GFAP) deficient mice (GFAP-/-), which found these mice had an increased intracerebral load of T. gondii and areas of tachyzoite-induced tissue necrosis (Stenzel et al., 2004),

supports the idea that the GFAP layering of cysts may aid in stabilizing cysts, perhaps preventing cyst rupture. The layers of glial filaments were observed to displace the host-cell mitochondria from the surface of the parasitophorous vacuole, and thus the glial filament wrapping has also been suggested to play a role in establishing the anaerobic environment, helping to facilitate tachyzoite-to-bradyzoite formation (Halonen *et al.*, 1998).

The cytokine IFN-y, alone or in combination with TNF- α , has been found to induce antitoxoplasmostatic activity in astrocytes. The effect of cytokines on bradyzoite induction and/or conversion has also been investigated in a few studies. IFN- γ -treated astrocytes, for example, were able to support cyst development, and astrocytes were able to maintain intact intracellular cysts for up to 3 months in culture (Jones et al., 1986). In organotypic cultures, however, IFN-γ severely inhibited the growth of T. gondii in astrocytes, but this inhibition was not linked to tachyzoite-to-bradyzoite conversion (Luder et al., 1999). Results of these studies indicate that astrocytes may function as immune effector cells as well as functioning as host cells supporting prolific development of the tachyzoite stage and bradyzoite/ cyst development. It is unclear whether IFN-ystimulates or supresses the induction of bradyzoite/cyst formation in astrocytes. Nonetheless, collectively these studies indicate that astrocytes may play a crucial role in controlling the replication and mediating persistence of the parasite in the brain.

Finally, in addition to having roles as effector cells controlling parasite replication and as host cells to the cysts, astrocytes have other immune functions that are possibly involved in toxoplasmic encephalitis (Wilson and Hunter, 2004). For example, upon infection with *Toxoplasma* there is a general inflammatory response in the brain, characterized by cytokine production and astrocyte activation. Activated astrocytes produce chemokines, such as IP-10, that may play a role in the regulation of T-cell trafficking. Additionally, *Toxoplasma*-infected astrocytes produce the proinflammatory cytokines IL-1, IL-6, and TNF- α , which participate in host resistance against

development of TE. Moreover, IFN- γ -activated astrocytes express MHC class II and co-stimulatory molecules on their surfaces, suggesting that they may also function as antigen-presenting cells.

21.8.3 Microglial and astrocytemicroglial-neuronal interactions

Of the three main resident brain cells, microglia appear to function primarily to limit growth of T. gondii. IFN-γ-activated murine microglia produce NO, which is responsible for microbiostatic and microbiocidal control of parasite growth. Recent studies indicate that a more complex interaction among microglia, astrocytes, and neurons occurs in the control of T. gondii in the brain. For example, while Th1 cytokines are necessary for microglia activation and responsible for protection against T. gondii in the brain, products from activated microglia may also cause tissue damage and be detrimental to neuron functions. Nitric oxide (NO), one of the mediators against T. gondii produced by microglia, is also one of the most noxious compounds for CNS cells. Despite this, in chronic infections with T. gondii in which the parasite persists in the brain and NO is present, neuronal and tissue damage does not usually occur. Thus, immunomodulatory mechanisms are thought to be involved in the prevention of neuronal degeneration and pathological alterations in the CNS during the latent phase of the infection. IL-10 and LPXA4 have been shown to be important downregulators of IFN-y-mediated immune responses.

21.9 HOST GENES INVOLVED IN REGULATING RESISTANCE

Resistance against *T. gondii* is under genetic control in both acute and chronic stages of infection. Of interest, genes involved in resistance differ between these two stages. Susceptibility of inbred strains of mice to acute infection does not correlate with that to chronic infection (Suzuki *et al.*, 1993). A minimum of five genes are involved in determining survival of mice during the acute stage (McLeod *et al.*, 1989), and one of these genes

is linked to the major histocompatibility complex (H-2) (McLeod *et al.*, 1989).

During the chronic stage of infection, development of TE in mice is regulated by the gene(s) within the D region of the major histocompatibility complex (H-2) (Suzuki et al., 1991, 1994; Brown et al., 1995). Mice with the d haplotype in the D region are resistant to development of TE, and those with the b or k haplotypes are susceptible. Freund et al. (1992) found that polymorphisms in the *TNF*- α gene located in the D region of the H-2 complex correlate with resistance against development of TE and with levels of TNF- α mRNA in brains of infected mice. However, more recent studies using deletion mutant mice (Suzuki et al., 1994) and transgenic mice (Brown et al., 1995) demonstrated that the L^d gene in the D region of the H-2 complex, but not the *TNF*- α gene, is important for resistance against development of TE. Resistance of mice to development of TE is observed in association with resistance to formation of T. gondii cysts in the brain (Suzuki et al., 1991, 1994; Brown et al., 1995). McLeod et al. (1989) reported that although the L^d gene has the primary effect on numbers in the brain, the Bcg locus on chromosome 1 may also affect it.

In humans, HLA-DQ3 was found to be significantly more frequent in white North-American AIDS patients with TE than in the general white population or randomly selected control AIDS patients who had not developed TE (Suzuki et al., 1996b). In contrast, the frequency of HLA-DQ1 was lower in TE patients than in healthy controls (Suzuki et al., 1996b). Thus, HLA-DQ3 appears to be a genetic marker of susceptibility to development of TE in AIDS patients, and DQ1 appears to be a resistance marker. HLA-DQ3 also appears to be a genetic marker of susceptibility to development of cerebral toxoplasmosis in congenitally infected infants, since higher frequency of DQ3 was observed in infected infants with hydrocephalus than in infected infants without hydrocephalus, or in normal controls (Mack et al., 1999). The role of the HLA-DQ3 and -DQ1 genes in regulation of the susceptibility/resistance of the brain to T. gondii infection is supported by the results from a transgenic mouse study (Mack et al., 1999). Expression of the HLA-DQ1 transgene conferred

greater protection against parasite burden and necrosis in brains in mice than did the *HLA-DQ3* transgene (Mack *et al.*, 1999). Expression of the *HLA-B27* and *-Cw3* transgenes had no effects on the parasite burden (Brown *et al.*, 1994). Since the L^d gene in mice and the *HLA-DQ* genes in humans are a part of the MHC which regulates the immune responses, the regulation of the responses by these genes appears to be important to determine the resistance/susceptibility of the hosts to development of TE.

21.10 GENETIC FACTORS OF T. GONDII DETERMINING DEVELOPMENT OF TE AND VIRULENCE

The strain of T. gondii is an important determining factor in the development of TE in murine models (Suzuki et al., 1989b; Suzuki and Joh, 1994). Following infection of mice with either the ME49, Beverley, or C56 strain of the parasite, the ME49 strain formed significantly greater numbers of T. gondii cysts in their brains than did the other strains, with no difference in the numbers of cysts between the Beverley and C56 strains (Suzuki and Joh, 1994). Following treatment with anti-IFN-y, mice infected with the ME49 strain developed significantly greater numbers of areas of acute focal inflammation in their brains than did those infected with the other strains (Suzuki and Joh, 1994). Since the ME49 strain formed the largest numbers of cysts in the brains and induced the most severe encephalitis, the number of cysts in the brain appears to be an important factor in determining the susceptibility of the host to development of TE. In addition, following treatment with anti-IFN- γ , mice infected with the Beverley strain developed foci of acute focal inflammation in their brains whereas animals infected with the C56 strain did not develop such inflammatory changes (Suzuki and Joh, 1994). Mice infected with the Beverley or C56 strain had similar numbers of cysts in their brains before they were treated with anti-IFN-y mAb; therefore, a factor(s) which is related to the strain of T. gondii but not related to numbers of cysts in the brain is also important in

determining the susceptibility of the host to development of TE.

Strains of T. gondii have been classified into three genotypes, types I, II, and III, based on polymorphisms of their genes (Sibley and Boothroyd, 1992). It is noteworthy that both the ME49 and Beverley strains, which induced severe inflammatory changes in brains of mice following immunosuppressive treatment, belong to the same genotype, type II, whereas the C56 strain, which did not induce such inflammatory changes, belongs to type III (Sibley and Boothroyd, 1992). The genotypes of the parasite may be an important factor for determining the susceptibility to development of TE. In relation to this, type II strains have been reported to be predominant in T. gondii strains isolated from AIDS and non-AIDS immunocompromised patients and those with congenital infections (Howe et al., 1997; Honore et al., 2000; Ajzenberg et al., 2002), whereas isolates from outbreaks of acute toxoplasmosis, which showed a tendency to cause severe ocular disease, were found to be type I (Sibley and Boothroyd, 1992; Lehmann et al., 2000). Thus, parasite genotype may influence development of clinical illness in humans. Recently, Robben et al. (2004) reported that parasite genotype affects the production of IL-12 by infected macrophages. Rodgers et al. (2005) recently reported that the genotype of the parasite also affects cytokine responses (IFN-y, IL-2, IL-4, and IL-10) by T cells when immune T cells are stimulated with either type I or type II tachyzoites. Even within the same type II parasite, an infection of mice with different strains resulted in different serum levels of IL-6, IL-10, IL-12, IFN- γ , and TNF- α (Araujo and Slifer, 2003); therefore, genetic variations of T. gondii affect the immune responses of the host. Overall, this is probably an important variable in how different parasite genotypes determine the outcomes of infection and development of TE.

21.11 IMMUNE EFFECTOR MECHANISMS IN OCULAR TOXOPLASMOSIS

The primary target of ocular toxoplasmosis is the neural retina, but infection may involve choroids,

sclera, optic nerve, and retinal pigment epithelial (RPE) cells (Norose *et al.*, 2003; Nagineni *et al.*, 1996). Inflammation associated with retinal infection may cause damage and disruption of the choroidoretinal interface, as well as necrosis of the neural retina and RPE. Toxoplasmosis also causes retinochoroiditis in AIDS patients (Holland *et al.*, 1988; Chakroun *et al.*, 1990). Ocular toxoplasmosis in these patients usually occurs from a reactivation of a latent infection, but can also occur from newly acquired infections with *T. gondii* (Holland *et al.*, 1988; Chakroun *et al.*, 1990).

The immune response to ocular toxoplasmosis is primarily mediated via T cells with some involvement of humoral immunity. CD8+ T cells and B cells limit tachyzoite proliferation in the eye, while CD4+ cells mediate the inflammatory response in the brain (Lu et al., 2004). Several studies indicate that cytokines regulate growth of T. gondii in ocular toxoplasmosis. Similar to TE, IFN- γ , and TNF- α are the dominant cytokines controlling growth of the parasite in the eye (Gazzinelli et al., 1994; Olle et al., 1996; Norose et al., 2003). In retinal pigment epithelial cells, an IDO-dependent mechanism is involved in the mechanism of IFN-\gamma-induced inhibition (Nagineni et al., 1996; Juan et al., 1999). In another model of ocular toxoplasmosis, IFN-y-induced inhibition was found to be via a NO-dependent mechanism (Shen et al., 2001). The IFN-γ-induced cell signaling was found to be mediated via STAT1 and the transcription factors interferon regulatory factor (IRF1) and IFN consensus sequence binding protein (ICSBP), similar to the IFN- γ signaling pathway in other cells (Juan et al., 1999). Thus, similar to TE, multiple IFN-y-induced mechanisms are likely to be involved in the inhibition and pathogenesis of ocular toxoplasmosis.

21.12 IMMUNE EFFECTOR MECHANISMS IN CONGENITAL TOXOPLASMOSIS

T. gondii can be transmitted across the placenta to the fetus, causing congenital toxoplasmosis and an associated encephalitis (CNS infection)

(Martin, 2001). Depending upon the gestational age of infection, clinical disease ranges through abortion, hydrocephaly, intracranial calcification, and retinal abnormalities. Even if asymptomatic at birth, retinal and CNS abnormalities may subsequently develop, with the risk of long-term sequelae. CD8+ T cells and IFN-γ from the mother are protective against congenital toxoplasmosis, while maternal CD4+ T cells and antibody, on the other hand, had no effect on transmission to the fetus (Abou-Bacar et al., 2004). Several in vitro studies have investigated immune effector mechanisms involved in congenital toxoplasmosis. T. gondii invades the umbilical vein endothelial cells, and is then disseminated throughout the fetus. In sheep, treatment of umbilical vein cells with IFN-y blocks the growth of T. gondii via a NO- and ROI-independent mechanism (Dimier and Bout, 1996). In human endothelial cells, IFN-y induces inhibition of T. gondii via a NO-, ROI-, and IDO-independent mechanism (Woodman et al., 1991). Thus similar to the chronic infection in the CNS and ocular toxoplasmosis, IFN-\gamma-induced inhibition of T. gondii in umbilical endothelial cells is probably important in the prevention of transplacental transmission of congenital toxoplasmosis.

21.13 CONCLUSIONS

IFN- γ -mediated immune responses play a central role in resistance against *T. gondii* in the brain. IFN- γ production by multiple types of cells, including T cells, is required for the resistance. Microglia are one type of the IFN- γ -producing cells that probably play an important role in controlling the parasite. IL-12, Bcl-3, NF- κ B(2), c-Rel, and CD40-CD40L ligand interaction are important for upregulation and maintainence of IFN- γ production by T cells for prevention of TE (Table 21.1). On the other hand, IL-10 and Lipoxin A4 appear to play a crucial role in downregulating the IFN- γ -mediated responses preventing the development of immune-response-mediated pathology.

IFN- γ activates microglia and astrocytes to inhibit intracellular replication of tachyzoites (Figure 21.1). To date three mechanisms have been identified to



FIGURE 21.1 Control of Toxoplasma gondii in the brain requires two effector populations.

Molecule	Function	Cells	References
IL-12	Maintaining IFN-γ production by T cells	Dendritic cells	Fischer et al., 2000; Yap et al., 2000
Bcl-3	Maintaining IFN- γ production by T cells	T cells	Franzoso <i>et al</i> ., 1997
NF- $\kappa B(2)$	Maintaining IFN- γ production by T cells	T cells	Caamano <i>et al.</i> , 2000
c-Rel	Upregulating IFN- γ production by T cells	T cells, dendritic cells	Mason <i>et al.</i> , 2004
CD40L	Upregulating IFN-γ production by T cells (in human but not in mouse)	T cells	Subauste <i>et al.</i> , 1999; Reichmann <i>et al.</i> , 2000
IFN-γ	Activation of effector cells to control <i>T. gondii</i>	γδ T cells (?) Microglia and	Suzuki <i>et al.</i> , 1997; Leiva <i>et al.</i> , 1998; Lepage <i>et al.</i> , 1998 Suzuki <i>et al.</i> 2005
		macrophages $\alpha\beta$ T cells	Suzuki <i>et al.</i> , 1989a; Brown and McLeod, 1990; Gazzinelli <i>et al.</i> , 1992; Kang and Suzuki, 2001;
			Wang <i>et al.</i> , 2004, 2005
iNOS	Production of NO to control <i>T. gondii</i> in effector cells	Microglia (mouse) Astrocytes (human)	Chao <i>et al.</i> , 1993a; Freund <i>et al.</i> , 2001 Peterson <i>et al.</i> , 1995
IDO	Tryptophan degradation to control <i>T. gondii</i> in effector cells	Astrocytes (human)	Daubener <i>et al.</i> , 1993
		Brain vascular (endothelial cells human)	Daubener <i>et al.</i> , 2001
IGTP	Unidentified mechanism to control <i>T. gondii</i> in effector cells	Astrocytes (mouse)	Halonen <i>et al.</i> , 1998, 2001; Halonen and Weiss, 2000

TABLE 21.1 IFN-γ-mediated resistance against T. gondii in the brain

control *T. gondii* in IFN- γ -activated effector cells in the brain: NO production by iNOS, tryptophan degradation by IDO, and unknown mechanisms mediated by IGTP. In addition, astrocytes and microglia play an important regulatory role in the IFN- γ -mediated immune responses by producing cytokines, such as TNF- α , IL-1, IL-6, and IL-10, and chemokines. Neurons appear to be involved in these interactions. It is important to elucidate the detailed mechanisms in the interactions between T cells, glial cells, and neurons, and the molecular basis of their protective functions, in order to gain a better understanding of the immunopathogenesis of cerebral toxoplasmosis.

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Innate Immunity in *Toxoplasma gondii* Infection

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22.1 INTRODUCTION

The adult gastrointestinal tract is the largest mucosal area of the body in contact with the external environment. The formidable task of this single-cell layer of mucosal epithelia is to maintain a physical barrier to exogenous stimuli and yet allow for the selective entry of essential nutrients. The gastrointestinal tract is populated by a resident microflora, essential for immunological intestinal homeostasis and as a source for nutrients. Maintenance of resident bacterial numbers and simultaneous protection against potential pathogens (including *Toxoplasma gondii*) acquired by the oral route, is provided by numerous nonimmunological factors, including barriers that are both physical (gut motility, epithelial cell layer) and chemical (gastric acid, pancreatic proteolytic enzyme, bile, mucus). This chapter discusses the role of individual cell compartments in the gutassociated lymphoid tissue (GALT) innate response to this parasite.

22.2 ENTEROCYTES

Epithelial cells provide the first line of innate immunological host defense against oral *T. gondii* infection. During natural infections, *Toxoplasma* initially crosses the intestinal epithelium, disseminates into the deep tissues, and traverses biological barriers in the placenta as well as the blood–brain and the blood–retina barriers (Barragan and Sibley, 2003). The tight junctions that provide complex enterocyte-enterocyte interaction constitute a physical barrier against the penetration of intestinal microorganisms. This intercellular barrier is formed by the tight junctional proteins claudin and occludin, which associate with different peripheral plasma membrane proteins such as the zonula occludens. However, despite this physical barrier, Toxoplasma actively crosses polarized cell monolayers (such as intestinal epithelium) and this ability is linked to parasite motility and virulence in the mouse model (Barragan and Sibley, 2002). Parasite transmigration requires viable and actively motile parasites, but the integrity of host-cell barriers is not altered during parasite transmigration. T. gondii seems to use more than one ligand in a multistep process to penetrate the host cells. The SAG1 protein and several other proteins have been associated with the adhesion process of this apicomplexan parasite. Attachment and invasion of host cells by T. gondii involves the exocytosis of the micronemal homodimeric protein MIC3 (Cerede et al., 2005) and the microneme protein MIC2, which are thought to participate in gliding motility and hostcell invasion (Barragan et al., 2005). The intercellular adhesion molecule 1 (ICAM-1), which is upregulated on cellular barriers during Toxoplasma infection, might interact with parasite-derived MIC2 (Barragan et al., 2005).

In all likelihood, enterocytes play a crucial role as sentinels against parasite invasion. Enterocytes have the immunological response capacity to act as 'immunocytes' by upregulation of activation markers associated with antigen presentation. The first line of defense protein, the alpha defensins, may be important immune effector molecules, although the role of such molecule(s) has not yet been explored in response to T. gondii infection. One additional mechanism utilized by the host for effective control and removal of intestinal acquired parasites might be the induction of nitric oxide (NO), an antimicrobial molecule secreted by the intestinal epithelium. T. gondii invades and proliferates in enterocytes, and activation of the enterocytes with interferon-gamma (IFN- γ) inhibits T. gondii replication. In contrast to what is observed in other parasites, such as Giardia,

neither nitrogen or oxygen derivatives nor tryptophan starvation appear to be involved in the inhibition of parasite replication by IFN-y. NO secreted by infected enterocytes does not appear to be involved as an effector response to the parasite (Yap and Sher, 1999). In chimeric mice, it was shown that expression of iNOS by cells of hematopoietic origin provided for host resistance. Expression of iNOS by non-hematopoietic cells was insufficient to induce resistance against T. gondii. Experiments using Fe²⁺ salt, carrier, and chelator indicate that intracellular T. gondii replication is iron-dependent, suggesting that IFN-y-treated enterocytes inhibit T. gondii replication by limiting the availability of intracellular iron to the parasite (Dimier and Bout, 1998).

Toxoplasma gondii infection of the intestine following oral challenge in certain strains of inbred and outbred mice, as well as rodents, pigs, and non-human primates, can induce a severe form of intestinal inflammation. In C57BL/6 mice, this pathology shares both morphologic and histologic characteristics with human IBD, such as loss of intestinal epithelial architecture, shortened villi, massive influx of inflammatory cells into the lamina propria, and scattered patches of necrosis. When unregulated, this inflammatory process results in the early mortality of the susceptible host. Female animals die earlier than males following oral infection, suggesting that both gender and sex hormones are important factors for determining susceptibility of the small intestine to T. gondii infection (Liesenfeld et al., 2001).

At day 7 post-infection, massive necrosis of the villi and infiltration with polymorphonuclear neutrophils (PMNs), macrophages, dendritic cells (DCs), and lymphocytes are observed (Liesenfeld *et al.*, 1996). Migration of CD11c+ and CD11b+ monocytes, DCs, macrophages, and PMNs into the lamina propria at day 7 post-infection has been reported following oral infection with parasite tissue cysts (Courret *et al.*, 2005). Chemokines secreted at the basolateral surface of intestinal epithelial cells (IECs) play a critical role in the initiation and modulation of the immune response to various pathogens, including *T. gondii*. IECs from inflamed intestines secrete inflammatory cytokines

T. gondii infection of enterocytes can initiate a series of innate immunologic events that lead to a robust inflammatory process in the gut. These early events culminate in the establishment of the appropriate conditions for long-term protective immunity against reinfection and perhaps reactivation. Tissue samples isolated from the small intestine of T. gondii-infected mice display a significant increase in chemokine secretion, including monocyte chemotactic protein 1 (MCP-1/CCL2) and IFN-y inducible protein (IP-10/CXCL10), and, to a lesser extent, both macrophage inflammatory proteins 1α and β (MIP- 1α and β /CCL3 and CCL4), as well as MIP-2/CXCL2 and Regulated on Activation and Normally T-cell Expressed Substance (RANTES/ CCL5) (Mennechet et al., 2002).

Owing to the short half-life of ex vivo enterocytes, a differentiated enterocyte cell line, the mIC_{cl2}, has been used to assess the expression and secretion of these various chemokines. The C57BL/6 (H-2^b)-derived enterocyte cell line mIC_{cl2} shares most of the features of epithelial cells. It produces a confluent monolayer of cuboid cells separated by tight junctions, develops dense, short apical microvilli, forms domes, and exhibits several important properties, including polarization and differentiated functions of intestinal crypt cells. (Bens et al., 1996). In response to infection in vitro, these cells produce chemokines - in particular MCP-1, MIP-2, and MIP-1 α and β – that enhance the migration of immunologically active cells, including PMNs, macrophages and monocytes, DCs, and T cells (Buzoni-Gatel et al., 2001).

22.3 NEUTROPHILS

Neutrophils are early-response cells that release several proinflammatory cytokines and chemokines in response to *T. gondii*. PMN depletion experiments revealed that PMNs may have reactive oxygen intermediate independent antimicrobial functions (Bliss *et al.*, 2001; Denkers *et al.*, 2004). PMNs also exhibit an immunoregulatory role such

that neutrophil-deficient mice co-infected with T. gondii and Plasmodium berghei exhibit a reduced Th1 response but no concomitant increase in Th2 cytokines. Toxoplasma infection in neutropenic mice results in depression of both type I and type II cytokines, giving rise to lethal systemic pathology associated with elevated parasite levels (Bliss et al., 2001). PMN has been identified as a source of several pro-inflammatory cytokines during infection with T. gondii. Both human and mouse neutrophils produce IL-12 and TNF- α as well as several chemokines when stimulated in vitro with T. gondii antigens (Bliss et al., 1999; Denkers et al., 2004). IL-12-secreting PMNs traffick to the peritoneal cavity following intraperitoneal parasite infection (Bliss et al., 2000). This PMN trafficking is dependent upon their expression of the chemokine receptor CXCR2, and absence of this receptor is linked with increased susceptibility of the challenged host (Del Rio et al., 2001). In humans, CXCR2 is the receptor of IL-8 - a chemokine well-known for its chemotactic activity on PMNs. Mice do not express a homologous molecule for IL-8, but MIP-2 and KC chemokines bind to CXCR1 and are likely to mediate neutrophil chemotaxis in murine models.

Chemokine receptor CCR1-deficient mice have defects in neutrophil trafficking and proliferation. In comparison with parental wild-type mice, CCR1^{-/-} mice exhibited dramatically increased mortality to *T. gondii* in association with an increased tissue parasite load. The influx of PMNs to the peripheral blood and to the liver was reduced in CCR1^{-/-} mice during early infection, suggesting that CCR1- dependent migration of neutrophils to the blood and tissues may have a significant impact in control-ling parasite replication-dependent host resistance to intracellular infection (Khan *et al.*, 2001).

In response to *T. gondii* infection, epithelial cells secrete chemokines that recruit PMNs. In addition to chemokines, IL-17, a granulopoiesis cytokine produced by CD4+ T cells and neutrophils, induces CXC chemokines that may also contribute to the migration of PMNs to infected sites during early infection (Kelly *et al.*, 2005). Thus PMNs recruited to the site of infection by release of chemokines such as MCP-1 or IL-8 (MIP-2) can participate in the attraction and migration of other

immune cells, such as dendritic cells, which may participate as a link between the innate and adaptive immune responses.

22.4 DENDRITIC CELLS

One of the primary roles of Dentritic Cells (DCs) is to present antigen, although the mechanism for this in the intestinal compartment is not fully understood. Within the intestine, DCs might have two different localizations. DCs with typical features of immature cells have been described in the Peyer's patches (PP) as forming a dense layer of cells in the subepithelial dome. Access of the parasite to the DCs from the PP may be via microfold cells (M cells). These unique cells are found in the epithelium surrounding the PP, and have been associated with microbial entry into the host. DCs are also scattered throughout the lamina propria. Compared with spleen DCs, DCs isolated from the lamina propria exhibit a characteristic phenotype. Most of the intestinal DCs (70 percent) are CD11c+ CD11b+, whereas only 6–8 percent express CD11c+ CD8 α + CD11b-. The homeostatic mechanisms whereby the intestinal DCs remain steady state in the presence of microbial exposure is not understood. A possible explanation is that epithelial cells may condition mucosal dendritic cells through the constitutive release of thymic stromal lymphopoietin and other mediators (Rimoldi et al., 2005).

At day 5 post-infection with T. gondii most of these lamina propria DCs are mature, as indicated by high-level expression of MHC class II, CD40, CD80, and CD86. It has been proposed that DCs from the lamina propria might gain access to the intestinal contents by using unique proteins to separate the tight junctional borders between the enterocytes without disrupting the monolayer integrity (Rescigno et al., 2001). This process would allow for the direct sampling by DCs of pathogens within the gut lumen. Alternatively, pathogens that cross the epithelium may also be captured directly by the DCs that process antigen for presentation. An additional way for the DCs from the lamina propria to sample the antigens is via the infected enterocytes. In this model,

apoptotic enterocytes are digested by the DCs and processed for antigen expression.

When hosts, including humans, ingest tissue cysts or oocysts containing T. gondii, free parasites are released in the gut lumen. They subsequently enter enterocytes, where they multiply and initiate the infection. Enterocytes loaded with parasites secrete chemokines, which recruit leukocytes in the lamina propria extravascular space. Parasites then disseminate to several distant tissues, including the brain - a major site supporting parasite latency (Dubey, 1997; Dubey et al., 1997). This event has important clinical implications, since T. gondii as a chronic infection is associated with the encysted bradyzoite, which slowly replicates under the control of unique hostdependent immune signals. T. gondii can efficiently enter and survive within DCs (Channon et al., 2000). The functional plasticity as well as the migratory property of DCs (mostly CD11c+ cells) can then be utilized by pathogens for dissemination through the body.

Among parasite carrier and motile candidates are DCs that are known to survey tissue integrity and to transport virus, fungi or bacteria from epithelia to draining lymph nodes. Although known for their T-cell stimulatory properties, DCs have the capacity to disseminate *T. gondii*. Among the other blood leukocyte lineages, the neutrophils, lymphocytes, and monocytes that are permissive to parasite growth *in vitro* (Channon *et al.*, 2000), the monocytes, especially those within the inflamed lamina propria, provide a 'Trojan horse' for parasite transport throughout the host.

Recent studies in one of our laboratories (DBG) have demonstrated that, following intragastric inoculation of cysts in mice, CD11c+ dendritic cells from the intestinal lamina propria, the Peyer's patches, and the mesenteric lymph nodes were parasitized, whereas parasites were associated with the CD11c- CD11b+ monocytes in the peripheral circulation. These parasitized cells are involved in disease induction in the brains of naïve recipient mice, as demonstrated by adoptive transfer experiment. *Ex vivo* analysis of parasitized cells showed that single tachyzoites, non-replicating parasites, could be identified at the cell periphery, often

surrounded by the host-cell plasma membrane. By several approaches, including (1) vital staining of leukocytes, (2) antibody labeling, or (3) chimeric mice in which the hematopoietic cells expressed the green fluorescent protein, it was determined that *T. gondii*-infected CD11b+ leukocytes can traffick to the brain extravascular space (Courret *et al.*, 2005). Additional studies identified CD11c+ and 33D1+ cells localized at inflammatory sites in infected brain (Fischer *et al.*, 2000).

Parasite infection of enterocytes results in the upregulation and expression of CD40, CD1d, class II, suggesting that enterocytes may act as APCs and be involved in the activation of lymphocytes. Because of the limited expression of CD80 and CD86 mandatory for efficient lymphocyte stimulation, DCs and macrophages that infiltrate the infected epithelium are likely to be principally involved as APCs for CD4+ T-cell activation from the lamina propria. In addition to their crucial role as APCs, DCs display an antimicrobial function. IFN- γ activation of DCs triggers oxygen-dependent inhibition of *T. gondii* (Aline *et al.*, 2002).

In addition to PMNs, DCs (Aliberti et al., 2003) as well as macrophages (Oliveira et al., 2000) produce IL-12 following parasite infection. A selective role of DCs in parasite-induced IL-12 production in vivo was evaluated when live tachyzoites or a soluble tachyzoite extract (STAg) were injected into mice. IL-12p40-producing cells were observed within a few hours in the T-cell regions of the spleen. This population was identified as consisting almost entirely of CD11c+ DCs. Staining of the same spleen sections with anti-CD11c monoclonal antibody revealed a shift of DCs in the spleen as a consequence of T. gondii exposure. There appeared to be a large number of cells leaving the red pulp and marginal zone and clustering in the T-cell areas. Splenic DCs were also shown to be highly responsive to STAg in vitro, producing significant levels of IL-12.

Interleukin-12 (IL-12) is the major cytokine triggering IFN- γ synthesis by NK and T lymphocytes during *T. gondii* infection. IL-12-deficient mice are equally susceptible to acute toxoplasmosis as IFN- $\gamma^{-/-}$ deficient mice. The implication of this is that IL-12 is the major initiation signal for host resistance to the parasite. IL-12 is also assumed to be responsible for T-helper 1 (Th1) effector choice in *T. gondii* infection, although no default to a Th2 cytokine production phenotype is seen in IL-12-deficient animals exposed to the parasite (for review, see Sher *et al.*, 2003).

22.5 MACROPHAGES

Macrophages are also crucial in innate immunity against T. gondii infection. They possess potent antimicrobial function, and efficiently process and present peptide antigens for T-cell activation. IFN-y is the major inducer of classical activation of macrophages. Activated mouse macrophages acquire antimicrobial activities that might be dependent on two factors. The first is the deprivation of tryptophan, a required amino acid essential to parasite growth. Tryptophan degradation occurs following induction of indoleamine 2,3-dioxygenase. Stimulation of inducible nitric oxide synthase (iNOS) production is another antimicrobial mechanism elicited by IFN-y-activated macrophages. iNOS induction leads to the production of reactive nitrogen intermediates that are toxic to the parasite. Activated macrophages synthesize TNF-a which, in addition to TNF- α produced by the CD4 T cells from the infected lamina propria, enhances iNOS production (Liesenfeld et al., 1999). The respective importance of these two mechanisms in altering parasite replication may vary among infected tissues (intestine, lung, brain) (Fujigaki et al., 2003). Although subtle regulation of NO production by the parasite itself (Luder et al., 2003) as well as by the presence of IL-13 and IL-4 in the infected intestine through a STAT6-dependent pathway (Rutschman et al., 2001) might occur, the overproduction of NO, IFN- γ , and TNF- α contributes to the general inflammation in the intestine of infected mice and, if uncontrolled, leads to death (Khan et al., 1997; Liesenfeld et al., 1999). The cellular factors that are required for the IFN- γ effect are largely unknown, although IGTP and LRG-47, members of the IFN-\gamma-regulated family of p47 GTPases, are required for resistance to acute T. gondii infections in vivo. It has been demonstrated that

IGTP largely co-localizes with endoplasmic reticulum markers, while LRG-47 is mainly restricted to the Golgi body. These results suggest that IGTP and LRG-47 are able to regulate host resistance to acute *T. gondii* infections through their ability to inhibit parasite growth within the macrophage (Butcher *et al.*, 2005a).

CD40/CD154 interaction is involved in the regulation of macrophage production of IL-12 and T-cell production of IFN-γ. Infection of C57BL/6 mice with T. gondii results in an upregulation of CD40 expression on accessory cell populations at local sites of infection, as well as in lymphoid tissues. CD40/CD154 ligation is essential to initiate the intestinal inflammatory response observed after oral infection of C57BL/6 mice (Li et al., 2002), and CD40/CD40L interaction is crucial in resistance to T. gondii (Reichmann et al., 2000). CD40/CD154 interaction may also promote the antiparasitic function of macrophages in the absence of priming with IFN-y and lack of production of reactive nitrogen intermediates. CD40stimulated macrophages acquired anti-T. gondii activity that was not inhibited by a neutralizing anti-IFN-y monoclonal antibody but was ablated by the neutralization of TNF- α (Andrade *et al.*, 2005). Moreover, while the induction of anti-T. gondii activity in response to CD40 stimulation was unimpaired in macrophages from IFN- $\gamma^{-/-}$ mice, macrophages from TNF receptor 1/2-/- mice failed to respond to CD40 engagement. In contrast, CD40 stimulation did not induce NOS2 expression and did not trigger production of reactive nitrogen intermediates. CD40 can induce the antimicrobial activity of macrophages against an intracellular pathogen despite the lack of two central features of classically activated macrophages: priming with IFN-y, and production of reactive nitrogen intermediates.

T. gondii has adopted unique strategies to subvert macrophage antimicrobial functions. The parasite interferes with host-cell microbicidal activities through sophisticated manipulation of intracellular macrophage signaling pathways. These subversive activities are probably dictated by the need to evade microbicidal effector function, as well as to avoid proinflammatory pathology that can destabilize the host–parasite interaction (Denkers and Butcher, 2005). Some of the molecular mechanisms underlying the parasite suppressive effect on macrophage proinflammatory cytokine production have been decrypted.

Thus macrophage invasion by live *T. gondii* parasites is accompanied by rapid and sustained activation of host STAT3, which correlates with suppression of LPS-triggered TNF- α and IL-12 synthesis. Parasite-induced STAT3 phosphorylation and suppression of LPS-triggered TNF- α and IL-12 was intact in IL-10-deficient macrophages, ruling out a role for this anti-inflammatory molecule in this pathway. Thus, *T. gondii* exploits host STAT3 to prevent LPS-triggered IL-12 and TNF- α production (Butcher *et al.*, 2005b).

22.6 B CELLS

Participation of B cells in the anti-inflammatory processes is debated. In a model of IFN- γ -deficient mouse infection, the B-2 subpopulation exhibited immunomodulatory functions, enhancing production of IL-4 and IL-10 and downregulating NO release in macrophages from the peritoneal cavity (Mun *et al.*, 2003a).

In the model of inflammatory bowel disease developed in B6 mice orally infected with cysts, we describe different roles for B cells in the regulation or induction of the inflammation. Thus B-cell-depleted mice infected with T. gondii have an increased production of TNF-α and IFN-γ by CD4+ and CD8+ T cells, and IL-12 by dendritic cells. This tends to be abrogated when B-cell-depleted mice are transferred before infection with naïve B cells, and at a lower extent with D7-primed B cells. Moreover, analysis of double chimeric mice, whose B cells are only CD1d-cells, (Henard et al., submitted) supports the theory that the inhibitory effect of B cells depends at least partially on CD1d expression. This observation has led us to speculate that B cells act on NKT cells, consequently lowering the inflammatory response (Laurence Mennard, in preparation).

22.7 SIGNALING PATHWAYS

Because of their major role in microbial recognition, the involvement of receptor pathways involving the

Toll-like receptor (TLR)/IL-1R superfamily in triggering both DC IL-12 production and host resistance to T. gondii has recently been addressed. Innate immune recognition relies on a limited number of germ-line encoded receptors, such as TLRs which recognize Pathogen Associated Molecular Patterns (PAMPs) of microbial origin (Quesniaux et al., 2004). To date, 11 members of the TLR family have been characterized. Although TLRs are expressed in a broad range of tissues, the greatest variety of TLR mRNAs is found in professional APCs, suggesting a key role of TLRs in innate immunity that is essential in the development of the acquired immune response characterized by polarization of naïve CD4+ helper T cells toward the Th1 or Th2 phenotype. Mice lacking myeloid differentiation factor 88 (MyD88), an adapter molecule used by all TLRs, as well as IL-1R and IL-18R, exhibited a near complete abrogation of the STAg-induced IL-12 response, and when challenged with T. gondii the knockout (KO) animals displayed a loss in resistance to infection equivalent to that of IL-12-deficient mice (Scanga et al., 2002). TLR-2 has been shown to contribute to the host resistance of mice to very high challenge doses of T. gondii (Mun et al., 2003b), as well as to STAg-induced chemokine production neutrophils. Nevertheless, TLR-2-deficient DCs as well as neutrophils produce normal amounts of IL-12 following STAg stimulation (Scanga et al., 2002; Del Rio et al., 2004).

Profilins are a class of small actin-binding proteins present only in eukaryotic cells, which have a regulatory role in the polymerization of actin. Profilin is a molecule that is produced by T. gondii, and is present in abundance in soluble antigen preparations that are produced using parasite cultures. The profilin molecule from Toxoplasma gondii generates a potent interleukin-12 (IL-12) response in murine DCs that is dependent on MyD88 T. gondii profilin activates DCs through TLR11 (Yarovinsky et al., 2005). This recognition event is important in mouse host defense, although not all differences observed in MyD88 KO mice could be seen in TLR11 KO mice. Although there is still no evidence for a Toll receptor involved in the recognition of T. gondii during human infection, and humans do not express TLR11, it can be imagined that TLR11 might have a crucial role in human disease indirectly due to the complicated parasite life cycle involving the cat and mouse.

Recently we have identified a role for TLR9 in the Th1-type inflammatory response that ensues following oral infection with T. gondii. The classically defined ligand for TLR9, bacterial unmethylated CpG DNA, has an immunomodulatory effect at the cellular level, and TLR9-/- mice are unresponsive to immunostimulatory CpG DNA (Hemmi et al., 2000; Rutz et al., 2004; Wagner, 2004). Human TLR9conferred responsiveness to CpG-DNA occurs as well via the recognition of species-specific CpG motifs (Bauer et al., 2001). Following the engagement of CpG DNA with TLR9, a MyD88-dependent signaling pathway activates NF-kB, which ultimately leads to the secretion of pro-inflammatory Th1 cytokines such as IL-12. Whereas TLR1, TLR2, and TLR4 are cell-surface-expressed, current opinion suggests that TLR9 has a predominant intracellular localization in macrophages and DCan advantageous position for the recognition of intracellular pathogens.

Following oral infection with T. gondii, susceptible B6 but not TLR9-/- (B6 background) mice develop a Th1-dependent acute ileitis compared with TLR9-/- mice (B6 background) that are free of gut inflammation. TLR9-/- mice have higher parasite burdens than control WT mice, suggesting depressed IFN-y-dependent parasite killing. IL-12producing DCs were reduced in TLR9-/- mice compared with the WT controls, which corresponded with a reduction in total T-cell frequency as well as IFN- γ -producing T cells from the lamina propria. Infection of chimeric mice having deletion of TLR9 in either the hematopoietic or nonhematopoietic compartments indicates that TLR9 expression in both compartments is involved (Minns et al., 2006).

The involvement of a second type of receptor and signaling pathway was revealed by studies investigating the role of chemokines in the migration of DCs to splenic T-cell areas following STAg injection. It was found that spleens of mice lacking the CC chemokine receptor CCR5 not only displayed impaired DC migration but also exhibited diminished DC IL-12 production (Aliberti *et al.*, 2000).

Moreover, DCs from naïve CCR5 KO mice showed highly defective IL-12 responses when stimulated with STAg in vitro. In agreement with these observations, CCR5 KO mice displayed decreased IL-12 production and survival following live T. gondii infection. Further, similar reductions in IL-12 response and survival were seen in mice treated with pertussis toxin (PTX), which uncouples the G-protein signaling pathway used by chemokine receptors (Aliberti and Sher, 2002). PTX treatment also abolished the residual IL-12 production observed in DCs from MyD88 KO mice, suggesting that the DC IL-12 response depends on both G-protein-coupled and MyD88-dependent signaling pathways. T. gondii expresses a secreted molecule associated in the parasite with secretory granules. This secreted protein, C-18, represents one of the multiple ligands in STAg responsible for DC IL-12 induction.

C-18 was shown to be a CCR5 rather than a TLR ligand. Thus, recombinant C-18 binds CCR5 with moderate affinity and triggers chemokine receptor signaling as measured by Ca^{2+} flux (Aliberti *et al.*, 2003). Because of binding to CCR5, C-18 was shown to be an effective HIV co-receptor antagonist blocking both viral-induced fusion and cell infectivity (Golding *et al.*, 2003, 2005; Yarovinsky *et al.*, 2004).

During T. gondii infection, chemokine receptors and TLRs may function together in APC activation. If the Th1-like immune response is not controlled, oral infection of B6 mice will culminate in a lethal ileitis within 7-9 days post-infection. The IL-12 response of splenic DCs to STAg in vivo was found to be extremely rapid, peaking in 36 hours, and was accompanied by a concomitant increase in cell-surface CD40 expression (Seguin and Kasper, 1999; Schulz et al., 2000; Subauste et al., 1999; Subauste and Wessendarp, 2000; Villegas et al., 2000). An immediate fall-off in the response was then observed, with a reduction to baseline by 24 hours. This reduction in IL-12 production occurred concurrently with the development of non-responsiveness to secondary administration of STAg. This phenomenon was found to last for a period of approximately 1 week, and was not due to DC apoptosis or suppression by IL-10. The latter

observations suggest that, following its initial stimulation by *T. gondii*, the DC IL-12 response is actively turned off.

Based on the observation that this dendritic cell response is accompanied by downregulation of CCR5, a receptor that (as described above) participates in STAg-induced IL-12 production, the mechanisms stimulated by T. gondii that would result in down-modulated CCR5 expression were examined. Lipoxin (LX) A4, an arachidonic acid metabolite generated by a 5-lipoxygenase (LO)dependent pathway, was identified as a major inhibitor of DC IL-12 production (Aliberti et al., 2002). T. gondii-infected 5-LO-deficient mice showed enhanced IL-12 production by CD11c+ as well as CD11c cells in brain sections, suggesting that LXA4 regulates IL-12 production by other APCs in addition to DCs in vivo. In addition to its inhibition of pro-inflammatory cytokine production, IL-10 has a major downregulatory effect on IL-12 and IFN- γ signaling that is believed to result from its induction of several members of the suppressors of cytokine signaling (SOCS) family, particularly SOCS-1 and SOCS-3. While treatment with an LX analog was shown to induce SOCS-2, this activity has not been formally linked with the down-modulatory effects of the eicosanoid in vivo, especially its suppression of pro-inflammatory cytokine production (Bannenberg et al., 2004; Aliberti, 2005).

DC production of IL-10 itself does not appear to be a major mechanism responsible for dampening IL-12 production, because DCs from IL-10 KO mice produce normal levels of IL-12 *in vitro* and *in vivo* following STAg (Gazzinelli *et al.*, 1996; Wille *et al.*, 2004).

22.8 NK AND NKT CELLS

In addition to IL-12, macrophages and dendritic cells produce IL-15. IL-15 exhibits pleiotropic functions at the interface between innate and adaptive immunity. IL-15 is a 14-kDa cytokine that shares common features with IL-2 and exhibits stimulatory effects on T-cell proliferation. However, IL-15 can be distinguished from IL-2 by

its broad cell-type distribution, its potent in vivo anti-apoptotic effects, and, most relevant to this review, as a bridge between the innate and adaptive immune response. IL-15 is necessary for the differentiation and/or homeostatic maintenance of the three subsets of lymphocytes linked to innate immune response: NK, NKT, and CD8aa intraepithelial lymphocytes (IEL). IL-15 is also mandatory for the survival of memory CD8 T lymphocytes. IL-15 induces the effector functions of NK and CD8 T lymphocytes, promotes the selection of high-avidity cytotoxic CD8 T cells and their expression of the co-receptor $CD8\alpha\beta$ (reviewed in Fehniger and Caligiuri, 2001). Finally, IL-15 stimulates the maturation of dendritic cells and thereby promotes antigen presentation.

The role of IL-15 produced by hematopoietic cells after infection by T. gondii is controversial (Doherty et al., 1996; Khan et al., 2002; Lieberman et al., 2004). In contrast, we have recently observed, using hematopoietic chimeric mice, that IL-15 produced by infected enterocytes as early as 6 hours postinfection initiates the inflammatory immune response that leads to the development of the lethal ileitis in C57BL/6 mice (Julie Shulthess, in preparation). IL-15 is critical for the differentiation and/or homeostasis of several murine innate immune cell subsets, including natural killer, NKT cells, and CD8aa intraepithelial lymphocytes as well as the generation and maintenance of specific memory CD8 TCRaß cells. In addition, IL-15 plays redundant functions with other cytokines to promote maturation of dendritic cells, proliferation of T and B cells, cytotoxicity of NK and CD8+ T cells, and production of proinflammatory cytokines.

Among cells targeted by IL-15, NK and NKT cells play a major role during the early phase of the *T. gondii* infection. NK cells are known to produce IFN- γ and to exhibit cytotoxic activity, thus influencing the parsiticidal mechanisms. NF-kappa B activity in NK cells is responsible for cell expansion and production of IFN- γ required for resistance to *T. gondii* (Tato *et al.*, 2003). After infection of mice, a population of NK cells that expresses activated CD44 emerges. Both IL-2 and IL-15 lead to the upregulation and activation of CD44, enhancing the ability of activated NK cells to produce IFN- γ (Sague *et al.*, 2004). Recently Combe *et al.* (2005) demonstrated the pivotal role of NK cells in the induction of CD8 T-cell immunity in the absence of CD4 T cells.

Natural-killer T cells represent a minor subset of T lymphocytes that share receptor structures with conventional T cells and NK cells. Murine NKT cells express intermediate levels of a TCR using a semi-invariant Va14-Ja281 TCR-chain paired with a limited number of β chains such as V β -8, -7, or -2 TCR, together with NK cell receptors (NKR-P1, Ly-49, and NK1.1 in C57BL/6 mice). These cells are located mainly in the liver, spleen, thymus, and bone marrow, and recognize Ag in the context of the monomorphic CD1d Ag-presenting molecule. Our findings suggest a potentially critical role for these early responder cells in the initiation and regulation of the lethal inflammatory process. The implication of NKT cells was demonstrated by the observation that NKT-cell-deficient mice ($J\alpha 281^{-/-}$) are more resistant than C57BL/6 mice to the development of lethal ileitis. Ja281-/- mice failed to overexpress IFN-y in the intestine early after infection. This detrimental effect of NKT cells is blocked by treatment with α -galactosylceramide, which prevents death in C57BL/6 but not in Ja281-/mice. This protective effect is characterized by a shift in cytokine production by NKT cells toward a Th2 profile, and correlates with an increased number of mesenteric Foxp3 lymphocytes. These results highlight the participation of NKT cells in the parasite clearance by shifting the cytokine profile toward a Th1 pattern and simultaneously to immunopathological manifestation when this Th1 immune response remains uncontrolled, and provide evidence that NKT cells are important in regulation of Th1/Th2 differentiation (Ronet et al., 2005). The parasite antigen able to trigger NKT function is not yet determined, but it is well known that NKT cells recognize Ag in the context of the monomorphic CD1d Ag-presenting molecule. CD1d and the invariant TCR chain are essential for the normal development of NKT. CD1 molecules present hydrophobic lipid Ags. However, in contrast to mice that are genetically impaired for NKT cells $(J\alpha 281^{-/-} mice)$ and that exhibit resistance to the

development of lethal ileitis in C57B6 mice, CD1d-deficient mice were more susceptible to the infection and apparently do not control their inflammatory response (Smiley et al., 2005). In C57BL/6 mice, CD4+ cells can cause intestinal pathology during T. gondii infection. Compared with WT mice, infected CD1d-deficient C57BL/6 mice had higher frequencies and numbers of activated (CD44high) CD4+ cells in mesenteric lymph nodes. Depletion of CD4+ cells from CD1ddeficient mice reduced weight loss and prolonged survival, demonstrating a functional role for CD4+ cells in their increased susceptibility to T. gondii infection. Another explanation might be that in addition to NKT depletion, regulatory cells such as IEL and B cells are also reduced in CD1d^{-/-} mice (Mizoguchi et al., 2002; Allez and Mayer, 2004).

During both the acute and chronic phases of T. gondii infection, protective immunity relies primarily on IFN-y secretion by NK, NKT, and T cells, rather than on cytotoxicity-based effector functions. The increased survival of T. gondiiinfected severe combined immunodeficiency disease (SCID) versus IFN-y-deficient animals (for review, see Sher et al., 2003) clearly demonstrates autonomous and potent activation of the innate immune system by this parasite, resulting in temporary control of tachyzoite growth. This response appears to be directed by IL-12-producing DCs that in turn promote IFN synthesis by NK and NKT cells. In T-cell-sufficient mice, it has been generally assumed that IL-12 serves as a bridge between innate and adaptive immunity by promoting the development of Th1 effector cells, thus ensuring lasting control of infection. Consistent with this concept is the finding that mice deficient in IL-12p40 succumb to acute infection with the same kinetics as infected IFN KO mice (Sher et al., 2003). However, it is not really clear whether IL-12 is really necessary to IFN- γ production by NKT cells. IL-15 production by infected enterocytes or direct stimulation by the parasite glycolipid might be sufficient to promote IFN-γ production by NKT or NK cells. In addition, T. gondii-induced polarization can occur even in absence of IL-12.

22.9 INTESTINAL ADAPTIVE IMMUNE RESPONSE

Activation of the innate immune system results in antigen presentation and activation of the antigenspecific T- and B-cell intestinal response.

22.9.1 Intraepithelial lymphocytes (IELs)

IELs are located between the epithelial cells, below the intercellular tight junctions. Most of the IELs are CD8+ T lymphocytes; they bear an oligoclonal repertoire of T-cell antigen receptor (TCR) and express the unusual integrin $\alpha E\beta$ 7, which is involved in adherence to epithelial cells by binding to E-cadherin. Infection of the gut with mucosal pathogens can result in the migration and activation of IELs. IEL migration towards the *T. gondii*-infected enterocytes requires the expression of the chemokine receptor CCR5 in response to the secretion of MIP-1 α (Luangsay *et al.*, 2003).

IELs provide a number of important immunological functions, including cytotoxic activity, secretion of cytokines, and modulation of epithelial cell death and regeneration. T. gondii antigen-primed IELs are cytotoxic for T. gondii-infected enterocytes (Chardes et al., 1994; Buzoni-Gatel et al., 1997). After T. gondii infection, C57BL/6 mice died of acute ileitis due to the uncontrolled production of Th1-like cytokine. Adoptive transfer of antigen-primed IELs into naive mice prior to infection rescues the recipient mice from death (Buzoni-Gatel et al., 2001). T. gondii antigen-primed IELs produce substantial amounts of TGF-B, which downregulate the production of IFN-y from the CD4 lymphocytes in the lamina propria through a Smad 2,3-dependent pathway (Mennechet et al., 2004).

22.9.2 Cells from the lamina propria

In intestinal toxoplasmosis, the development of a Th1-like T-cell response, orchestrated by IFN- γ -producing CD4 T cells from the lamina propria, leads to the inhibition of parasite replication, but also may damage the intestinal barrier. CD4 T cells from the *T. gondii*-infected lamina propria produce

copious amount of IFN- γ and TNF- α , which enhance the production of chemokines by infected enterocytes and increase the inflammatory response (Mennechet *et al.*, 2002).

Studies indicate that production of secretory IgA antibodies is associated with early infection in mice (Chardes *et al.*, 1992, 1993). The lamina propria is indeed populated with numerous B cells that differentiate into IgA plasmacytoid cells. In addition the natural presence of TGF- β into the intestine contributes to IgA switch. However, the protective role of these secretory IgA antibodies is still debated. Specific antibodies are not considered to be the major factor in recovery from infection, although they may play a role in protection against re-infection and are useful for an early diagnosis.

22.10 PARASITE ANTIGENS THAT TRIGGER THE INNATE RESPONSE

T. gondii is a complex eukaryotic organism with multiple surface proteins as well as cytoplasmic antigens that can elicit a host inflammatory response. The role of specific Toxoplasma antigens in the induction of the innate response is only partially understood. The surface of T. gondii comprises a family of developmentally regulated glycosylphosphatidylinositol (GPI)-linked proteins (SRSs), of which surface antigen 1 (SAG1) is the prototypic member. SAG1 protein is exclusively expressed on the tachyzoite. The biological role for this superfamily of surface proteins remains mostly enigmatic, although there is evidence for a role in parasite attachment. SAG1 induces the dominant antibody response during infection, and a strong, systemic Th1-like T-cell response characterized by high-titer IFN-γ production by CD4 and CD8 T lymphocytes.

A SAG1 null mutant was engineered by homologous recombination and used to infect C57BL/6 mice. This mutant was shown *in vitro* to adhere to and replicate in fibroblasts at the same or even at a better rate than the control parental strain. *In vivo*, we were able to demonstrate that this antigendeficient parasite is unable to induce ileitis following intralumenal infection. Although this mutant can replicate in both the host and *in vitro* cell culture, infection is associated with a decrease in both innate and adaptive inflammatory immune responses.

We investigated the ability of this antigendeficient parasite to induce a lethal intestinal inflammatory response in susceptible mice. C57BL/6 mice orally infected with Δ sag 1 parasites failed to develop lethal ileitis. In vitro, the mutant parasites replicated in both enterocytes and dendritic cells. In vivo, infection with the mutant parasites was associated with a decrease in the production of both chemokines and cytokines within several compartments of the gut-associated cell population, including enterocytes, dendritic cells, and CD4+ T cells isolated from the lamina propria. RAG-deficient (RAG-/-) mice are resistant to the development of the lethal ileitis after oral infection with the parasite. Adoptive transfer of antigen-specific CD4+ effector T lymphocytes isolated from C57BL/6 infected mice into RAG-/mice conferred susceptibility to the development of the intestinal disease. In contrast, CD4+ effector T lymphocytes originated from mice infected with the mutant strain Δ sag1 failed to transfer the pathology. In addition, resistant mice (BALB/c) that fail to develop lethal ileitis following oral infection with T.gondii were rendered susceptible following intranasal pre-sensitization with the SAG1 purified protein. This process was associated with a shift toward a Th1 response, as indicated by increased production of IFN-y but not IL-10 or TGF- β in the intestine of these mice. These findings demonstrate that a single microbial antigen (SAG1) of T. gondii can elicit a lethal inflammatory process in an experimental model of pathogendriven ileitis (Rachinel et al., 2004).

22.11 CONCLUSIONS

The development of *T. gondii*-specific Th1 cells in the absence of IL-12 favors the concept that signals upstream and independent of IL-12 production are critical for microbial-induced Th1 effector choice.

What are the other signals by which DCs could promote Th1 subset selection? The encounter of

microbes or their products with DCs can lead to the upregulation of major histocompatibility complex (MHC) class II as well as co-stimulatory molecules (e.g. CD40) that together should result in APC–T-cell interaction at the high signal strength level previously shown to be associated with Th1 polarization.

Whatever the signals and the pathways used to shift the immune response, a Th1-like immune response is absolutely necessary to control parasite replication. If left unmodulated, this Th1-like immune response can lead to lethal host damage such as the ileitis seen in C57BL/6 mice (Figure 22.1).



FIGURE 22.1 A model of the GI mucosal immune response to Toxoplasma gondii. When parasites invade the mucosal intestinal epithelium they first face a physical barrier formed by enterocytes bound together with tight junctions (1). Parasites have developed multiple strategies to adhere, sometimes to invade the enterocytes, and to spread beyond the epithelium. When enterocytes are infected by the parasites, physiological and morphological disturbances occur and enterocytes might secrete cytotoxic molecules such as nitric oxide (NO) (2). In addition, enterocytes respond to the infection by secretion of chemokines and cytokines, which attract polymorphonuclear leukocytes (PMNs) (3), macrophages MΦ (4), and dendritic cells (DCs) (5). When stimulated, these cells from the innate immune system can be directly microbicidal. They are also source of cytokines such as IL-12, which triggers the adaptive CD4 immune response (6). To be elicited, specific immune response needs antigen presentation, mainly through DCs. DCs sample the antigen by different pathways; one of them is direct antigen capture into the lumen by elongation of the dendrites through the tight junctions (7). Activated T cells, in addition to NK and NKT cells (8) stimulated by cytokines produced by infected enterocytes such as IL-15, secrete IFN-γ, which activates MΦ, DCs, and enterocytes for parasite clearance. B cells (9) are also triggered to secrete antibodies that can cross the epithelial barrier by active transcytose and reach the parasite in the lumen. Besides microbicidal activities, $IFN-\gamma$, if not controlled, may damage the intestinal integrity. Intraepithelial lymphocytes (IEL) (10) are cytotoxic for infected enterocytes and may produce TGF- β , which limits IFN- γ production. This figure is reproduced in color in the color plate section.

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Adaptive Immunity and Genetics of the Host Immune Response

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- 23.1 Introduction
- 23.2 Mouse genetic studies
- 23.3 Studies of Lewis and Fisher rats
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23.1 INTRODUCTION

Immunity to *T. gondii* is complex, with aspects of innate and adaptive immune responses playing important effector roles (Remington and Merigan, 1968; Anderson and Remington, 1974; Hoff and Frenkel, 1974; Frenkel and Caldwell, 1975; Anderson *et al.*, 1976; Lindberg and Frenkel, 1977; McLeod and Remington, 1977a; McLeod *et al.*, 1985a, 1985b, 1989a, 1989b; Murray *et al.*, 1985a, 1985b; Khan *et al.*, 1988a, 1988b, 1996a, 1996b; Danneman *et al.*, 1989; Yano *et al.*, 1989; Adams *et al.*, 1990; Brown and McLeod, 1990; Araujo, 1991, 1992; Parker *et al.*, 1991; Gazzinelli *et al.*, 1992a; Sher and Coffman, 1992; Chardes and Bout, 1993; Denkers *et al.*, 1993a, 1993b, 1993c, 1997a, 1997b, 2003a, 2003b; Saavedra and Herion, 1993; Aosai *et al.*, 1994; Bala *et al.*, 1994; Chardes *et al.*, 1994; Deckert-Schluter *et al.*, 1994a, 1994b, 1996, 1998a, 1998b; Hunter *et al.*, 1994a, 1994b, 1995a, 1995b, 1995c; Shirahata *et al.*, 1994; Brown *et al.*, 1995a; Amichay *et al.*, 1996; Montoya *et al.*, 1996; Purner *et al.*, 1996; Saavedra *et al.*, 1996; Liesenfeld *et al.*, 1997; Dobbin *et al.*, 2002; Lieberman and Hunter, 2002; Liesenfeld, 2002; Kang *et al.*, 2003; Mun *et al.*, 2003a, 2003b; Abou-Bacar *et al.*, 2004a; Kasper *et al.*, 2004; Mennechet *et al.*, 2004; Rachinel *et al.*, 2004) (Tables 23.1, 23.2). The innate immune system is also important in driving and dictating the quality of the adaptive immune response (Yano *et al.*, 1989; Sher *et al.*, 1991, 1992, 1993; Curiel *et al.*, 1993; Bendelac, 1995; Bendelac *et al.*, 1995; Denkers *et al.*, 1996, 1997a, 1997b; Gazinelli *et al.*, 1996a; Kos and Engleman, 1996; Scharton-Kersten *et al.*, 1996a, 1997a, 1997b; Del Rio *et al.*, 2001, 2004; Caamano and Hunter, 2002; Sacks and Sher, 2002; Maeckner *et al.*, 2005; Chan *et al.*, 2006).

The ability of T. gondii to infect any cell means that disease can be systemic and affect many organs, but in some cases certain organs, including the brain, eye, or intestine, have the predominant pathology. These individual organs have been studied in detail using animal, most often murine, models of disease. The strong influence of host genetics on this disease process has been evident from early studies, and has been exploited to identify immunological functional correlates of protection and pathology (Johnson, 1984; McLeod et al., 1989a, 1989b, 1995, 1996; Brown and McLeod, 1990; Blackwell et al., 1993, 1994; Brown et al., 1995b; Montoya and Remington, 1997; Blackwell, 1998; Mack et al., 1999; Mordue et al., 2001; Ottenhoff et al., 2002; Vilches and Parham, 2002; Trowsdale and Parham, 2004; Morley et al., 2005). The selection of genetically different strains of mice with specific susceptibilities to different disease manifestations has allowed a careful dissection of immune responses in various organs, and has provided valuable insights into disease manifestations in humans with different disease patterns. Most notably, these include toxoplasmic encephalitis (TE) (Ghatak and Zimmerman, 1973; Hoff et al., 1976; Jones et al., 1977; Masur et al., 1978; Ferguson and Hutchison, 1987a, 1987b; Holland et al., 1988; Israelski et al., 1989; Suzuki et al., 1989, 1991a; Foulon et al., 1990; Suzuki and Remington, 1990, 1993; Gazzinelli et al., 1992a, 1992b, 1992c, 1993a; Kwon et al., 1992; Suzuki et al., 1993a, 1993b; Deckert-Schluter et al., 1994c, 1995, 1996, 1998a, 1998b, 1999; Suzuki and Joh, 1994; Brown et al., 1995b; Minamidani et al., 1996; Suzuki, 1997; Schluter et al., 1998; Luder et al., 1999, 2003a,

2003b; Kang and Suzuki, 2001; Kang *et al.*, 2003; Kwok *et al.*, 2003) and ocular toxoplasmosis (OT) (Gazzinelli *et al.*, 1994a; Roberts and McLeod, 1999; Roberts *et al.*, 2000).

The influence of T. gondii genetics on the pathogenesis of both murine and human T. gondii infection has become increasingly evident in recent years. In experimental studies, the route and lifecycle stage used to initiate infection has been varied according to convenience or scientific rationale, or to induce particular disease manifestations, and must also be noted when considering studies in the literature. It is also important to try to correlate findings of animal models with clinical findings and studies of human materials (Frenkel, 1955, 1990; Hogan et al., 1958; Eichenwald, 1960; Hogan, 1961; Desmonts, 1966; Vainisi and Campbell, 1969; Desmonts and Couvreur, 1974, 1984; Koppe et al., 1974, 1986; Hoerni et al., 1978; Murray et al., 1979, 1980, 1985a, 1985b; Ryning et al., 1979; Wilson and Remington, 1979; McLeod et al., 1980, 1984, 1985a, 1985b, 1988, 1990, 2006; Wilson et al., 1980a, 1980b; Luft et al., 1983, 1984a, 1984b; Couvreur et al., 1984, 1991, 1993; Nathan et al., 1984; Wilson and Haas, 1984; Wilson, 1985; Catterall et al., 1986; Dutton et al., 1986; McLeod and Mack, 1986; Daffos et al., 1988; Silveira et al., 1988; Dutton, 1989; Hohlfeld et al., 1989; Yano et al., 1989; Brown and McLeod, 1990; Huskinson et al., 1990; Mitchell et al., 1990; Sibalic et al., 1990; Bulow and Boothroyd, 1991; Hunter et al., 1992; Roberts and Alexander, 1992; Berger et al., 1993; Curiel et al., 1993; Wong et al., 1993; Brezin et al., 1994; McAulev et al., 1994; Biggs et al., 1995; Channon and Kasper, 1996; Decoster, 1996; Purner et al., 1996; Vogel et al., 1996; Buzoni-Gatel et al., 1997; Mets et al., 1997; Burnett et al., 1998; Subauste et al., 1998a, 1998b; Gormley et al., 1999; Mack et al., 1999; Channon et al., 2000; Lee et al., 2000; McLeod and Dowell, 2000; Blader et al., 2001; Labalette et al., 2001; Silveira et al., 2001; Caamano and Hunter, 2002; Fardeau et al., 2002; Rothova, 2003; Holland, 2004; Wallon et al., 2004; Cavailles et al., 2006a, 2006b; Garweg et al., 2005; Vallochi et al., 2005).

In the past ten years there have been a number of scholarly, comprehensive reviews which describe



FIGURE 23.1 Processing of exogenous *T. gondii* antigen, presentation on MHC class II and activation of CD4+T lymphocytes.

- 1. In the endoplasmic reticulum (ER), class II MHC (MHC II) molecules are bound by the invariant chain.
- Export of the class II MHC-invariant chain complex from the ER, through the Golgi and to class II MHC endocytic compartments.
- 3. Endosomes containing *T. gondii*-derived proteins fuse with the class II MHC endocytic compartments. The invariant chain is proteolytically cleaved, allowing internalized peptides to bind class II MHC molecules. In humans, Human Leukocyte Antigen (HLA) DM molecules facilitate peptide loading.
- 4. Class II MHC molecules carrying peptide cargo are exported to the surface of the Antigen Presenting Cell (APC).
- 5. Peptides are presented on class II MHC to CD4+ T lymphocytes, which interact via their surface α/β -TCR and CD4 molecules. The CD3 complex becomes associated with α/β -TCRs and act as a docking site for tyrosine kinases that transmit activating intracellular signals. CD28 on the surface of T cells interacts with CD80 and/or CD86 on the APC to provide co-stimulation for the T cell.

Continued

- 6. CD40 is constitutively expressed on dendritic cells and interacts with CD40 ligand (CD40L) expressed on the surface of activated T cells. This increases expression of CD80–86 on dendritic cells, and enhances T-cell co-stimulation.
- 7. Engagement of CD40 activates the dendritic cell. Production of cytokines such as IL-12 by dendritic cells favors the differentiation of CD4+ T cells into Th1 cells that are characterized by IFN- γ production but have low or undetectable IL-4 production.
- 8. T cells express CTLA-4 at the later stages of activation. Engagement of this with CD80/86 induces negative signals to the T cell which terminate their activation.

(Includes concepts from Figure 5 in McLeod *et al.* (1996), and Figure 4.1 in Lewis and Wilson (2005), with permission.)

This figure is reproduced in color in the color plate section.



FIGURE 23.2 For legend see opposite page.

cell-mediated immunity, newborn immunity, dendritic cells, and antigen processing and presentation, and how each of these areas is relevant to toxoplasmosis (Wilson, 1985; Wilson et al., 1986; Parker et al., 1991; Ohtake et al., 1992; Gazinelli et al., 1993a, 1993b, 1993c; Olle et al., 1994; McLeod et al., 1995, 1996; Peacock et al., 1995; Gazzinelli et al., 1996a, 2004; Hunter et al., 1996; Pelloux and Ambroise-Thomas, 1996; Bruning et al., 1997; Denkers et al., 1997a, 1997b, 2003a, 2003b; Montoya and Remington, 1997; Denkers and Gazzinelli, 1998; Yap and Sher, 1999; McLeod and Dowell, 2000; Ropert and Gazzinelli, 2000; Butcher et al., 2001; Luder and Seeber, 2001; Butcher and Denkers, 2002; Scott and Hunter, 2002; Teixeira et al., 2002; Aliberti et al., 2003, 2004; Sinai et al., 2004; Taylor et al., 2004; Denkers and Butcher, 2005; Lewis and Wilson, 2005; Courret et al., 2006). References included in these reviews are incorporated in the reference list at the end of this chapter, for completeness. This chapter does not reiterate the detailed summaries of the literature already presented in these outstanding reviews, but instead includes relevant references, summary figures, and tables from these earlier works, as well as tables created for this chapter that provide overviews and outline the mechanisms involved in the T. gondii immune response (Figures 23.1-23.6; Tables 23.1, 23.2). These tables develop the concepts that the immuno response contributes to both protection and pathogenesis of disease, e.g. retinal lesions in both the immunocompetent and immunocompromised person (Figure 23.7). We have focused this chapter on work from our own laboratory groups, papers published recently that have shaped and extended concepts in the earlier reviews, and papers that have provided important mechanistic insights into the immune response to T. gondii.

FIGURE 23.2 Processing of endogenous antigen presentation on MHC class I and activation of CD8+ T lymphocytes to kill *T. gondii*-infected cells:

- 1. Proteins escaping or released from the *T. gondii* parasitophorous vacuole are proteolytically cleaved in the proteosome and enter the ER via TAP (translocator associated with antigen processing) molecules. These peptides are loaded onto class I MHC (MHC I) molecules in the ER.
- 2. The class I MHC peptide complex is exported from the ER, through the Golgi and ultimately to the surface of the cell.
- 3. Peptides presented on class I MHC are recognized by CD8+ T lymphocytes via their surface α/β -TCR and CD8 molecules. The CD3 complex associated with α/β -TCRs acts as a docking site for tyrosine kinases that provide activating intracellular signals. CD28 on the surface of T cells interacts with CD80 and/or CD86 to provide co-stimulation for T cells. CD40 is expressed constitutively on dendritic cells and on ligation with CD40 ligand (CD40L), expressed on the surface of activated cells, increases CD80–86 expression on dendritic cells, and enhances T-cell co-stimulation. Engagement of CD40 activates the dendritic cell to produce cytokines such as IL-12 see Figure 23.1). Dendritic cells use a process called 'cross-presentation' to transfer proteins taken up as part of necrotic or apoptotic debris into the class I endogenous processing pathway.
- 4. Cytolytic CD8+T cells recognize specific *T. gondii*-derived peptides presented by class I MHC molecules. Ligation of FasL on the surface of CD8+T cells with Fas on the surface of target cells induces apoptosis of target cells.
- 5. Following recognition of specific *T. gondii*-derived peptides presented by class I MHC molecules, CD8+ T cells can also kill target cells by release of perform and granzyme.
- 6. CD8+ T cells also release IFN- γ , which can induce IDO and iNOS in target cells, which are effective in killing *T. gondii* tachyzoites.
- 7. At the later stages of activation, T cells express CTLA-4 which, when engaged with CD80/86, delivers negative signals to the T cell that favor termination of activation (see Figure 23.1).

(Includes concepts from Figure 4.1 in McLeod *et al.* (1996) and Figure 4.1 in Lewis and Wilson (2005), with permission).

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FIGURE 23.3 Proposed afferent interactions of dendritic cells with Toxoplasma, NK, CD8+T, and CD4+T cells.

- 1. *T. gondii* releases a number of molecules that have immunological effects. GIPL binds TLR-4, profilin binds TLR-11, and Cp-18 binds CCR5 and HSP70, which induces dendritic cell maturation through an unknown mechanism (not all of these interactions may happen in all mammals, and TLR-11 apparently is not functional in humans).
- 2. The net effect of these molecules is dendritic cell activation and maturation with the likely production of IFN, IL-12, IL-15, IL-18, IL-23, and IL-27. These mediators act on NK cells and CD4+ and CD8+ T cells.
- 3. NK cells produce IFN-γ which, together with IL-12, favors Th1-cell as opposed to Th2-cell maturation.
- 4. CD4+ T-cell production of IL-2 further activates NK cells and favors the expansion of cytolytic CD8+ T cells that can kill *T* gondii-infected cells, by a number of methods (see Figure 23.2).

(Includes concepts from Figure 5 in McLeod *et al.* (1996) and Figure 4.2 in Lewis and Wilson (2005), with permission.)



FIGURE 23.4 Proposed regulatory interactions of dendritic and mononuclear phagocytic cells with *Toxoplasma*, NK, CD8+ T, and CD4+ T cells (Th1 and Th2), NKT, and regulatory T cells. Note differentiation of CD4 cells into Th1 and Th2 cells, and interactions with NKT cells and regulatory T cells. NKT cells recognize glycolipids in the presence of CD1. (Includes concepts from Figure 5 in McLeod *et al.* (1996) and Figure 4.2 in Lewis and Wilson (2005), with permission.) This figure is reproduced in color in the color plate section.

23.2 MOUSE GENETIC STUDIES

23.2.1 Mortality

A role for the H-2 complex (MHC) in mediating protection or conferring susceptibility to mice, measured as survival following intraperitoneal infection with *T. gondii*, was first demonstrated by Williams and colleagues (Williams *et al.*, 1978). In this study, susceptibility was found to be associated with the H-2a and H-2b haplotypes, while resistance was associated with the H-2d and H-2k haplotypes. Susceptibility as measured by mortality is a complex trait, and the investigators, even in this early study, recognized that other genes were involved. Jones and colleagues recognized that mouse strain influenced brain cyst numbers (Jones *et al.*, 1977).

To develop a model more biologically like the naturally acquired infection, the natural history and pathology of infection in a variety of strains of mice and Toxoplasma were characterized, and patterns of susceptibility based on host genotype and of various parasite isolates were noted (McLeod et al., 1984). The ME49 strain was selected for further studies because it caused little mortality and a high cyst burden, and when administered on the eleventh day of gestation led to 100 percent transmission to the fetus in outbred Swiss-Webster mice. Thus, this model could be used for testing effects of attenuated vaccines - i.e. the temperature-sensitive mutant 4 (Ts-4) vaccine strain - on the congenital transmission of T. gondii (McLeod et al., 1984). These studies provided a foundation for work to characterize genetic susceptibility measured as survival and cyst



FIGURE 23.5 Differentiation of antigenically naïve CD4+ T cells into Th1, Th2, unpolarized, and follicular helper effector and memory T cells. Antigenically naïve CD4+ cells express high levels of the CD45RA isoform of the CD45 surface protein tyrosine phosphatase. They are activated by antigen presented by antigen-presenting cells (APCs) to express CD40 ligand and interleukin (IL)-2, and to undergo clonal expansion and differentiation, which is accompanied by expression of the CD45RO isoform and loss of the CD45RA isoform. Most effector cells die by apoptosis, but a small fraction of these cells persist as memory cells which express high levels of CD45RO. Exposure of expanding effector cells to IL-12 family cytokines, IL-18, and interferon (IFN)- γ favors their differentiation into Th1 effector cells that secrete IFN- γ , whereas exposure to IL-4 favors their differentiation into Th1 effector cells that secrete IL-4, IL-5, and IL-13. Many memory cells are non-polarized and do not express either Th1 or Th2 cytokines. They may be enriched for cells that continue to express the CCR7 chemokine receptor which favors their recirculation between Th2 cytokines. They also may be enriched for cells that continue to express the CCR7 chemokine receptor which favors their recirculation between the blood and the lymph nodes and spleen. T follicular helper cells, which express high levels of CXCR5, move into B-cell follicle areas, where they express CD4O ligand and provide help for B-cell responses. The signals that promote the accumulation of memory T follicular helper cells and their capacity to produce cytokines are poorly understood. Memory cells rechallenged with antigen undergo rapid clonal expansion into secondary effector cells that mediate the same functions as the initial memory population. Most secondary effector cells eventually die by apoptosis. (Adapted from Figure 4.6 in Lewis and Wilson (2005), with permission.)

This figure is reproduced in color in the color plate section.

formation using inbred and, later, recombinant congenic, gene-deficient, and transgenic strains of mice (McLeod *et al.*, 1989a; 1989b; Brown and McLeod, 1990; Brown *et al.*, 1995b; Johnson *et al.*, 2002a, 2002b; Smiley *et al.*, 2005). The ability of mice to survive for the first 30 days after infection when the ME49 strain of *T. gondii* was orally administered was found to be under polygenic control and to be regulated by a minimum of five genes, one of which maps to the H-2 region (Figures 23.8, 23.9) (McLeod *et al.*, 1989b). An important observation in this work is that there is not a simple association between the development of brain cyst burden, and mortality (McLeod *et al.*, 1984, 1989a, 1989b).

The route of infection was discovered to influence mortality in experimental toxoplasmosis (Johnson, 1984; McLeod *et al.*, 1989a). For example, C57BL/6 mice were found to be susceptible to oral infection, but resistant to intraperitoneal infection (Johnson, 1984; McLeod *et al.*, 1989a). In contrast, LACA mice exhibited the reverse characteristics (Johnson, 1984). Interestingly, dendritic cells disseminate *T. gondii* early on from the intestine to the brain (Courret *et al.*, 2006). As a separate aspect of pathogenesis associated with peroral acquisition, studies have revealed that C57BL/6 mice develop fatal enteritis when infected by the

oral route. The characteristics of the inflamatory response and parasite antigens (e.g. SAG1) involved in this inflammatory bowel disease following peroral infection have been defined (Liesenfeld *et al.*, 1996, 1999; Alexander *et al.*, 1997; Alexander and Hunter, 1998; Alexander *et al.*, 1998; Suzuki *et al.*, 2000a; Nickdel *et al.*, 2004). The genes involved in the susceptibility of C57BL/6 mice to oral infection were studied with genetic mapping of survival of A × B and B × A mice following peroral infection. Subsequent analyses of this



FIGURE 23.6 (A) Putative stages of human $\alpha\beta$ -T cell receptor-positive ($\alpha\beta$ -TCR+) thymocyte development. Prothymocytes expressing CD7 are produced in the bone marrow or fetal liver. These enter the thymus via vessels at the junction between the thymic cortex and medulla. In the thymus, these cells differentiate to more mature $\alpha\beta$ -TCR+ thymocytes (defined by their pattern of the $\alpha\beta$ -TCR-CD3 complex, CD4, CD8, and CD38). Rearrangement of the TCR α and TCR β chain genes occurs in the outer cortex. Positive selection occurs mainly in the central thymic cortex and involves the interaction of thymic epithelial cells. Negative selection occurs mainly in the medulla, and involves the interaction of thymic dendritic cells. Medullary thymocytes emigrate into the circulation and colonize the peripheral lymphoid organs. These T cells are CD4+ and CD8+ with high levels of the $\alpha\beta$ -TCR-CD3 complex. These cells are referred to as 'recent thymic emigrants' (RTEs), and probably lack CD38 surface expression. (Modified from Figure 4.3 in Lewis and Wilson (2005), with permission.)

(B) Transmission and severity of illness in each trimester. In general, as gestation progresses the frequency of maternal to fetal transmission increases and the severity of illness in the newborn infant diminishes. This figure is reproduced in color in the color plate section.

ADAPTIVE IMMUNITY AND GENETICS OF THE HOST IMMUNE RESPONSE

С	Gestation	Birth	6 months	1 year
NK cell	6 weeks, present in liver	15% of total lymphocytes Double absolute number present in adults 50% of adult cytolytic activity Overall increase in number but decrease in function		Full cytolytic activity reached in late infancy
Monocyte/ macrophage & dendritic cell	4 weeks, detected in yolk sac then liver and bone marrow	Monocytes number > adults IL-1 equals adult Diminished IL-6 and TNF- α Cord blood DC less effective than adult in T-cell support		Chemotaxis < adult for 6-10 years
Neutrophil	Limited storage pool. 14-16 weeks, precursors seen 22-24 weeks, 10% of circulating leukocytes	50-60% of circulating leukocytes 40-45% adhesive capability to endothelium Poor chemotactic ability Normal superoxide anion Decreased hydroxyl radicals		
Eosinophil	18-30 weeks, 10-20% of total granulo- cytes	Post-natal peak at 3-4 weeks		
T cell	7 weeks, detected in yolk sac and liver 8 weeks, lymphoid colo- nization and reduced TCR diversity and TdT enzyme activity 10 weeks, lymphoid tissues, liver and bone marrow 14 weeks, all three thymocytes in proper location 16 weeks, improved TCR diversity and TdT activity	Virgin subset T cells Normal IL-2 synthesis and IL-2R expression Reduced TNF-α, IL-3, IL-4, IL-5, IFN-γ, and GM-CSF CTL activity variable Diminshed production of IFN-γ No DTH skin reaction to antigens and diminished reaction after stimulation	Peak T-cell number	Adult T-cell number, age 4 Peak size of thymus, age 10 Decreased skin reaction persists to age 1
B cell	8 weeks, maternal IgG crosses placenta (majority <i>in utero</i>) 15 weeks, IgM-secreting plasma cells present 17 weeks, some circula- ting fetal IgG may first be seen 20-30 weeks, Igg and Iga plasma cells first appear	T1-type 1 antigen response T-dependent response primary	T1-type 2 antigen response to encap- sulated organisms Maximum IgM at 2-6months	Circulating IgG is nearly all from infant (nadir at 3-4 months)
CD type	NK CD16 at 6 weeks ges- tation	NK CD56 50% NK CD57 decreased		
	T-cell thymocytes express CD7 at 7 weeks gestation T-cell thymocytes express CD3, CD7, CD4, and CD8 at 12-14 weeks gestation T-cell thymocytes express CD4 or CD8 -14 weeks gestation	Most Cd4 [†] T cells express CD45RA ⁺ marker (naïve)	CD45RA ⁺ cells → CD45RO ⁺ with age and antigen stimulation	

FIGURE 23.6 (C) Putative development of human T cells, NK cells, monocyte/macrophage/dendritic cells, neutrophils, eosinophils, and B cells. (Adapted from Table 2 in McLeod and Dowell (2000), and Figure 4.3 in Lewis and Wilson (2005), with permission.)

MOUSE GENETIC STUDIES

T-cell surface		Corresponding	
molecule	T-cell distribution	ligand(s)on APCs	APC distribution
CD2	Most T cells; higher on memory cells, lower on adult naïve and neonatal T cells	LFA-3 (CD58), CD59	Leukocytes
CD4	Subset of $\alpha\beta$ T cells with predominantly helper activity	MHC class II β chain	Dendritic cells, Mø, B cells, others (see text)
CD5	All T cells	CD72	B cells, Μφ
CD8	Subset of $\alpha\beta$ T cells with predominantly cytotoxic activity	MHC class 1 heavy chain	Ubiquitous
LFA-1 (CD11a/ CD18)	All T cells; higher on memory cells, lower on adult naïve and neonatal T cells	ICAM-1 (CD54) ICAM-2 (CD 102) ICAM-3 (CD50)	Leukocytes (ICAM-3 > ICAM-1, -2) and endothelium
			(ICAM-1, ICAM-2); most ICAM-1 expression requires activation
CD28	Most CD4 ⁺ T cells, subset of CD8 ⁺ T cells	CD80 (B7-1) CD86 (B7-2)	Dendritic cells, Mø, activated B cells
ICOS	Effector and memory T cells; not on resting naïve cells	B7RP-1 (B7h)	B cells, Mφ, dendritic cells, endothelial naïve cells
VLA-4 (CD49d/ CD29)	All T cells; higher on memory cells, lower on adult naïve and neonatal T cells	VCAM-1 (CD 106)	Activated or inflamed endothelium (increased by TNF, IL-1, IL-4)
ICAM-1 (CD54)	All T cells; higher on memory cells, lower on adult virgin and neonatal T cells	LFA-1 (CD11a/ CD18)	Leukocytes
CTLA-4 (CD152)	Activated T cells	CD80 CD86	Dendritic cells, Mø, activated B cells, activated T cells
CD40 ligand (CD154)	Activated CD4 ⁺ T cells; lower on neonatal CD4 ⁺ T cells	CD40	Dendritic cells, Mø, B cells, thymic epithelial cells
PD-1	Activated CD4 ⁺ and CD8 ⁺ T cells	PD-L1, PD-L2	Dendritic cells, Mø, B cells, regulatory T cells

TABLE 23.1 Selected pairs of surface molecules involved in T cell-antigen-presenting cell (APC) interactions

CTLA-4, cytotoxic T lymphocyte antigen-4; ICAM, intercellular adhesion molecule; ICOS, inducible co-stimulator; IL, interleukin; LFA, leukocyte function antigen; M ϕ , mononuclear phagocytes; MHC, major histocompatibility complex; PD, programmed death [molecule]; VCAM, vascular cell adhesion molecule; VLA-4, very late antigen-4.

Adapted from Lewis and Wilson (2005), with permission.

original typing $A \times B$ and $B \times A$ mice (McLeod *et al.*, 1989b; Johnson *et al.*, 2002a) in the context of further information about the mouse genome and more sophisticated analytical programs (Dai *et al.*, 1994; Collazo *et al.*, 2002; Fujigaki *et al.*, 2002; Khan *et al.*, 2002; Khan *et al.*, 2002; Khan *et al.*, in press) has demonstrated that genes or gene regions involved include regions

on chromosomes 1, 8, 7, and 11 (Figure 23.9; Table 23.3) (Johnson *et al.*, 2002a).

Mortality following intraperitoneal infection has also been linked to the H-13 locus through examination of recombinant inbred mice originating from BALB/c \times C57BL/6J mice. Of the seven recombinant lines obtained, the three most susceptible were *Text continued on p. 641*

Gene deficiency	Mouse background	Parasite strain	Route of infection	Effect on parasite	Effect on pathology	Effect on survival	Immunological effects	Reference
IL-2	C57BL/6	ME49	i.p.	No difference in parasite number in brain	None reported	Reduced	Reduced IL-12p40 and IFN-γ	Villegas <i>et al.</i> (2002)
IL-4	C57BL/6	Beverley (type II)	Oral	No difference in intestine	Decreased necrosis in intestine	Enhanced	Increased plasma IFN-γ and IL-12; increased IL-10 transcripts in intestine	Nickdel <i>et al.</i> (2004)
IL-4	129/J	Me49 (type II)	Oral	Increased tachyzoites and cysts in brain	Increased areas of focal inflammation	Reduced	No difference in transcripts for IFN-γ, TNF-α, IL-6, and IL-10; reduced IFN-γ production by antigen stimulated splenocytes	Suzuki <i>et al.</i> (1996a)
IL-4	129/Sv× C57BL/6	Beverley (type II)	Oral	Reduced cysts in brain	Decreased encephalitis; decreased necrotic lesions	Reduced	Increased IFN-γ (day 7), reduced IL-10 (day 28)	Roberts <i>et al.</i> (1996a)
IL-5	C57BL/6	Me49 (type II)	i.p.	Increased parasite number in brain	Not reported	Reduced	Reduced splenocyte IL-12 production	Zhang and Denkers (1999)
IL-5	C57BL/6	Beverley (type II)	Oral	No difference in intestine	Decreased necrosis in intestine	Enhanced	Increased plasma IFN-γ and IL-12; reduced eosinophilia	Nickdel <i>et al.</i> (2001)
IL-6	129/Sv× C57BL/6	Me49 (type II)	i.p.	Increased % of infected peritoneal cells	Increased necrosis in brain	Not reported	Reduced transcripts for IFN-γ and increased transcripts for IL-10 in brain	Suzuki <i>et al.</i> (1997)
IL-6	129/SvJ	Beverley (typeII)	Oral	Increased cyst number and uncontrolled tachyzoites replication in brain	Increased brain pathology	Reduced	Reduced serum IL-6 levels	Jebbari <i>et al</i> . (1998)

 TABLE 23.2A
 The effect of interleukin or interleukin receptor gene deficiency on murine T. gondii infection

IL-6	129/SVJ	Beverley (type II)	Intraperi- toneal	Increased tachyzoite number	Increased inflammation in the eye	Not reported	Decreased ocular IL-1 mRNA transcripts; increased ocular TNF-α mRNA transcripts	Lyons <i>et al.</i> (2001)
IL-10	C57BL/6	Me49 (type II)	Oral	Not reported	Increased necrosis in ileum	Reduced	Increased IFN-γ mRNA transcripts	Suzuki <i>et al.</i> (2000a)
IL-10	BALB/c	Me49 (type II)	Oral	Not reported	Focal necrosis in ileum	Reduced	Not reported	Suzuki <i>et al</i> . (2000a)
IL-10	C57BL/6	Me49 (type II)	i.p.	None observed in brain	Increased inflammation and necrosis in brain	Reduced	Increased CD4+ T cells in brain; partial depletion of CD4+ T cells; decreased inflammation and increased survival	Wilson <i>et al.</i> (2005)
IL-10	C57BL/6	Me49 (type II)	Oral	Reduced number (brain)	Increased liver pathology	Increased	Increased serum IL-12(p70), IL-12p(40) and IFN-γ	Gazzinelli <i>et al.</i> (1996b)
IL-10	C57BL/6	Me49 (type II)	i.p.	Increased numbers (intestine and liver)	Not reported	Reduced	Not reported	Wille <i>et al.</i> (2004)
IL-10	BALB/c	RH (type I)	i.p.	Not reported	Not examined	None	No difference in serum IL-12p40 or IFN-γ levels	Wille <i>et al.</i> (2001)
IL-10	C57BL/6	RH	Intra- ocular	None reported	Increased necrosis and inflammation in the eye	Not reported	Increased serum IFN- γ	Lu <i>et al</i> . (2003)
IL-10	BALB/c	RH	Intra- ocular	None reported	Increased necrosis and inflammation in the eye	Not reported	Increased serum IFN- γ	Lu <i>et al.</i> (2003)
p40 (IL-12/ IL-23)	C57BL/6	ts-4	i.p.	Not reported	Not reported	Reduced	Surviving mice have increased resistance to increased doses of ts-4 or infection with 76K strain of <i>T. gondii</i>	Ely <i>et al.</i> (1999)
p40 (IL-12/ IL-23)	BALB/c	ts-4	i.p.	Not reported	Not reported	Reduced	Reduced serum IFN-γ	Lieberman <i>et al.</i> (2004)

MOUSE GENETIC STUDIES

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Continued

Gene deficiency	Mouse background	Parasite strain	Route of infection	Effect on parasite	Effect on pathology	Effect on survival	Immunological effects	Reference
p35 (IL-12)	BALB/c	ts-4	i.p.	Increased numbers (peritoneal exudate)	Not reported	Reduced	Reduced serum IFN-γ	Lieberman et al. (2004)
p40 (IL-12/ IL-23)	C57BL/6	ME49	i.p.	Increased number in brain and lung	Not reported	Reduced	Not reported	Villarino <i>et al</i> . (2003)
p40 (IL-12/ IL-23)	C57BL/6	Me49 (type II)	i.p.	Not reported	Not reported	Reduced	Exogenous IL-12 restores resistance but reacti- vation occurs on withdrawal	Yap <i>et al.</i> (2000)
p40 (IL-12/ IL-23)	C57BL/6	Me49 (type II)	i.p.	Increased % of infected peritoneal cells	Not reported	Reduced	Not examined	Scharton- Kersten <i>et al.</i> (1997b)
p40 (IL-12/ IL-23)	C57BL/6	ME49	i.p.	Increased numbers (peritoneal exudate)	Not reported	Reduced	None reported	Lieberman <i>et al.</i> (2004)
p35 (IL-12)	C57BL/6	ME49	i.p.	None	Not reported	Reduced	None reported	Lieberman <i>et al.</i> (2004)
IL-15	C57BL/6	Me49 (type II)	i.p.	Not reported	None	None	Similar levels of IFN-γ production and T-cell activation	Lieberman <i>et al.</i> (2004)
IL-18	C57BL/6	Me49 (type II)	Oral	No difference in lung; increased number in liver; reduced number in intestine	Reduced necrosis in intestine and liver	Increased	Reduced serum IFN-γ; reduced intestinal IFN-γ production	Vossenkamper <i>et al.</i> (2004)
p19 (IL-23)	C57BL/6	ME49	i.p.	None	Not reported	None	No difference in serum IFN-γ or IL-12 levels	Lieberman <i>et al</i> . (2004)
WSX-1 (IL-27R)	C57BL/6	ME49	i.p.	Increased numbers (peritoneal exudate)	Increased inflammation and necrosis in liver and lungs	Reduced	Increased serum IL-12 and IFN-γ, increased T-cell activation	Villarino <i>et al.</i> (2003)

 TABLE 23.2A
 The effect of interleukin or interleukin receptor gene deficiency on murine T. gondii infection—cont'd

Gene deficiency	Mouse background	Parasite strain	Route of infection	Effect on parasite	Effect on pathology	Effect on survival	Immunological effects	Reference
ITLR1	129/Ola× C57BL/6	PTGluc (type II)	i.p.	None	None	None	None examined	Hitziger <i>et al.</i> (2005)
TLR2	129/Ola× C57BL/6	PTGluc (type II)	i.p.	None	None	None	None examined	Hitziger <i>et al.</i> (2005)
TLR2	C57BL/6	Fukaya	Oral	None	Damaged renal function; glomerular and extracellular matrix swelling, advancing glomerular tissue proliferation, thickened Bowman's capsules and vacuolization of tubules	Not reported	None reported	Kudo <i>et al.</i> (2004)
TLR2	C57BL/6	Fukaya	Increased inflam- mation in lungs	No difference in brain or lung	Increased pathology in lungs	Reduced	Reduced PEC production of IL-12 and IFN- γ ; increased PEC production of IL-4 and IL-10; reduced iNOS and IDO expression and NO production	Mun <i>et al.</i> (2003a)
TLR4	129/Ola× C57BL/6	PTGluc (type II)	i.p.	None	None	None	None examined	Hitziger <i>et al.</i> (2005)
TLR4	C57BL/6	Fukaya	Oral	Increased numbers (kidney)	Damaged renal function	Not reported	None reported	Kudo <i>et al.</i> (2004)
TLR4	C57BL/6	Fukaya	None	No difference in brain or lung	Not reported	None	C57BL/6	Mun <i>et al</i> . (2003a)
TLR6	129/Ola× C57BL/6	PTGluc (type II)	i.p.	None	None	None	None examined	Hitziger <i>et al.</i> (2005)
TLR9	129/Ola× C57BL/6	PTGluc (type II)	i.p.	Increased dissemination	None	None	None examined	Hitziger <i>et al.</i> (2005)

 TABLE 23.2B
 The effect of TLR gene deficiency on murine T. gondii infection

Gene deficiency	Mouse background	Parasite strain	Route of infection	Effect on parasite	Effect on pathology	Effect on survival	Immunological effects	Reference
MyD88	129/Ola× C57BL/6	PTGluc (type II)	i.p.	None	None examined	Reduced	None examined	Hitziger <i>et al.</i> (2005)
MyD88		Fukaya	Increased inflam- mation in lungs	No difference in brain or lung	Increased pathology in lungs	Reduced	Reduced PEC production of IL-12 and IFN-γ; increased PEC production of IL-4 and IL-10; reduced iNOS IDO expression and NO production	Mun <i>et al.</i> (2003a)
MyD88	129/Ola× C57BL/6	ME49	i.p.	Increased parasite number in liver, lung, heart, spleen and brain	None reported	Reduced	Reduced plasma IL-12p40 and IFN-γ	Scanga <i>et al.</i> (2002)
c-Rel	C57BL/6	Me49	Oral	Increased numbers (peritoneal macrophages)	More severe encephalitis	Reduced	Decreased T cell activation, proliferation and IFN-γ production	Mason <i>et al.</i> (2004b)
STAT1	129Sv/Ev	Me49	i.p.	Increased numbers (peritoneal exudate)	No information	Reduced	Reduced iNOS, IGTP, and LRG-47; failure to upregulate IL-12Rbeta2; reduced IFN-γ- producing CD4 + and CD8+T cells; increased IL-4, Arg1, Ym1, and Fizz1	Gavrilescu et al. (2004)

 TABLE 23.2C
 The effect of cell signaling gene deficiency on murine T. gondii infection

STAT1	SvEv	Me49	i.p.	Not reported	Not reported	Reduced	Failure to upregulate MHC; reduced NO production; reduced IFN-inducible GTPase proteins	Lieberman <i>et al</i> . (2004)
STAT1	129/Sv/Ev	ME49	i.p.	Not reported	Not reported	Reduced	Not reported	Collazo <i>et al.</i> (2002)
STAT4	129/Sv/ C57BL/6	Me49 (type II)	i.p.	Not reported	Not reported	Reduced	Reduced plasma IFN-γ	Cai <i>et al</i> . (2000a)
NF- kappaB(2)	C57BL/6	Me49 (type II)	i.p.	Increased % infected PEC	Increased toxoplasmic encephalitis	Reduced	Reduced IFN-γ production and loss of CD4+ and CD8+ T cells; increased expression of Fas and apoptosis in spleen	Caamano <i>et al</i> . (2000)
NF- kappaB/ p52	C57BL/6	Me49 (type II)	i.p.	Increased % infected PEC	Not reported	Reduced	Not reported	Franzoso <i>et al</i> . (1998)
Rel-B	C57BL/6	Me49 (type II)	i.p.	No difference in brain cyst burdens	Necrotizing myocarditis and interstitial pneumonia in moribund animals	Reduced	Reduced IFN-γ levels and NK cell function	Caamano <i>et al</i> . (1999)
ICSBP	C57BL/6	Me49 (type II)	i.p.	Increased cysts in brain; increased numbers of tachyzoites in brain	Not reported	Reduced	Reduced serum IFN-γ and IL-12	Scharton- Kersten <i>et al.</i> (1997b)
LRG-47	C57BL/6× 129SvJ	ME49	i.p.	Increased cysts in brain	Not examined	Reduced	Modest increase in serum IFN-γ and IL-12p40	Collazo <i>et al.</i> (2001)

Continued
Gene deficiency	Mouse background	Parasite strain	Route of infection	Effect on parasite	Effect on pathology	Effect on survival	Immunological effects	Reference
IRG-47	C57BL/6× 129SvJ	ME49	i.p.	None	Not examined	Reduced	None reported	Collazo <i>et al.</i> (2001)
IRF	C57BL/6	PLK	i.p.	Not determined	Not determined	Reduced	Increased splenocyte IL-10 mRNA transcripts; similar splenocyte IL-2, IL-12, and IFN-γ mRNA transcripts	Khan <i>et al.</i> (1996a)
IRF	129	ME49	i.p.	Increased parasite number in liver, lung, and spleen	Not reported	Reduced	Reduced IDO and iNOS expression in lungs	Silva <i>et al.</i> (2002)
IGTP	C57BL/6× 129Sv	ME49	i.p.	Increased % of infected peritoneal exudates cells	Not reported	Reduced	Cannot be made resistant by bone marrow donation from WT mice	Collazo <i>et al.</i> (2002)
IGTP	C57BL/6× 129Sv	Me49 (type II)	i.p.	Not reported	Not reported	Reduced	Increased plasma IFN-γ and IL-12; increased iNOS transcripts in liver	Taylor <i>et al.</i> (2000)

 TABLE 23.2C
 The effect of cell signaling gene deficiency on murine T. gondii infection—cont'd

Gene deficiency	Mouse background	Parasite strain	Route of infection	Effect on parasite	Effect on pathology	Effect on survival	Immunological effects	Reference
IFN-γ	C57BL/6 BALB/c	Fukaya strain cysts	Peroral	Increased bradyzoite number in the eye; increased cyst burden in the eye	Increased inflammation in the eye	Not reported	None reported	Norose <i>et al.</i> (2003)
IFN-γ	C57BL/6	ME49	i.p.	Not reported	Not reported	Reduced	Not reported	Collazo <i>et al.</i> (2002)
IFN-γ	C57BL/6	ME49	i.p.	Increased parasite number in brain	Not reported	Reduced	None reported	Silva <i>et al.</i> (2002)
IFN-γ	C57BL/6	ME49	i.p.		Not reported	Reduced	Reduced IDO and iNOS expression in lungs	Silva <i>et al.</i> (2002)
IFN-γ	C57BL/6	Fukaya	i.p.	Increase parasite number in brain, spleen, gut, and liver	Not reported	Not reported	Reduced IDO expression in lung and brain	Fujigaki <i>et al.</i> (2002)
IFN-γ	C57BL/6	Me49 (type II)	i.p.	Increased cyst burden in brain	Not reported	Reduced	Not examined	Scharton- Kersten <i>et al.</i> (1997b)
IFN-γ	BALB/c	Fukaya	Oral	Increased number (brain, liver, lung, gut, and spleen)	Not reported	Reduced	Not examined	Norose <i>et al.</i> (2003)
IFN-γ	C57BL/6	Fukaya	Oral	Increased number (brain, liver, lung, gut, and spleen)	Not reported	Reduced	Not examined	Norose <i>et al.</i> (2003)
IFN-γ	BALB/c	Me49 (type II)	i.p.	Increased percent of infected peritoneal cells	Not reported	Reduced	Similar levels of serum IL-12	Scharton- Kersten <i>et al.</i> (1996a)
IFN-γ	C57BL/6	Fukaya	Oral					Kobayashi <i>et al.</i> (1999)

TABLE 23.2D The effect of IFN-γ, TNF-α, LT-α, iNOS or receptor gene deficiency on murine T. gondii infection

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Gene deficiency	Mouse background	Parasite strain	Route of infection	Effect on parasite	Effect on pathology	Effect on survival	Immunological effects	Reference
IFN-γ	BALB/c	Fukaya	Oral					Kobayashi <i>et al</i> . (1999)
IFN-γ	C57BL/6	Fukaya	Oral	Increased numbers (kidney)	No change to renal function	Not reported	None reported	Kudo <i>et al.</i> (2004)
IFN-γ	BALB/c	ME49	Oral	None (brain)	No change to renal function	Not reported	None reported	Wang <i>et al</i> . (2004)
TNF-α	C57BL/6	DX	Oral	Increased parasite numbers in brain	Necrosis in brain; extracellular parasites	Reduced	Reduced intracerebral splenic IFN-γ production	Schluter <i>et al.</i> (2003)
LT-α	C57BL/6	DX	Oral	Increased parasite numbers in brain	Necrosis in brain; extracellular parasites	Reduced	Reduced intracerebral splenic IFN-γ production	Schluter <i>et al.</i> (2003)
TNF/LT-α	C57BL/6	DX	Oral	No difference in parasite number in liver	Necrosis in brain; extracellular parasites	Reduced	Reduced intracerebral splenic IFN-γ production	Schluter <i>et al.</i> (2003)
iNOS	C57BL/6	ME49	i.p.	Increased parasite numbers in liver, lung, heart, and spleen	Not reported	Reduced	None reported	Silva <i>et al.</i> (2002)
iNOS	C57BL/6	ME49	i.p.	Not reported	Not reported	Reduced	Not reported	Collazo <i>et al.</i> (2002)
iNOS	C57BL/6	ME49	i.p.	Increased parasite numbers in lung and brain	Not reported	Reduced	None reported	Silva <i>et al</i> . (2002)

TABLE 23.2D The effect of IFN-γ, TNF-α, LT-α, iNOS or receptor gene deficiency on murine T. gondii infection—cont'd

iNOS	C57BL/6× 129	76K	Oral	Increased parasites in brain and liver	Reduced intestinal and liver pathology	Increased (moder- ately but parasite dose dependent)	Reduced IL-10 and IFN- γ mRNA transcripts in spleens; similar levels of serum IFN- γ and TNF- α	Khan <i>et al.</i> (1988b)
iNOS	C57BL/6 × 129	Me49 (type II)	i.p.	No difference in % infected peritoneal cells; increased brain cyst burdens	Increased necrosis in brain	Reduced	IFN-γ, IL-12 neutrophil- dependent, but iNOS- independent mechanism responsible for early survival	Scharton- Kersten <i>et al.</i> (1997a)
IFN-γR	SvEv	Me49	i.p.	Not reported	Not reported	Reduced	Not reported	Lieberman <i>et al</i> . (2004)
IFN- $\alpha/\beta/R$	SvEv	Me49	i.p.	None	Not reported	None	Not reported	Lieberman <i>et al</i> . (2004)
IFN-γR	129/Sv	DX	Oral	Increased parasite number in intestine, lymphatic tissue, liver, and spleen	Necrotizing hepatitis	Reduced	Reduced expression of MHC II on macro- phages; decreased TNF-α iNOS and IL-1β transcripts	Deckert- Schluter <i>et al.</i> (1996)
IFN-γR	129/Sv	DX	Oral	Increased parasite numbers in brain	Encephalitis present, but no comparison with WT control mice reported	Not reported	Failure to upregulate ICAM-1, MHC class I and II in brain endothelial cells; failure to upregulate LFA-I, ICAM-1, MHC class I and II in microglial cells; reduced TNF-α expression in brain	Deckert- Schluter <i>et al.</i> (1999)
TNFRp55	C57BL/6	ME49	i.p.	Increased parasite numbers, in lung, heart, spleen, and brain	None reported	Reduced	None reported	Silva <i>et al.</i> (2002)

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Gene deficiency	Mouse background	Parasite strain	Route of infection	Effect on parasite	Effect on pathology	Effect on survival	Immunological effects	Reference
TNFRp55	129/Sv × C57BL/6	DX	Oral	Increased parasite numbers in brain	Encephalitis present, but no comparison with WT control mice reported	Not reported	No difference in ability to upregulate ICAM-1, MHC class I and II in brain endothelial cells; no difference in ability to upregulate LFA-I, ICAM-1, MHC class I and II in microglial cells; reduced TNF- α expression in brain	Deckert- Schluter <i>et al.</i> (1999)
TNFRp55	C57BL/6	ME49	i.p.	Increased parasite numbers in liver, lung, and spleen	Not reported	Reduced	None reported	Silva <i>et al.</i> (2002)
TNFRp55	129Sv× C57BL/6	DX	i.p.	Increased parasites in brain and lung	Increased severity of brain pathology including encephalitis and necrosis; pneumonia	Reduced	Reduced levels of mRNA transcripts for iNOS in brain	Deckert- Schluter <i>et al.</i> (1998a)
TNFRp75	129Sv× C57BL/6	DX	i.p.	No difference		No difference	Similar levels of mRNA transcripts for iNOS in brain	Deckert- Schluter <i>et al.</i> (1998a)

TABLE 23.2D The effect of IFN-γ, TNF-α, LT-α, iNOS or receptor gene deficiency on murine T. gondii infection—cont'd

TNFRp55/ Rp75	129Sv × C57BL/6	DX	i.p.	Increased parasites in brain and lung	Increased severity of brain pathology including encephalitis and necrosis; pneumonia	Reduced	Reduced levels of mRNA transcripts for iNOS in brain	Deckert- Schluter <i>et al.</i> (1998a)
TNFRp55/ Rp75	C57BL/6× 129	Me49 (type II)	i.p.	Increased cyst number	Increased severity of TE including necrosis	Reduced	iNOS and IFN-γ induction in brain and peritoneal cavity unimpaired	Yap <i>et al</i> . (1998a)
TNFRp55/ Rp75	129/Sv× C57BL/6	DX	Oral	Not reported	Encephalitis present, but no comparison with WT control mice reported	Not reported	No difference in ability to upregulate ICAM-1, MHC class I and II in brain endothelial cells; no difference in ability to upregulate LFA-I, ICAM-1, MHC class I and II in microglial cells; reduced TNF-α expression in brain	Deckert- Schluter <i>et al.</i> (1999)

Gene deficiency	Mouse background	Parasite strain	Route of infection	Effect on parasite	Effect on pathology	Effect on survival	Immunological effects	Reference
CD1d	BALB/c	Me49 (type II)	Oral	Increased numbers	Not reported	None	None reported	Smiley <i>et al.</i> (2005)
CD1d	C57BL/6	Me49 (type II)	Oral	Increased numbers	Increased intestinal pathology; increased weight loss	Reduced	Increased serum IFN-γ	Smiley <i>et al.</i> (2005)
CD4	C57BL/6	RH	Intraocular	Increased number of tachyzoites	Decreased necrosis and inflammation in the eye	Not reported	No increase in IFN- γ or TNF- α as seen in WT	Lu <i>et al.</i> (2004)
CD4	C57BL/6	76K	Oral	Increased cysts (brain)	Reduced pathology in liver and gut; increased pathology in brain	Enhanced	Reduced IFN-γ expression in liver, lung, gut, and spleen	Casciotti et al. (2002)
CD4	C57BL/6	ME49	oral	Increased cysts (brain)	Not reported	Reduced	Reduced splenocyte production of IFN-γ	Johnson and Sayles (2002)
CD8	C57BL/6	RH	Intra- ocular	Increased number of tachyzoites in the eye	Increased necrosis and inflammation in the eye	Not reported	Similar levels of IFN-γ or TNF-α as seen in WT	Lu <i>et al.</i> (2004)
CD28	C57BL/6	Me49 (type II)	i.p.	No difference in brain cyst burdens	Reduced severity of encephalitis	Increased	Reduced IFN-γ levels; reduced CD4+ T cells in brain	Reichmann <i>et al</i> . (1999)

 TABLE 23.2E
 The effect of CD gene deficiency on murine T. gondii infection

CD28	C57BL/6	ME49	i.p.	Increased tachyzoites in peritoneal exudate	No difference in brain	None	Chronically infected mice have defective memory response and are susceptible to challenge with the RH strain of <i>T. gondii</i>	Villegas <i>et al.</i> (2002)
CD40	C57BL/6	76K	Oral	Increased parasite number in spleen; no difference in intestine	Reduced intestinal pathology	Increased	Reduced transcripts for IL-1β, IL-18, IL-6, and IFN-γ, MIP1β, MIP2, MCP3, MCP1, IP10, and RANTES in ileum; reduced serum IL-12 (p70)	Li <i>et al.</i> (2002)
CD154	C57BL/6	76K	Oral	Increased parasite number in spleen; no difference in intestine	Reduced intestinal pathology	Increased	Reduced transcripts for IL-1 β , IL-18, IL-6, and IFN- γ , MIP1 β , MIP2, MCP3, MCP1, IP10, and RANTES in ileum; reduced serum IL-12 (p70)	Li <i>et al.</i> (2002)

Gene deficiency	Mouse background	Parasite strain	Route of infection	Effect on parasite	Effect on pathology	Effect on survival	Immunological effects	Reference
CCR1	C57BL/6	76K	Oral	Not reported	Increased necrotic lesions in liver, reduced necrotic lesions in intestine	Reduced	Reduced PMN influx into blood and liver	Khan <i>et al.</i> (2001)
CCR2	C57BL/6J	PTG	i.p.		Not examined	Reduced	Reduced recruitment of Gr-1+ monocytes	Robben <i>et al.</i> (2004)
CCR5	B6129F2/J	Not speci- fied	Oral	Increased parasite numbers in intestine	Increased inflammation in intestine	Not reported	CD8+ lymphocytes have reduced ability to migrate <i>in vitro</i>	Luangsay <i>et al</i> . (2003)
CXCR2	BALB/c	ME49	i.p.	Increased tachyzoites in peritoneal exudate	Not reported	Not reported	Reduced serum IFN-γ, defective neutrophil recruitment	Del Rio <i>et al.</i> (2001)
CXCR2	BALB/c	RH	i.p.	Increased tachyzoites in peritoneal exudate cells	Not reported	Not reported	Defective neutrophil recruitment	Del Rio <i>et al.</i> (2001)

 TABLE 23.2F
 The effect of chemokine receptor gene deficiency on murine T. gondii infection

Gene deficiency	Mouse background	Parasite strain	Route of infection	Effect on parasite	Effect on pathology	Effect on survival	Immunological effects	Reference
gamma(c)	C57BL/6	Me49 (type II)	i.p.	Increased cysts in brain	Not reported	Reduced	Similar IFN-γ and IL-12 production measured in serum and produced by spleen cells and peritoneal cells; reduced NK cell activity to YAC-1 cells	Scharton- Kersten <i>et al.</i> (1998)
TCR- Vgamma1	C57BL/6	RH (type I)	Oral	None	No difference in ileitis and liver pathology	None reported	Increased macrophage and granulocyte infiltration of liver; increased serum TNF-α and IFN-γ	Egan <i>et al.</i> (2005)
NKT (Jalpha28)	C57BL/6	Me49 (type II)	Oral	Not reported	Increased weight loss	Reduced	None reported	Smiley <i>et al.</i> (2005)
NKT (Jalpha28)	C57BL/6	76K	Oral	Increased parasite number in intestine	Reduced severity intestinal pathology	Reduced	Reduced IFN-γ production in the intestine; increased number of FoxP3 lymphocytes	Ronet <i>et al.</i> (2005)
МСР	C57BL/6J	PTG	i.p.	None	Increased neuro- pathology	Reduced	Reduced recruitment of Gr-1+ monocytes	Robben <i>et al.</i> (2004)
ICE	C57BL/6	PTGluc (type II)	i.p.		None	None	None examined	Hitziger <i>et al.</i> (2005)
Perforin		ME49	Oral	Not reported	None (brain)	None	Increased splenocyte IFN-γ production	Wang <i>et al.</i> (2004)

 TABLE 23.2G
 The effect of various gene deficiencies on murine T. gondii infection

 TABLE 23.2G
 The effect of various gene deficiencies on murine T. gondii infection—cont'd

Gene deficiency	Mouse background	Parasite strain	Route of infection	Effect on parasite	Effect on pathology	Effect on survival	Immunological effects	Reference
Fibrinogen	C57BL/6	ME49	Oral	Not reported	Hemorrhagic foci in liver	Reduced	No difference in IFN-γ, iNOS, IL-10, or TNF-α transcripts or IFN-γ protein levels or nitric oxide levels in liver	Johnson <i>et al.</i> (2003)
Fibrinogn- like protein 2 (Fgl2, fibro- leukin)	C57BL/6	Me49	Oral	None in peritoneal exudate	None	None	None	Hancock <i>et al.</i> (2004)
Ucp2	C57BL/6	Me49 (type II)	i.p.	Reduced parasites in brain	None reported	Enhanced	Not reported	Arsenijevic <i>et al.</i> (2000)
SLAM- associated protein (SAP)	C57BL/6 or 129Sv/ Ev/Tac	ME49	i.p.	Not reported	Not reported	None	Increased IFN-γ production	Czar <i>et al.</i> (2001)

Α1-α	C57BL/6/ 129Sv/SLJ	RH	i.p.	Increased SAG2 expression in lung and brain	Not reported	Enhanced	Reduced peritonitis	Orlofsky <i>et al.</i> (2002)
Beta 2m	C57BL/6×129	RH	subcu- taneous	Increased number of tachyzoites	Not determined	None	Expansion of spleen NK1.1	Denkers <i>et al</i> . (1993a)
B cell	C57BL/6	Me49 (type II)	oral	Increased number of cysts and tachyzoites in brain	Necrosis in brain of muMT mice only	Reduced	Similar levels of IFN-γ, IL-10, and iNOS, but increased TNF-α mRNA transcripts in brains	Kang <i>et al</i> . (2000)
B cell	C57BL/6	RH	Intra- ocular	Increased dissemination of parasites in multiple ocular tissues	Increased necrosis and inflammation in the eye	Not reported	No increases in serum Ig levels	Lu <i>et al</i> . (2004)



А

FIGURE 23.7 (A) Toxoplasmic retinochoroiditis in immunocompetent patients. *Upper left*: active toxoplasmic retinochoroiditis in a patient with acute acquired infection (provided by Dr Jack S. Remington, Stanford University). Bar = 1.5 mm. *Upper right*: acute lesion in a 5-day-old infant born prematurely. There is complete necrosis in all layers of the retina (r), with numerous inflammatory cells (i) and focal calcification (c). Bar = 100 mm. *Lower left*: quiescent toxoplasmic retinochoroidal scar in a congenitally infected patient. Bar = 1.5 mm. *Lower right*: retinochoroidal scar in a 2-year-old child. The hyperpigmentation at the edge of a retinochoroidal scar seen with ophthalmoscopy as in *lower left* is the result of disruption and proliferation of the retinal pigment epithelium (p). The retina adjacent to this also shows disruption of the normal architecture (r). Scattered chronic inflammatory cells persist in the lesion (i). Bar = 100 µm. (Adapted from Roberts and McLeod 1999, with permission.)

(B) Toxoplasmic retinochoroiditis in immunocompromised patients. Upper left: fundus photograph with red-free light showing irregular margins and presence of multiple satellite lesions. Upper right: multiple cysts of T. gondii in necrotic retina (hematoxylin-eosin, ×440). Upper center: mononuclear inflammation within the optic nerve, with several T. gondii cysts on the left. Mid-center: two young cysts (YC) within a host retinal cell. The cell on the left has many microtubules (mt) and Nissl body-like structures (open arrows), suggesting a neuroretinal cell. (Adapted from Yeo et al. 1983, with permission.) Lower center left: Fundus appearance of right eye in an AIDS patient with bilateral miliary toxoplasmic retinitis, showing multiple small, round, white, inflammatory retinal lesions, as well as hemorrhage and edema in the macula. (Adapted from Berger et al. 1993, with permission.) Lower center right: fundus appearance of left eye from an 8-year-old child treated with systemic corticosteroids, showing active retinal inflammatory lesion adjacent to an old retinochoroidal scar along the inferonasal vascular arcade. (Adapted from Morhun et al. 1996, with permission.) Bottom: histologic section from an adult receiving long-term corticosteroid therapy, showing focal zones of inner retinal necrosis adjacent to the vessels. Arrows showing cysts and released organisms lie at the interface of intact and necrotic retina. V, vitreous cavity; L, blood-vessel lumen (hematoxylin-eosin, ×700). (Adapted from Nicholson and Wolchok 1976, with permission.) This figure is reproduced in color in the color plate section.

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FIGURE 23.7 B. For legend see opposite page.



FIGURE 23.8 Survival and brain-cvst burden in AXB/BXA recombinant inbred strains of mice: cumulative mortality (A) and cyst number (B) 30 days after the peroral infection of AXB and BXA recombinant inbred strains of mice. The first number within parentheses represents the number of mice studied for mortality, the second number represents the number of mice studied for brain-cyst number. In this study, genetics of two traits, survival and brain-cyst number after peroral Toxoplasma gondii infection, were studied by using recombinant inbred strains of mice derived from resistant A/J (indicated by the letter A) and susceptible C57BL/6J (indicated by the letter B) progenitors, F1 progeny of crosses between A/J and C57BL/6J mice, and congenic mice (B10 background). The continuous variation in the percentage survival indicated that control of this trait involved multiple genes. Analysis of strain distribution pattern of survival of AXB/BXA recombinant mice indicated that survival is regulated by a minimum of five genes. One of these genes appears to be linked to the H-2 complex; another is related to an as yet unmapped gene controlling resistance to *Ectromelia* virus, and another to the *Wnt1* locus. The high-versus low-magnitude phenotypes indicate that cyst formation is regulated by one or only a few genes. Associations of defined traits with resistance or susceptibility to Toxoplasma cyst formation were also analyzed. Cyst number is regulated by a locus on chromosome 17 within 0-4 cM of the H-2 complex (P = 0.001). Mice with the H-2 haplotype are resistant and those with the H-2b haplotype are susceptible. This analysis also indicated that the Bcg (*Nramp*) locus on chromosome 1 may affect cyst number (map distance = 12 cM, P = 0.05). Resistance to cyst formation is a dominant trait. (Adapted from McLeod et al. (1989b), with permission.)



FIGURE 23.9 Qualitative trait analysis (likelihood analysis) of genes influencing parasite burden and survival of AXB/BXA mice perorally infected with *T. gondii* (ME49 strain). Chromosome number appears at the upper left of each section. Peaks with accompanying red line and typography indicate loci with high association with outcome (i.e. survival) following peroral infection. Significance of associations indicated with red typography is P < 0.003. Candidate loci for susceptibility or resistance and their chromosome locations are indicated in Table 23.1, along with possible human orthologs. (Adapted from Johnson *et al.* 2002a, with permission.)

This figure is reproduced in color in the color plate section.

found to contain the H-13 allele of the susceptible C57BL6J parental strain, while the four more resistant strains had the H-13 allele of the resistant BALB/c parent (Williams *et al.*, 1978). The relative importance of the balance of MHC class II genes was shown in the work of Johnson *et al.* (2002a), in which the presence of only Ia or Ie led to increased mortality, whereas the presence of both did not lead to this imbalance (see Figure 23.10A). Mortality is also influenced by gender, age, and other factors in addition to genetics (see Figures 23.10B–G) (Gardner and Remington, 1978a, 1978b).

The work of a number of investigators has demonstrated the importance of both Th1 and Th2 type cytokines in the pathogenesis of *T. gondii* infection (Nathan *et al.*, 1984; Pfefferkorn, 1984; Pfefferkorn *et al.*, 1986; Eisenhauer *et al.*, 1988; Black *et al.*, 1989; McLeod *et al.*, 1989a; Chang *et al.*, 1990;

Raymond et al., 1990; Suzuki and Remington, 1990; Gazzinelli et al., 1991, 1993a, 1993b, 1994a, 1994b, 1994c, 1996a; Sibley et al., 1991; Suzuki et al., 1991b, 1993a, 1988, 1989, 1994a, 1994b, 1994c 1996a, 1997, 2000a, 2000b; Shirahata et al., 1992, 1993, 1994; Subauste et al., 1992, 1995; Bermudez et al., 1993; Hunter et al., 1993, 1994a, 1994b, 1995a, 1995b, 1995c, 1996, Thomas et al., 1993; Brown et al., 1995a; Channon and Kasper, 1996; Pelloux et al., 1996; Roberts et al., 1996a, 1996b; Romagnani, 1996; Johnson and Sayles, 1997; Never et al., 1997; Dimier and Bout, 1998; Roberts and McLeod, 1999; Cai et al., 2000b; Subauste, 2002; Araujo et al., 2001; Lyons et al., 2001; Nickdel et al., 2001, 2004; Silva et al., 2002; Rozenfield, 2003; Sher et al., 2003; Abou-Bacar, 2004b; Wang et al., 2004, 2005). Recently, Shaw and colleagues (2006) demonstrated that Tyk2 Janus kinase functions as a nodal regulator

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Survival after T. gondii infection
1: IL10@69.9cM [IL10@lq31–32] and <i>Tgf</i> β2@101.5cM [TGFβ2@1q41] ^b
7: NA [NA]
8: NA [NA]
11: Sc11 Syntenic [<i>IL4, IRF11L3, CSF2,1L5, IL9@5q23–32</i>] and [<i>NOS2, SCYA1-5@17q12</i>]

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^aChromosome number is shown in bold with candidate genes involved in resistance to other pathogens at approximately the same locations. Distance in centimorgans from the centromere is indicated. Shown inside brackets are human ortholog of these candidate genes with their locations indicated by cyto-band.

^bThere are other associations of genes at these approximate loci (see Figure 23. 6), but these are less significant, P > 0.001. Adapted from Johnson *et al.* (2002a), with permission.

of pro-inflammatory versus anti-inflammatory cytokine balance during toxoplasmosis. They found that Tyk2 Janus kinase regulates Th1/IL-10 cytokine balance by mediating IL-12 as well as IL-10 receptor signaling and cellular responses. Tyk2-deficient or mutant mice are susceptible to *T. gondii* infection. A dichotomy was also noted in the regulation of interferon- γ (IFN- γ) versus IL-10 secretion by CD4+ T cells primed by *Toxoplasma* vaccination. Primed lymphocytes respond to *in vitro* antigen restimulation by immediate synthesis of IFN- γ , whereas IL-10 secretion requires an *in vivo* reactivation step regulated by Th1 proinflammatory cytokines, i.e. IL-12/Tyk2/IFN- γ receptor signaling (Shaw *et al.*, 2006).

The plethora of mice engineered to be deficient in immunologically important genes (see Table 23.2) has established, or in some cases confirmed, a role for many immunological components. This raises the possibility that naturally occurring polymorphisms in these genes or promoters may also account for observed differences in murine models and in humans.

23.2.2 Encephalitis

Parasite burden in the brain and the immune mechanisms in the brain that are important in controlling or contributing to encephalitis have been extensively studied (Jones *et al.*, 1977; Suzuki *et al.*, 1989, 1991a,



FIGURE 23.10 (A) Survival following peroral infection of mice without murine class II genes, with murine class II transgenes, and wild-type controls. The difference in survival between *Ia–Ie–* mice and mice with only one of these genes (*Ia* wild type or *Ie* transgene) was significant (P < 0.05) in this experiment. In a replicate experiment the trends were the same, although not all differences achieved statistical significance. Survival of the *Ia–Ie–* and the *Ia+Ie+* mice was not significantly different (P > 0.05).



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FIGURE 23.10 cont'd (B), (C) Lack of protective effect of the L^d gene on survival with differing doses or routes of *Toxoplama gondii*. (B) L^d does not increase or significantly decrease survival with high or low inocula (P > 0.05). (C) L^d does not increase or significantly decrease survival in peroral or parenteral infection (P > 0.05). Age in months is indicated after strain of mouse. p.o., peroral; i.p., intraperitoneal.

(D)–(G) Influence of Dm2 mutation on survival of young and old BALB/c mice. The Dm2 mutation diminishes survival of younger and older mice following peroral infection (D, F), and of young mice following parenteral infection (E, G). Older mice of both the wild-type BALB/c and with the Dm2 mutation are more susceptible than younger mice (P <0.05). (F) Age in months is indicated after the strain of mouse.

(H)–(K) Influence of route and age on survival of C3H.L^d mice. Increase in age (months of age indicated after strain of mouse) only slightly increases susceptibility to peroral infection (P > 0.05) (H, J), but markedly increases susceptibility to parental infection (P < 0.05) (I, K). L^d does not significantly increase or decrease survival.

(L) Influence of gender on survival of Dm2 mice. Female mice of the Dm2 strain are markedly more susceptible than males (P < 0.001).

(Adapted from Johnson et al. (2002b), with permission.)

1993b, 1994a, 1994b, 1994c, 1996a, 1996b, 2000a, 2000b; Suzuki and Remington, 1990; Gazzinelli et al., 1992a, 1993b; Deckert-Schluter et al., 1994a, 1996, 1998a, 1998b, 1999; Brown et al., 1995b; Luder et al., 1999, 2003a, 2003b). These have revealed the importance of both host and parasite genetics. MHC genes would appear to play the predominant role, but the NRAMP gene (previously ITY/BCG/ Lsh) and loci on chromosomes 1, 4, 17, and 18 have also been implicated in playing an additional role (McLeod et al., 1989b; Blackwell et al., 1994; Johnson et al., 2002a) (Figures 23.9, 23.11; Table 23.3). Careful mapping of inbred, congenic, genedeficient (knockout), and transgenic strains of mice has shown definitively that the Ld gene, a mouse class I MHC gene, plays an important role in protection of certain strains of mice against toxoplasmic encephalitis induced by type II T. gondii strains (Johnson et al., 2002b) (Figures 23.10H-L, 23.12-23.14; Table 23.4). The Ity/BCG/Lsh 1 NRAMP gene exhibits a similar (although minor) effect on tissue cyst production (Figure 23.11). Mice deficient in immunologically important genes (Table 23.2) have also provided many useful insights, and in some cases confirmed a protective or exacerbatory role for many other immunological components in toxoplasmic encephalitis. In other cases the absence of a certain gene has produced in surprising results, and polymorphisms in these genes or their promoters is likely to account for the observed differences between murine models.

T. gondii effects on the brain and the immune response are considered in depth in Chapter 21. It has been demonstrated that congenital and adult acquisition of infection by mice leads to distinct patterns, much like disease patterns in humans (Deckert-Schluter et al., 1994a). Specifically, in NMRI mice following prenatal infection with lowdose DK strain T. gondii, newborn mice had foci of necrosis, intracerebral calcifications, and ventriculitis, resembling human congenital toxoplasmosis. Inflammatory responses included macrophages, granulocytes, and astrocytes (Deckert-Schluter et al., 1994a). In chronic toxoplasmic encephalitis, recruitment of T cells stopped and apoptosis of CD4+ and CD8+ T cells occurred; nonetheless, intracerebral T cells that had already been recruited



FIGURE 23.11 Studies with congenic mice demonstrate a major influence of the H-2 complex and minor influence of the Bcg (Nramp) locus on the number of brain cysts following peroral infection. Number of cysts (per 10 µl) in brains of B10 congenic mice 30 days after peroral infection with Toxoplasma gondii. Circles represent mice with the H-2a haplotype. Solid symbols represent mice that are Bcg resistant, and open symbols represent mice that are Bcg susceptible. Data are from two replicate experiments with similar results. Differences between H-2a and H-2b mice were highly significant regardless of their Bcg type ($\tilde{P} < 0.001$). The smaller differences between B10.A.Bcg^r and B10.AsgSn/J mice also were significant (P < 0.01). In these experiments, control A/J mice had low cyst numbers and C57BL/6J mice had high cyst numbers, as in all other experiments. (Adapted from McLeod et al. (1989b), with permission.)

persisted (Schluter *et al.*, 1997; Deckert-Schluter *et al.*, 1999). Suzuki has noted that the immune responses to different strains of *T. gondii* in the brain of mice are not always the same during acute and chronic infection (Suzuki *et al.*, 1993b), and different cell types may participate in earlier and later immune responses. Suzuki also recently identified V β 8 CD8+ Ld-restricted T cells that are *T. gondii* antigen-specific in the brains of chronically infected, genetically resistant BALB/c mice. This population of T cells can transfer protection to naïve mice (Wang *et al.* 2005).

In a study of chemokines in C57BL/6 (susceptible to toxoplasmic encephalitis) adult mice, CR G-2/IP-10, MuMiG, RANTES, MCP-1, MIP-1 α , and



FIGURE 23.12 Mapping studies which indicate that resistance to brain parasite burden and encephalitis are regulated by the L^d gene.

(A) MHC haplotypes of mouse strains used to determine the controlling locus for cyst formation following peroral *Toxoplasma gondii* infection.

(B) Schematic diagram indicating the location of these MHC loci on mouse chromosome 17. Control of resistance to cyst burden following peroral infection with *T. gondii* had been mapped previously to a region of mouse chromosome 17 of approximately 140 kb (McLeod *et al.*, 1989b; Brown and McLeod, 1990). This region is contiguous with and contains the class 1 gene L^d. Resistance to development of toxoplasmic encephalitis had also been reported to be controlled by genes in this region of H-2 by Suzuki *et al.* (1991c). *TNF-* α , *D*, and *L* genes, as well as unidentified genes, are in this region. Studies were performed to identify the gene(s) in the 140-kb region that confers resistance to cysts and encephalitis (Brown *et al.*, 1995b).

MIP-1ß reached higher maximum levels earlier when compared with BALB/c (resistant to toxoplasmic encephalitis) mice. In both murine models, astrocytes and microglia produced CRG-2/IP10 and MCP or RANTES and MuMiG respectively, and leukocytes transcribed CRG-2/IP10, MCP-1, and RANTES. Genetic factors exerted a strong impact on intracerebral chemokines. In a separate study, chemokines were differentially expressed by astrocytes (GRG-2/IP-10, MCP-1), microglia (RANTES), and inflammatory leukocytes, and were critically regulated by IFN-y. IFNγ-deficient mice did not produce CRG-2/IP10, MuMiG or RANTES, and expressed reduced amounts of MIP-1 alpha, MIP-1 beta and MCP-1 mRNA, diminishing the recruitment of leukocytes across the blood-brain barrier. These investigators stated that 'T cells are the single source of IFN- γ gamma in toxoplasmic encephalitis and thus lead to parasites in brain parenchyma'. Using peroral infection of B6C (H-2 $\{b \times d\}$) mice with T. gondii expressing β-galactosidase and monitoring CD8 T cells with MHC class I tetramer staining, Kwok et al. (2003) found that in primary infection only tachyzoites induced CD8+ T cells, but in secondary infection tachyzoites and bradyzoites transiently increased intracerebral CD8+ T cells specific for β-galactosidase. At 23 days after infection, both brain and spleen had high levels of these specific CD8+ T cells; however, after that time the numbers of these cells diminished in spleens but T cells remained at high levels in the brain. These cells produced IFN-y and were cytolytic.

Inactivation of the VCAM gene in VCAM (flox/ flox Mx Cre) mice resulted in lack of induction of

VCAM-1 on cerebral blood vessel endothelial cells (but not choroid plexus epithelial cells or ependyma), and resistance to T. gondii was abolished in conjunction with diminished B cell response, T. gondii-specific intracerebral T cells, and microglial activation; however, leukocytes continued to home across cerebral blood vessels (Deckert *et al.*, 2003). LT- α and TNF- α were essential for the control of intracerebral toxoplasmosis, including that caused by the T. gondii temperature-sensitive 4 (ts-4) mutant (Schluter et al., 2003). Antioxidant systems in *T. gondii* and cytoplasmic catalase in protection against oxidative injury were also studied (Ding et al., 2005). Using CD45 congenic and chimeric mice, there was microglial cytokine production (TNF-α, IL-Iβ, IL-10, IL-15 in normal brain; IL-12 p40, iNOS, increased IL-1β and TNF-α, continuous IL-10, IL-15 and induction of MHC class I and II molecules, and ICAM, and LFA 1). Depletion of CD4+ and CD8+ T cells showed that cytokine expression was regulated by CD8+ T cells, and expression of cell surface molecules was less dependent on T cells. T lymphocytes regulated microglia in brain (Schluter et al., 2001). In a separate study, interleukin 10 downregulated intracerebral immune response in chronic T. gondii encephalitis, and it is possible that it may contribute to parasite persistence in the brain (Deckert-Schluter et al., 1995, 1999; Schluter et al., 1991, 1997). In another study, IFN-γ receptor mediated signaling, but not TNF receptor type I or 2 mediated signaling, was crucial for the activation of cerebral blood vessel endothelial cells and microglia in murine Toxoplasma encephalitis. This was demonstrated in mice without IFN-y receptors

In this study, relative resistance to *T. gondii* organisms and cyst burden in brain, and toxoplasmic encephalitis 30 days following peroral *T. gondii* infection, were correlated with presence of the L^d gene in inbred, recombinant, mutant, and C3H.L^d transgenic mice. Mice that were resistant to cysts and encephalitis had little detectable brain cytokine mRNA expression, whereas mice that were susceptible had elevated levels of mRNA for a wide range of cytokines, consistent with their greater amounts of inflammation. This work definitively demonstrates that and L^d-restricted response decreases the number of organisms and cysts within the brain and thereby limits toxoplasmic encephalitis and levels of IFN- γ , TNF- α , IL-2, IL-6, IL-10, TGF- β , IL-1 α , IL-1 β , and macrophage inhibitory protein mRNA in the brain 30 days after peroral infection. *Boxed X* indicates that the haplotype at this locus has not yet been determined. *Hatched square* indicates deletion of the L^d gene. (Adapted from Brown *et al.* (1995b), with permission.)



FIGURE 23.13 For legend see page 650.

MOUSE GENETIC STUDIES



FIGURE 23.13 For legend see page 650.



FIGURE 23.13 (A) Representative examples of brains of resistant and susceptible strains of mice 30 days after peroral infection. Resistant mice with the L^d gene had minimal inflammation and parasite burden, and susceptible mice with the L^d gene had greater inflammation and parasite burden. Brains of resistant BALB/c (a, b) and C3H.L^d (c, d) mice. Brains of susceptible C3H/HeJ (e, f), B10.RKDB (g, h), and C57BL/l0J (i, j) mice. In (a) and (c), H&E-stained sections (×250) from the resistant mice demonstrated only mild meningeal and perivascular inflammation (marked by arrows). These figures are representative of mild inflammation graded as = 2 by the pathologist. In (b) and (d), immunoperoxidase-stained sections representative of resistant mice did not demonstrate presence of T. gondii tachyzoites either in the meninges or in association with vessels. Arrow in (b) marks a *T. gondii* cyst, seen only rarely in sections from resistant mice. In (e), (g), and (i), H&E-stained sections (×250) from the susceptible mice demonstrated substantial meningeal and perivascular inflammation and, in contrast to the cyst-resistant strains, there was also substantial parenchymal inflammation (each marked by arrows). These figures with larger amounts of inflammation are representative of those graded as = 3 by the pathologist. In (f), (h), and (j), immunoperoxidase stains of representative brain sections from susceptible mice demonstrated many extracellular *T. gondii* tachyzoites or bradyzoites (marked by arrows) within foci of inflammatory cells. The morphologically distinct cyst (e.g. in the upper right-hand corner of b and g) were also demonstrated both with and without accompanying inflammatory cell infiltrates. Results with BALB/c-H-2^{dm2} mice were similar (data not shown). The polyclonal anti-Toxoplasma antisera used for immunoperoxidase staining recognizes both tachyzoites and bradyzoites. (B) Representative examples of CD4+ and CD8+ T cells in brains of resistant BALB/c and C3H.L^d (a-d) and susceptible C3H/HeJ, BALB/c-H-2^{dm2} and C57BL/10J (e–j) mice that were perorally infected 30 days earlier. CD4+ and DC8+ T cells were demonstrated by immunoperoxidase stain. (a) BALB/c mouse, CD4+ cells. Note that there were only a small number of CD4+ cells (arrow). These cells were also present around a blood vessel and in the meninges (data not shown). (b) BALB/c mouse, CD8+ cells. Note that there were also occasional CD8+ cells (arrow). (c) C3L^d CD8+ cells. Arrow indicates CD8+ cell. (e) C3H/HeJ mouse, CD4+ cells. Arrow demonstrates substantial numbers of CD4+ cells in the brain parenchyma. (f) C3H/HeJ mouse, CD8+ cells. Arrow demonstrates CD8+ cells in the wall of a blood vessel in the brain parenchyma. (g) BALB/c-H-2^{dm2} mouse, CD4+ cells, Large numbers of CD4+ T cells were present. (h) BALB/c-h-2^{dm2} mouse, CD8+ cells. Very small numbers of CD8+ T cells were present. (i) C57BL/10 mouse, CD4+ cells. Large numbers of CD4+ cells were present in clusters in the brain parenchyma (arrow). (j) C57BL/10 mouse, CD8+

cells. Large numbers of CD8+ lymphocytes were present in brain parenchyma of control mice that had not been infected with *T. gondii* (data not shown). (C) TGF- β and actin mRNA in brains of resistant and susceptible (Dm2) mice. Ethidium bromide-stained gels demonstrate reverse transcriptase PCR products from brains of resistant and susceptible mice. In unin-

fected controls, cytokine message was absent (data not shown).

(Adapted from Brown *et al.* (1995b), with permission.)

Parts A and B of this figure are reproduced in color in the color plate section.



FIGURE 23.14 Splenocytes from *T. gondii*-infected C3H.L^d mice exhibit L^d-specific lysis when cultured *in vitro* with either of two type II strains, but not a type I strain, of *T. gondii*.

(A) and (B) Splenocytes were harvested from uninfected C3H.L^d mice or from mice infected 12 days earlier with cysts of the Me49 strain of *T. gondii* and stimulated for 6 days *in vitro* with R5(A) or PTg(B) organisms attenuated by gamma irradiation. Effectors were tested against uninfected targets (data not shown, as lysis was <10 percent at all E : T cell ratios) and against R5 strain (A) and PTg strain (B) *T. gondii*-infected P815 (H-2d) and Rl.l(H-2k) target cells. Spontaneous lysis of all target cells was 23 percent or less, with the exception of 40 percent for R5-infected Rl.l target cells. Rl.l target cells were 38–55 percent infected. P815 target cells were 65 percent infected.

(C) Splenocytes were harvested as described in (A) and (B) from mice infected 11 days earlier and stimulated with the UV-attenuated RH strain *T. gondii*. Effectors were tested against uninfected (open box: data not shown when lysis was <10 percent at all E : T cell ratios) and RH-infected (black box) P815 and Rl.l target cells. Spontaneous lysis of targets was 14 percent or less for all target cells. Target cells were 50–55 percent infected. (Adapted from Johnson *et al.* (2002b), with permission.)

		P815 Cells			R1.1 Cells			
Treatment of cultures	Ua	Ib	% lysis ^c	% reduction ^d	Ua	Ip	% lysis ^c	% reduction ^d
No Ab	10	72	62		0	34	34	
Isotype control	20	72	52	16	1	36	35	0
Antibody to L ^d	43	43	31	50	11	45	34	0

TABLE 23.4 Ab to L^d abrogates cytolytic activity of Me49-infected C3H.L^d effectors

^aPercent lysis of uninfected cells.

^bPercent lysis of R5-infected cells.

^cPercent lysis of infected cells minus percent lysis of uninfected cells.

^dPercent lysis of Ab-treated cells/percent lysis of untreated cells (100 percent). Spontaneous lysis of Ab-treated cells was 18 percent or lower for all target cells, with the exception of 41 percent for infected Rl.l cells.

Adapted from Johnson et al. (2002b), with permission.

in which expression of ICAM, LFA-1, and MHC class I and II antigens, but not ILI- α , IL-10, IL-12 p40 or IL-15, was independent of INF- γ receptor signaling (Deckert-Schluter *et al.*, 1996).

Signaling in certain strains of mice through TNFR1 but not TNFR2 induces protective nitric oxide (Deckert-Schluter et al., 1998a). Interestingly, the role of iNOS and NO in containing toxoplasmic encephalitis appears to depend on host genetics. In mice with the C57BL6 background, NOS and NO are essential for protection, but this is not the case in resistant BALB/c mice, 30 days after infection (Schluter et al., 1999). NO also plays an immunoregulatory role by inhibiting the proliferation of spleen cells early in infection (Hayashi et al., 1996a). The role of iNOS in some neurodegenerative diseases (Kroncke et al., 1998) suggests that chronic T. gondii infection in the brain might also be controlled in an iNOS/NO-independent manner for at least some humans, similar to the BALB/c mouse.

There is expression of certain cell surface molecules induced by *T. gondii* by both the central nervous system and immune cells. There are novel molecules expressed by neural and hematopoietic lineage cells (Deckert-Schluter *et al.*, 1998b); specifically, heat-stable antigen (HSA, CD24, nectadrin) and GL7 are hematolymphoid differentiation antigens that play a role in antigen presentation, cell adhesion, signal transduction and

activation in normal brain ependymal cells, choroid plexus macrophages, and some blood vessel endothelial cells. HSA can be detected in brain parenchymal cells by immunohistochemistry, and GL7 can be detected in choroid plexus epithelium. Toxoplasmic encephalitis did not modify this GL7 expression, but in acute and chronic toxoplasmic encephalitis HSA and GL7 were strongly induced in resident brain cells and activated astrocytes were the predominant HSA+ and GL7+ cell types. HSA+ microglia were present, but were a small fraction of the total microglia and increased only a limited amount in toxoplasmic encephalitis. HSA and GL7 may have anatomically and functionally diverse immunological and nonimmunological roles.

23.3 STUDIES OF LEWIS AND FISHER RATS

Recent genetic mapping studies (Cavailles *et al.*, 2006a, 2006b) with immunologic correlates have extended an initial fascinating observation that the Lewis rat strain shows remarkable resistance to infection with *T. gondii* (Gross *et al.*, 1993). This resistance is probably due to an initial innate gastrointestinal or other very early immune response in the intestine before the parasite can even disseminate (Figure 23.4). This refractoriness

to infection is intrinsic to bone-marrow derived cells, and is dependent on IFN-y. Using a genomewide search with F2 progeny of susceptible BN and resistant Lewis rats, resistance was found to be controlled by a single locus on chromosome 10 designated 'Toxo'. This effect was found to be independent of the background genes. Using rats from congenic sublines characterized by genomic recombination with Toxo1 the interval has been reduced to a 1.8-cM region homologous to human 17p13. In vitro functional studies demonstrated that this gene controls the ability of T. gondii to proliferate within macrophages. Using this functional correlate of resistance, the interval was further reduced in congenic sublines to a 0.3-cM region of 1.8 Mb that contains 26 identified rat genes. This forward genetics approach in combination with further functional studies is highly likely to identify the specific gene responsible for resistance of Lewis rats (Figure 23.15A), and should provide very useful insights regarding key protective mechanisms in this infection (Cavailles et al., 2006a, 2006b).

23.4 STUDIES IN HUMANS CONCERNING GENES THAT CONFER RESISTANCE OR SUSCEPTIBILITY AND USE OF MURINE MODELS WITH HUMAN TRANSGENES

Early studies that aimed to determine whether HLA haplotypes (Figure 23.16) conferred susceptibility to eye disease (Figures 23.15B-C) demonstrated no associations. More recent results have found that there are associations between HLA haplotypes and the severity of toxoplasmic encephalitis in AIDS patients and the manifestations of disease in congenitally infected children (Mack et al., 1999; Suzuki et al., 1996b). The importance of the HLA DQ3 gene in determining the susceptibility of patients with AIDS (Luft et al., 1984b) to toxoplasmic encephalitis has been demonstrated by Suzuki (Suzuki et al., 1996b) (Table 23.4). In adddtion, the HLA DQ3 gene has also been demonstrated to be an indicator of the susceptibility of congenitally infected children

developing hydrocephalus (Figure 23.16) (Mack et al., 1999). The observation that there is concordance in monozygotic twins but discordance of dizygotic twins for manifestations of congenital toxoplasmosis (Farquhar, 1950; Murphy and Flannery, 1952; Couvreur and Girre, 1976; Wiswell et al., 1984; Remington et al., 2005) emphasizes the importance of the genetic background of a fetus in determining the susceptibility to congenital toxoplasmosis. In these studies, infants with congenital toxoplasmosis and hydrocephalus had a statistically significant greater frequency of having the DQ3 allele. The observation that there were fewer than predicted children who were homozygous for the DQ3 allele suggested that homozygosity for this allele might lead to increased loss of fetuses infected with T. gondii (perhaps from spontaneous abortion) (Mack et al., 1999) (Figures 23.16A-C; Tables 23.5, 23.6). Furthermore, taking advantage of the fact that human MHC class II genes like DQ3 can function in mice, studies were performed that compared the susceptibility of mice expressing HLA DQ3 or HLA DQ1 to cerebral toxoplasmosis. Mice expressing the DQ3 gene were found to develop significantly more severe encephalitis than those expressing DQ1 (Figure 23.16). This indicates that this type of murine model could be used to further validate the association of certain human genes with susceptibility to diseases, such as occurred in this work (Mack et al., 1999) (Figure 23.17; Tables 23.5, 23.6).

Other studies (Brown and McLeod, 1990) have also demonstrated that human MHC class I transgenes can function in mice (Figure 23.18). Although these mice are yet to be used in models in which pathogenosis and protection in T. gondii infection are characterized, they are likely to provide valuable insights into the role of these genes in humans and the T. gondii peptides that HLA molecules are capable of presenting. Polymorphisms in a number of other genes have also recently emerged as being important in determining disease. Thus mutations in the gene for CD40, which is a ligand for B71, have been demonstrated to be associated with increased susceptibility of patients (Subauste et al., 1998b). More recently, there have been preliminary reports of a



FIGURE 23.15 (A) *Toxo1* controls the proliferation of *T. gondii* within macrophages. (a) BN or LEW macrophages were mixed with *T. gondii* for 1 hour, washed, and cultured for 20 hours. The figure represents the repartition of infected macrophages according to the number of parasites per parasitophorous vacuole. The columns and the bars show the mean result and the standard deviation of three independent experiments. (b) The intracellular growth of *T. gondii* on macrophage and fibroblast monolayers from BN, LEW, and congenic (BN.LEWc10-E, BN.LEWc10-CC, LEW.BNc10F, LEW.BNc10-C) lines was measured by monitoring titrated uracil incorporation into *T. gondii* RNA. From the two different LEW.BNc10 lines of the same BN genotype at *Toxo1*, the -F line was used for macrophage studies and the -C line was used for fibroblast studies. The columns and the bars show the mean result and the standard deviation of triplicates in one rat. These results are representative of two (fibroblasts) and three (macrophages) independent experiments. As a whole, studies on macrophages were performed on six BN, four LEW, and four rats of each congenic line with similar results. Dotted lines indicate the limits of *Toxo1* (boundary markers: D10Rat116 and D10Rat80). N, homozygous BN; L, homozygous LEW. (Adapted from Cavailles (2006a), with permission.)



FIGURE 23.15 (B) For legend see next page.

(B) Ocular histopathology in congenital toxoplasmosis. (a) Top: a well-demarcated area of retinal necrosis (n) at the posterior pole in the eve of a 22-week-gestation fetus (hematoxylin-eosin, original magnification ×250). Bottom: the edge of a large retinochoroidal scar from the eve of the 2-year-old child. The scar is well demarcated, with tubuloacinar proliferation of the retinal pigment epithelium (rpe) at the edge of the scar. The center of the scar is devoid of retina (hematoxylin-eosin, original magnification ×250). (b) Top: eye from the 32-week-gestation fetus showing a large hyperpigmented scar, with a white rim, in the superotemporal region of the eye (arrow). Bottom: the retina from the edge of the scar shows disorganization with formation of Flexner-Wintersteiner rosettes (arrows) (hematoxylin-eosin, original magnification ×400). (c) Left: retina from the 5-day-old infant eye showing retinal detachment with an exudate between retina and choroids. The inner retinal layer is edematous and inflamed (hematoxylin-eosin, original magnification ×100). Right: retina from the eye of a 22-23-week-gestation fetus showing gliosis of the inner retinal layers (hematoxylineosin, original magnification ×250). (d) Eye from a 23–23-week-gestation fetus showing a moderate inflammatory infiltrate within the primary vitreous (i) and surrounding the hyaloid artery (ha) (hematoxylin-eosin, original magnification $\times 20$). (e) Optic nerve from the eye of a 23-week-gestation fetus with congenital toxoplamosis. The nerve architecture is disrupted with an inflammatory cell infiltrate (as in (f)) (hematoxylin-eosin, original magnification ×100). (Adapted from Roberts et al. (2001), with permission.) This figure is reproduced in color in the color plate section

calcium channel gene upstream of INF- γ as being important in susceptibility to toxoplasmosis, as well as other intracellular infections such as tuberculosis and salmonella infections (Boulter *et al.*, 2005).

There are clearly patients who have differing manifestations of their *Toxoplasma* infections – some asymptomatic, some with adenopathy of varying duration, some with a flu-like illness, some with chronic fatigue, and some with eye disease. Small numbers of apparently immunologically normal individuals have damage to certain organ systems – for example, encephalitis (Townsend *et al.*, 1975; Couvreur and Thulliez, 1996; Carme *et al.*, 2002), pericarditis, or myocarditis (Remington *et al.*, 2005). Some of this variability may be due to parasite clonal type or inoculum, or the form of the parasite acquired, but almost certainly some is due to differences in genetic susceptibility.

Other genetic mutations, knockouts or alleles conferring susceptibility are shown in Table 23.2 (Denkers *et al.*, 1993a; Deckert-Schluter *et al.*, 1996, 1998a, 1999; Gazzinelli *et al.*, 1996b, 2004; Khan *et al.*, 1996a, 1997; Roberts *et al.*, 1996a; Scharton-Kersten *et al.*, 1996a, 1996b, 1997a, 1997b; Suzuki *et al.*, 1996a, 1997, 2000a; Franzoso *et al.*, 1998; Jebbari *et al.*, 1998; Yap *et al.*, 1998a, 2000; Caamano *et al.*, 1999, 2000; Ely *et al.*, 1999; Zhang *et al.*, 1999; Arsenijevic *et al.*, 2000; Kang *et al.*, 2000; Taylor *et al.*, 2000; Czar *et al.*, 2001; Del Rio *et al.*, 2001; Lyons *et al.*, 2001; Nickdel *et al.*, 2001, 2004; Wille *et al.*, 2001, 2002; Collazo *et al.*, 2002; Casciotti *et al.*, 2002; Fujigaki *et al.*, 2002; Johnson *et al.*, 2002a, 2002b; Scanga *et al.*, 2002; Silva *et al.*, 2002; Villegas *et al.*, 2002; Lu *et al.*, 2003, 2004; Mun *et al.*, 2003a; Schluter *et al.*, 2003; Gavrilescu *et al.*, 2004; Hancock *et al.*, 2004; Kudo *et al.*, 2004; Lieberman *et al.*, 2004; Mason, 2004a, 2004b; Vossenkamper *et al.*, 2004; Wang *et al.*, 2004; Egan *et al.*, 2005; Ronet *et al.*, 2005; Wilson *et al.*, 2005).

23.5 INFLUENCE OF PARASITE STRAIN ON IMMUNE RESPONSE AND DISEASE

Toxoplasma gondii strains can be divided into three main lineages (types I, II, and III) based on various genetic markers (Sibley and Boothroyd, 1992a; Howe and Sibley, 1995; Ajzenberg *et al.*, 2005). Studies in mice have shown that infection with each of the three lineages of *T. gondii* results in different outcomes: type I strains are highly virulent, whereas types II and III are relatively avirulent in mice (Kaufman *et al.*, 1958, 1959; Johnson *et al.*, 1979; Sibley and Boothroyd, 1992a, 1992b; Howe and Sibley, 1995). Type I strains differ genetically by 1 percent or less from type II and type III strains (Sibley and Boothroyd, 1992a, 1992b; Sibley, 2003b); however, the main determinants that dramatically affect the virulence of different



FIGURE 23.15 (C) Inflammatory cells and *Toxoplasma gondii* organisms present in ocular toxoplasmosis. (a)–(d) represent the same discrete ocular lesion from a 21-week-gestation fetus with a peripapillary lesion. (a) Disruption of the retinal pigment epithelium (RPE) with choroidal congestion and inflammation (hematoxylin-eosin, original magnification ×400). Immunohistochemical staining for T cells, CD3 (b) and T-cell subset CD4 (c) shows numerous positive lymphocytes within the choroids (arrows). (d) In this case, CD68-positive macrophages are numerous within the choroids underlying the area of RPE disruption (arrows). No staining was identified in the negative control or in sections stained with anti-CD8 (not shown). (e) and (f) demonstrate staining for *T. gondii*. (e) *Left*: retina from the 5-day-old infant showing a collection of intracellular *T. gondii* within the retina (arrow) (hematoxylin-eosin), original magnification ×400). *Right*: retina from the 5-day-old infant eye showing immunohistochemical staining for *T. gondii* antigen. Note the same small blood vessel (v) also identified in (e). *Continued*

Many extracellular *T. gondii* organisms are identified (arrows). In addition, the inset shows the presence of organisms in a perivascular location (L43 stain, original magnification ×100 and ×250). (f) *Left*: gliotic retina in an eye from a 22-week-gestation fetus showing extracellular organisms scattered throughout the retinal layers (arrows) (polyclonal antibody, original magnification ×250). *Right*: disrupted retina and necrotic debris in an eye from a 23-week-gestation fetus. Numerous extracellular *T. gondii* organisms (arrows) are present within the necrotic debris (L43 stain, original magnification ×400). The red staining product allows distinction from melanin pigment granules (pg) of disrupted RPE. (Adapted from Roberts *et al.* (2001), with permission.)

This figure is reproduced in color in the color plate section

T. gondii strains in the host and the pathogenesis of toxoplasmosis are only partially understood (Miller et al., 1999a, 1999b, 2000; Mordue et al., 2001; Robben et al., 2004; Saeij et al., 2005a, 2005b). Type II strains of T. gondii have been reported to be dominant in the United States, and are frequently isolated from AIDS patients with toxoplasmic encephalitis (Howe et al., 1997; Honore et al., 2000). Interestingly, other studies suggest that type I and type I/III or recombinant strains are involved in the development of ocular disease (Grigg et al., 2001a; Khan et al., 2005a). In one study, an atypical strain parasite was identified as responsible for a toxoplasmosis epidemic outbreak associated with a high rate of development of acquired ocular disease (Burnett et al., 1998). Infection with an attenuated parasite permits the development of latency with subsequent infection with a normally virulent strain (Yap et al., 1998b).

In contrast with the frequencies of clonal types of parasites observed in the United States and

much of Europe (Howe and Sibley, 1995; Howe et al., 1997), in a study of strains in the Minas Gerais region of Brazil there were mixed genotypes, with typical alleles of types I, II, or III at almost all loci assessed. There was a clear dominance of alleles characteristic of type I strains, followed by a high frequency of type III strains and a very low frequency of alleles from type II strains (Ferreira et al., 2004, 2005). Others have reported on atypical isolates in South America and Africa (Dardé et al., 1992; Glasner et al., 1992; Howe and Sibley, 1995; Fazaeli et al., 2000; Neto et al., 2000; Silveira et al., 2001; Ajzenberg et al., 2002, 2004; Dubey et al., 2002; Dubey et al., 2003a, 2003b, 2003c, 2004, 2005a, 2005b; Sibley 2003b; da Silva et al., 2005; Khan *et al.*, 2005b). The fact that the Brazilian T. gondii strains are more closely related to the type I lineage is noteworthy. These strains were studied in mice, and 85 percent were highly virulent, virulent, or of intermediate virulence, while only 15 percent of the strains were avirulent (Ferreira et al., 2004, 2005). These findings contrast with studies





(A) Map of location of polymorphic HLA class I and class I genes in the human MHC on chromosome 6. The number of known alleles of each gene, whether identified serologically or by sequence analysis, is indicated.



FIGURE 23.16 cont'd (B) Human and murine class II genes: (a) schematic representation of the relationships of human and murine MHC class II genes; (b) diagrams of expression of murine and human class II molecules on cells from mice used in the studies described herein. The Ea b gene is non-functional. A functional Ea k gene was inserted to produce E+ mice. Shaded area indicates not present.

performed in the USA and Europe, where most strains are avirulent of types II or III (Howe and Sibley, 1995; Howe *et al.*, 1997). Although speculative at this point, it is plausible that the high frequency of virulent strains closely related to type I lineage may be in part responsible for the high frequency of acquired ocular toxoplasmosis commonly found in Minas Gerais, Brazil (Glasner *et al.*, 1992; Silveira *et al.*, 2001; Portela *et al.*, 2004).

Clonal type I and type II *T. gondii* strains (Grigg *et al.*, 2001b) have been shown to stimulate host cells to produce pro-inflammatory cytokines

differently. For example, the RH strain, the prototype strain of the type I group, induces a robust pro-inflammatory response during the very early stages of infection. This immune response appears to be in part responsible for the pathology and lethality observed during the first week of infection (Mordue et al., 2001). In addition, type II strains and not type I strains were shown to elicit the production of IL-12 by host macrophages. IL-12 has a critical role in inducing IFN-y and host resistance to infection with T. gondii (Robben et al., 2004). Similar findings have been described by Suzuki et al. (1995, 2005). Thus, the ability to induce IL-12 may contribute to the control of parasite replication and relative avirulence of type II strains, as opposed to type I strains. The avirulent type I/III strains showed a similar pattern of induction of cytokines, such as IL-12 and IFN- γ , as compared to the ME49 strain, a type II strain (Fux et al., 2003). There have been suggestions that clonal type of parasite may be associated with magnitude of eye disease, IgA antibody to GPILs, or other disease manifested during infection in humans (Grigg et al., 2001a; Portela et al., 2004). In addition, Dobbin et al. (2002) found that HSP70 of T. gondii inhibits iNOS expression, NO production, and NFkB activity, which can have effects on cytokine production, but that these effects occur only with the virulent RH and ENT strains and not with the avirulent ME49 and e strains.

As previously noted for a type II strain (ME49), IL-12/IFN-y axis and iNOS were defined as main determinants of resistance during the acute infection with the Brazilian strains (Bahia-Oliviera et al., 2003). Different from the type II strain of T. gondii (ME49), peroral infection with the type I/III strains led to only a mild inflammatory infiltrate and no major lesions in the intestine of C57BL/6 mice. In addition, BALB/c (resistant to ME49) and C57BL/6 (susceptible to ME49) mice were shown, respectively, to be more susceptible and resistant to cyst formation and toxoplasmic encephalitis, when infected with type I/III strains. Studies with congenic BALB/c strain mice that had MHC haplotype 'b' and C57BL/6 containing MHC haplotype 'd' showed that whereas the 'd' haplotype was a critical element conferring host resistance to



FIGURE 23.16 cont'd (C) Parasite burden and histopathology in *T. gondii*-infected mice with differing class II phenotypes. (a, b) Numbers of cysts in wet mounts of brains from wild-type, knockout, and transgenic mice. (c) Comparison of magnitude of parasite burden in histopathologic preparations of tissue. In panel (f), antibody was polyclonal rabbit anti-*Toxoplasma* antibody produced by immunizing rabbits with tachyzoites of the C56 strain followed by tachyzoites of the RH strain of *T. gondii*. (d) Comparison of magnitude of parenchymal inflammatory activity. (f) Clusters of *T. gondii* cysts (arrow). (g) Necrosis (arrow) and parenchymal inflammation in the brain of an *Ia–Ie–* strain mouse. (h) Severe meningitis (arrow) in the brain of a DQ3 strain mouse. (i) Severe perivascular inflammation (arrow) in the brain of a DQ3 strain mouse. (Adapted from Mack *et al.* (1999), with permission.) This figure is reproduced in color in the color plate section



FIGURE 23.17 Hydrocephalus in congenital toxoplasmosis.

- (A) Pathologic specimen that demonstrates the periaqueductal and periventricular necrosis and inflammation (single arrow) which has obstructed the aqueduct of Sylvius and caused ventricular dilatation (double arrow). Photograph kindly provided by J.S. Remington, Stanford, CA.
- (B) Computed tomography of the brain of an infant with hydrocephalus with bilateral ventricular dilatation.
- (C) Magnetic resonance image of the brain of an infant with unilateral hydrocephalus (arrow) secondary to obstruction of the foramen of Monro.

(Adapted from Mack et al. (1999), with permission.)

Patient no.	CD4 cell count	No. of lesions on brain CT or MRI	Patient no.	CD4 cell count	No. of lesions on brain CT or MRI
1	30	Multiple	11	10	Single
2	20	Multiple	12	20	Multiple
3	90	Multiple	13	113	Multiple*
4	43	Multiple	14	45	Multiple*
5	35	Multiple	15	60	Multiple
6	20	Multiple	16	60	Multiple‡
7	NA	Single*	17	50	Multiple*
8	41	Multiple	18	4	Single
9	10	Single [†]	19	260	Single
10	71	Multiple	20	34	Multiple

TABLE 23.5A Findings in 20 AIDS patients with toxoplasmic encephalitis

NOTE. All patients were positive for *Toxoplasma* IgG antibodies, and all patients except nos. 13 (NA, not available) and 16 (died) had positive response to specific therapy. CT, computed tomography; MRI, magnetic resonance imaging.

*Brain biopsy revealed Toxoplasma gondii.

[†]Basal ganglia lesion.

‡Cerebrospinal fluid positive for *T. gondii* DNA by polymerase chain reaction.

Adapted from Suzuki et al. (1996b) with permission.
				% phenotype	frequency		
			Healthy co	ontrols		TE-n AIDS	egative local controls
			From the	eliterature			
HLA antigen	TE patients $(n = 20)$	Local (<i>n</i> = 136)	Serotyped* (<i>n</i> = 232)	DNA typed [†] ($n = 167$)	All healthy controls $(n = 535)$	Random (<i>n</i> = 15)	Toxoplasma seropositive \ddagger (n = 8)
DQ1	40.0 [§]	64.7	68.1	65.9	66.5 [§]	60.0	75.0
DQ2	30.0	38.2	40.5	38.9	39.6	26.7	25.0
DQ3	85.0	52.2	48.7	55.7	51.8∥	40.0	62.5
DQ4	5.0	11.0	4.7	5.4	6.5	20.0	12.5

TABLE 23.5B	Frequencies of HLA-DQ antigens in white North American AIDS patients with
toxoplasmic en	ncephalitis (TE) and controls

*11th International Histocompatibility Workshop.

[‡]Includes 1 patient from local TE-negative random group.

Significant differences (TE patients vs. combined healthy controls): ${}^{\circ}P = .028$ and corrected $P(P_c) = .108$, ||P = .007 and $P_c = .028$.

Adapted from Suzuki et al. (1996b), with permission.

parasites of the ME49 strain, the genetic background (and not MHC haplotype) of C57BL/6 mice was responsible for resistance of these mice to recombinant type I/III strains isolated in the Minas Gerais region of Brazil. These results indicate that MHC haplotype 'b' is a major determinant of susceptibility to cyst formation and toxoplasmic encephalitis induced during infection with type II, but not with type I/III strains of *T. gondii* (Fux *et al.*, 2003).

For mice with the same genetic background, different clonal types of parasites may not elicit the same immune response. For example, in C3H. Ld mice, CD8+ cytolytic T cells could lyse target

TABLE 23.6A Association of the DQ3 gene with presence of hydrocephalus in infants with congenital toxoplasmosis and their mothers

		Children with	toxoplasmosis	Mothers of children with toxoplasmosis		
	US population	Without hydrocephalus	With hydrocephalus	Without hydrocephalus	With hydrocephalus	
Number in group	232ª	45	23	41	21	
Gene frequency	0.487 ^a	0.444	0.783 ^b	0.537	0.762 ^c	

aLiterature source is [Darke and Dyer, 1993].

^bDifferences between *DQ3* gene frequencies of infants with and without hydrocephalus (P < 0.02) and infants with hydrocephalus and the USA population (P < 0.02) were significant.

^cThe difference between *DQ3* gene frequencies of mothers of infants with and without hydrocephalus were not significant (P > 0.05) but the difference between mothers of children with hydrocephalus and the US population was significant (P < 0.03).

Adapted from Mack et al. (1999), with permission.

 TABLE 23.6B
 Fewer than expected DQ3+/DQ3+ (homozygous) children with hydrocephalus

Group	п	Observed DQ3 + / +	Observed DQ3 + /-	Observed DQ3–/–	Expected DQ3 + /+	Expected DQ3 + /-	Expected DQ3–/–	Chi square Total
Children with hydrocephalus	23	2	16	5	4.35	11.30	7.35	3.97^{a}
Mothers of children with hydrocephalus	21	3	13	5	4.30	10.40	6.30	1.31
Children without hydrocephalus	45	2	18	25	2.69	16.62	25.69	0.31
Mothers of children without hydrocephalus	41	4	18	19	4.12	17.76	19.12	0.008

^aNote: Among infants with hydrocephalus, there were fewer than expected DQ3 + /+ , i.e. DQ3 homozygotes ($\chi^2 = 3.97$, P < 0.04), whereas differences for other groups were not significant (P > 0.05).

Adapted from Mack et al. (1999), with permission.



FIGURE 23.18 Effect of human MHC transgenes on parasite burden in brain. Cyst numbers in brains of control and HLA-transgenic mice. Presence (+) or absence (-) of transgene expression in littermates is indicated. (Adapted from Brown *et al.* (1995b), with permission.)

cells that were infected with the homologous type II strain with which the mice were perorally infected (Figure 23.19), but not MHC matched target cells infected with a heterologous clonal type (Johnson *et al.*, 2002b).

23.6 GENERAL ASPECTS OF IMMUNITY

23.6.1 Relationship of adaptive immunity to the innate immune system

Insights discovered using other systems in the study of innate and adaptive immunity, genetics as a tool to understand innate and adaptive immunity, and the interrelationship of innate and adaptive immunity have been useful when applied to understanding protections against and pathogenesis of infection with T. gondii in humans and other animals, including characterization of how T. gondii interacts with specific host cells and the interaction of specific T. gondii antigens in eliciting immune responses (McLeod and Remington, 1979; Teutsch et al., 1979; Handman and Remington, 1980; Krahenbuhl et al., 1980; Hauser and Remington, 1981; Hayward and Kurnick 1981; Frenkel and Taylor, 1982; Locksley and Klebanoff, 1983; Yeo et al., 1983; Scollay et al., 1984; Jones



FIGURE 23.19 C57BL6 mice develop toxoplasmic retinochoroiditis that can be exacerbated by inhibition of NO production by L-NAME treatment. In infected mice not treated with L-NAME, the inflammatory infiltrate is focal and in areas where the retina appears normal (A). In other areas, there is a mild inflammatory cell infiltrate (arrows), confined to the inner retinal layers and optic nerve head (B). In these mice there is only mild perivascular cuffing (pv) (C). In L-NAME treated mice, inflammatory infiltrate is severe and diffuse (D and E). There are numerous cells in the vitreous (v) and expanding choroids, as well as within the inner retinal layers and optic nerve head (arrows). Perivascular cuffing by inflammatory cells is more severe in L-NAME treated mice (F) than in untreated mice. (Adapted from Roberts *et al.* (2000), with permission.) This figure is reproduced in color in the color plate section.

et al., 1986; Potasman et al., 1986; Kobayashi and Suzuki, 1987; Masson and Tschopp, 1987; Schwartzman, 1987; Suzuki and Kobayashi, 1987; Suzuki et al., 1987, 1988, 1992, 1994a, 1994b, 1994c, 1995; Canessa et al., 1988; Ware and Kasper, 1987; Kappler et al., 1988; Kasper, 1989; von Boehmer et al., 1989; Fiorentino et al., 1991; Hakim et al., 1991; Makioka et al., 1991; Terpenning and Bradley, 1991; Freund et al., 1992; Furtado et al., 1992; Oswald et al., 1992; Robey et al., 1992; Lucet et al., 1993; Moore et al., 1993; Seder et al., 1993; Shirasawa et al., 1993; Thilaganathan et al., 1993, 1994; Watanabe et al., 1993; Wegman et al., 1993; Wong et al., 1993; Zenner et al., 1993; Denkers et al., 1994; Gross and Bohne, 1994; Maggi et al., 1994; Nossal, 1994; Soete et al., 1994; Yong et al., 1994; Buxton and Innes, 1995; Murphy et al., 1995; Wang and Hakanson, 1995; Weiss et al., 1995; Chon et al., 1996; Dobrowolski and Sibley, 1996; Halonen et al., 1996; Khan et al., 1996a; Ma et al., 1996; Morhun et al., 1996; Sibley and Howe, 1996; Steffen et al., 1996; Unanue, 1996; Khalili and Chang, 1997; Reis e Sousa et al., 1997; Ellis-Neyer et al., 1998; Lyons and Johnson, 1998; Manger et al., 1998; Nash et al., 1998; Ceravalo et al., 1999; Haque et al., 1999; Khan and Casciotti, 1999; Marshall et al., 1999; Mead et al., 1999; Mordue et al., 1999a, 1999b; Edelson and Unanue, 2000; Yamamoto et al., 2000, 2003; Liesenfeld et al., 2001; Mackay, 2001; Shen et al., 2001; Butcher and Denkers, 2002; He et al., 2002; Mennechet et al., 2002; Nguyen et al., 2003; Payne et al., 2003; Su et al., 2003; Tham et al., 2003; Cobbold et al., 2004; Kim et al., 2004; Couper et al., 2005; Mun et al., 2005; Rodgers et al., 2005; Fehervari et al., 2006; Montecino-Rodriguez et al., 2006; Saeij et al., 2005a).

Understanding of pathogenesis arises from the study of both disease and immune responses in humans and in animal models (Eichenwald,1960; Desmonts and Couvreur, 1974; Nicholson and Wolchok, 1976; O'Connor and Frenkel, 1976; Herb *et al.*, 1977; Frenkel *et al.*, 1978; Gardner and Remington, 1978a, 1978b; Desmonts and Remington, 1980; Wilson *et al.*, 1980a; Desmonts *et al.*, 1981; Dubey *et al.*, 1981, 1997; Hay *et al.*, 1981; Navia *et al.*, 1986; Dubey and Beattie, 1988; McAuley *et al.*, 1994; Dubey, 1997; Hennequin,

1997; Gavrelescu and Denkers, 2003; Kim *et al.*, 2004; McLeod *et al.*, 2006).

Studies in mice demonstrate the important role of cytokines such as IL-12, TNF- α , and IFN- γ , and reactive nitrogen intermediates (RNI) as mediators of host resistance to T. gondii infection (Denkers et al., 1997a, 1997b). Animals deficient in IL-12, IFN- γ , and inducible nitric oxide synthase (NOS2), treated with neutralizing antibodies/anti-cytokines, or specific inhibitors of NOS2 (on a C57B/6 background), are highly susceptible to infection with T. gondii (Suzuki et al., 1989; Gazzinelli et al., 1991, 1992b, 1993a, 1994a; Hayashi et al., 1996a, 1996b; Scharton-Kersten et al., 1996a, 1996b, 1997a, 1997b). Pathology associated with excessive immune stimulation of Th1 responses and high levels of IFN-γ has been also demonstrated during acute infection with T. gondii in a mouse model (Liesenfeld et al., 1996). Production of IL-10 is stimulated during infection with T. gondii and is involved in regulating production of IL-12, IFN- γ , and TNF- α , and its absence is associated with exacerbated pathology (Gazzinelli et al., 1996b; Neyer et al., 1997). Acquired immunity to T. gondii is associated with a Th1-type response (Gazzinelli et al., 1991, 1992a, 1994b, 1994c). During chronic toxoplasmosis, neutralization of IFN- γ or TNF- α , or inhibition of NOS2, results in the reactivation of disease and the development of encephalitis as well as uveitis (Gazzinelli et al., 1992a, 1993a, 1994a; Hayashi et al., 1996a, 1996b). The relative importance of some of these factors, including TNF- α and NOS2, appears to be dependent on the host genetic background (Johnson, 1984; Rozenfeld et al., 2003). Among other factors, specific MHC haplotypes are known to be important determinants of host resistance and susceptibility to early infection in mice (McLeod et al., 1989a, 1989b; Brown and McLeod, 1990; Suzuki et al., 1991c; Blackwell et al., 1993; Johnson et al., 2002a, 2002b) and appear to influence the relative contribution of some of these factors in different mouse strains. Consistently, both CD4+ T and CD8+ T lymphocytes are important components in host resistance to this parasite (Gazzinelli et al., 1991; Suzuki and Remington, 1988). Thus, one of the important contributions of innate immunity and of the Toll-like receptors (TLRs) is

in shaping protective T-cell-mediated (adaptive immunity) immunity. Some of these interactions between components of the innate and adaptive immune systems are shown in Figure 23.5.

In other systems major advances have been made in the assignment of individual TLRs to specific components of bacterial cell walls (LPS, lipoproteins, and peptidoglycan) that have potent proinflammatory activity. Characterization of TLR ligands is only beginning to be performed for protozoan parasites. Several studies have identified that glycosylphosphatidylinositol (GPI) anchors (or fragments from GPI anchors) from P. falciparum, L. major, T. gondii, T. brucei, and T. cruzi act as molecules that activate cells of both lymphocytic and monocytic lineages. Structural requirements for the bioactivity of protozoan-derived GPI anchors have been partially defined. Minor structural modifications of these glycolipids markedly affect their biological activity, including the induction of pro-inflammatory cytokines, chemokines, and apoptosis. Importantly, native GPI anchors purified from the tachyzoite stage of T. gondii as well as synthetic fragments mimicking the native GPI anchors were shown to promote NF-yB activation and stimulate the synthesis of TNF- α by a murine macrophage cell line (Debiere-Grockiego et al., 2003). Heat-shock protein and other partially purified protein preparations from T. gondii tachyzoites were shown to activate TLR4 and TLR2, respectively. More recently a profilin-like protein from T. gondii (PFTG) was also shown to activate TLR11 in murine cells, as discussed in section 23.6.2 (Yarovinsky et al., 2005). In humans the TLR11 gene has a stop codon in the middle, and humans may express a truncated form of TLR11 without the functional domain (A. Sher, personal communication, 2005); nevertheless, live tachyzoites have been shown to elicit IL-12 production by human dendritic cells, suggesting that other TLR agonists may be acting to activate TLRs in humans.

The strongest data suggesting that TLRs are important in the host resistance and pathogenesis of protozoan infections come from microbial challenge studies in MyD88-deficient mice. MyD88 is involved in the activation of all of the known TLRs with the exception of TLR3. In addition to TLRs, MyD88 has only been described as essential for IL-1 and IL-18 receptor functions (Adachi et al., 1998). Neither IL-1 nor IL-18 has been described as critical in host resistance to T. gondii infection (Graefe et al., 2003); hence an impaired response of MyD88-deficient mice to a pathogen provides reasonable evidence that a member of the TLR family is engaged. Indeed, MyD88-deficient mice infected with T. gondii had diminished production of IL-12 and IFN-y, enhanced parasitemia, and accelerated mortality (Scanga et al., 2002). Thus, TLRs appear to be involved in early resistance to infection with T. gondii. The identification of a single TLR involved in the in vivo host responses to protozoan parasites has been a more difficult task. Enhanced susceptibility to infection in mice deficient in a specific TLR, such as TLR2 and TLR4, is highly dependent on experimental conditions including parasite strain, size, and route of inoculum. In most cases, TLR deficiency has no major impact on resistance to protozoan infections (Gardner and Remington, 1977; Masur et al., 1978; Holland et al., 1988; Suzuki and Kobayashi, 1990; Orellana et al., 1991; Saavedra and Herion 1991; Herion and Saavedra, 1993; Denkers, 1996; Johnson et al., 1997; Mordue and Sibley, 1997; Yano et al., 1997; Buzoni-Gatel et al., 1999; Oliviera et al., 2000; Weiss and Kim, 2000; Adachi et al., 2001; Aosai et al., 2002; Caamano and Hunter, 2002; Strack et al., 2002a, 2002b; Wei et al., 2002; Mun et al., 2003a; Ding et al., 2004; Kropf et al., 2004; Oliveira et al., 2004; Denkers and Butcher, 2005; Sergent et al., 2005; Deckert et al., 2006). On the other hand, mice lacking TLR11 (the counterpart receptor for PFTG) showed a significant phenotype, with a great increase in cyst numbers (Yarovinsky et al., 2005), and TLR 9 has recently been found to be important in protection of mice (Minns et al., 2006). However, no published information concerning the role for TLRs in human toxoplasmosis is available. As mentioned above, in all humans tested to date, TLR11 gene has a stop codon and does not appear to function (Zhang et al., 2004). Thus, it is likely that alternative TLR(s) or another cognate receptor from innate immunity will be important in human toxoplasmosis.

23.6.2 Antigen presentation and processing, and MHC class I and class II molecules

T. gondii can enter all cell types and establish infection in a unique intracellular vacuole, as described elsewhere in this book (Wilson et al., 1980a; Mordue et al., 1999a, 1999b; Sibley, 2003a), which then elicits an immune response. Schematic diagrams of antigen presentation to the MHC class I and MHC class II pathways, the effector functions elicited, the cytokines produced, and some of the evidence for the importance of these mechanisms, are summarized in Figures 23.1-23.10, and Tables 23.1 and 23.2. Several T. gondii proteins (e.g. SAG1, GRA2, GRA6, GRA7, BAG1) have elicited antibodies, providing evidence for class II MHC processing and presentation and CD4+T cell help (see, for example, Couper et al., 2003; Letscher-Bru et al., 2003). The presence of CD4+ and CD8+ cytolytic and INF-\gamma-producing T cells (Figure 23.18) has provided evidence that there is MHC processing either through conventional MHC class I processing or via bystander uptake of antigen which enters an MHC class I pathway. Pores in the vacuolar membrane (Joiner et al., 1990; Schwab et al., 1994) and secretion from the parasite into the vacuole (Gubbels and Striepen, 2004) contribute T. gondii proteins (peptides) which can enter a class I MHC pathway. The antigens which can elicit these responses are discussed in more detail in Chapter 24 of this book.

As described above, primary infection by *T. gondii* stimulates an effective protective immune response through production of type 1 cytokines and continuous cell-mediated immunity. After innate resistance and IFN- γ -dependent mechanisms have controlled tachyzoite replication, both CD4+ and CD8+ T cells are essential to maintain bradyzoites in a latent stage. Control of acute and chronic phases of *T. gondii* infection requires both CD4+ and CD8+ T cells. There are only limited data about antigens that elicit cell-mediated immunity (Khan *et al.*, 1994), and endogenous immunodominant epitopes have not yet been characterized.

One approach to studying the generation of antigen-specific T-cell responses during toxoplasmosis has been to introduce model antigens for which the TCR specificity is known (Kumar and Tarleton, 2001; Pope et al., 2001; Ding et al., 2005). Many genetic tools and approaches have been developed to study T. gondii biology (Roos et al., 1994; Boothroyd et al., 1997). These genetic approaches are currently being utilized to study antigen presentation through both MHC-I and MHC-II, and naïve and effector or memory CD4+ and CD8+ T-cell responses in the context of acute and chronïc infection by T. gondii (Dzierzinski Toxoplasma Meeting, Corsica 2005; Dzierzinski, personal communication, 2005). Dziersinski and colleagues used a transgenic system of ovalbuminexpressing T. gondii parasites (Pepper et al., 2004). This system was shown to be appropriate to study CD4+ T-cell responses in vivo (Pepper et al., 2004). It is now being used to better understand antigen presentation by professional or non-professional antigen-presenting cells through MHC class I and/or MHC class II molecules, as well as CD8+ T-cell responses, in vitro and in vivo (F. Dzierszinski, M. Pepper, C.A. Hunter and D.S. Roos, unpublished). Taking advantage of another model antigen, Eα:I-A(b) complex-red fluorescent protein fusion (E α RFP) (Barlow et al., 1998; Itano and Jenkins, 2003), a second T. gondii transgenic system was generated to track antigen-presenting cells and follow MHC class II antigen presentation in vivo (M. Pepper, F. Dzierszinski, D.S. Roos and C.A. Hunter, unpublished). In order to better characterize antigen presentation and T-cell responses during toxoplasmosis, transgenic parasites were also generated expressing the model antigens under the control of different promoters, which vary in strength and stage specificity, and in multiple parasite strain backgrounds. A variety of assays and genetic screens are also currently being developed to determine T. gondii and host molecules implicated in parasite-host interactions (Dzierszinski, personal communication, 2005).

Transgenic ovalbumin-expressing parasites have also been utilized to study the antigen presentation proteins that enter the parasite secretory pathway (Gubbels *et al.*, 2005). These antigens were found to enter the classical class I MHC presentation pathway (Gubbels *et al.*, 2005). Presentation of ovalbumin secreted by the parasite required the peptide transporter TAP, and occurred primarily in actively infected cells rather than through bystander cells. Dendritic cells are a major target of *T. gondii* infection, and account for much of the antigen presentation by the spleen (Gubbels *et al.*, 2005). Further, Cre protein secreted by *T. gondii* is capable of mediating recombination in the nucleus of the host cell. Thus, using this heterologous system, it has been shown that proteins can escape from *Toxoplasma* into the parasitophorous vacuole and then enter a class I MHC presentation pathway and be recognized by CD8 T cells (Gubbels and Striepen, 2004).

Aliberti, Jankovic and Sher recently reviewed dendritic cell function in Toxoplasma gondii infection (Aliberti et al., 2004). Dendritic cells play a major role in the initiation of IL-12-driven host resistance. IL-12 synthesis by dendritic cells is carefully regulated to avoid overproduction. Dendritic cells play a critical role in determining the highly polarized T-helper 1 type response triggered by T. gondii. Dendritic cell function is initiated by T. gondii, and parasite-primed dendritic cells drive the Th1 effector choice. There is endogenous regulation of dendritic cell IL-12 production during T. gondii infection. IL-12 is the primary cytokine initiating IFN-γ synthesis by NK and T lymphocytes during T. gondii infection, and is essential for survival following T. gondii infection. Injection of live tachyzoites or STAg (soluble T. gondii antigen) led to dendritic cells clustering in T cell areas, producing significant levels of IL-12 (also in vitro). The dendritic cells that respond to STAg are of the CD8 $\alpha\beta\gamma$ T-cell subset. The response is a rapid (peaks in 3-6 hours) response, returning to baseline by 24 hours, and then is actively turned off for up to a week. Stimulation of mouse TLR11 is responsible. TLR11 activation of dendritic cells by a profilin-like protein is responsible for stimulation of dendritic cell IL-12 production in an MyD88-dependent manner. CCR5, which is activated by parasite cyclophilin-18, is MyD88independent.

Lipoxin A4 is an arachidonic acid metabolite generated by a 5-lipoxygenase (LO)-dependent pathway. LXA4 inhibts dendritic cell IL-12 production

(Lewis et al., 1990; Aliberti et al., 2002a, 2002b; Karp et al., 2004; Mandal et al., 2005) by inhibiting CCR5 expression. LXA4 synthesis is stimulated by STAg. Acute infection of mice leads to LXA4 production that reaches a plateau at 15 days and persists during the chronic phase of infection (Aliberti et al., 2002b). Mice deficient in LXA4 have more IL-12/IFN-γ synthesis, reduced parasite cyst numbers in the brain, but die 38 days after infection - probably from uncontrolled pro-inflammatory responses. In 5-LO-deficient mice, LXA4 administration prevented mortality and exogenous IL-10 prolonged survival; however, a relapse of infection in the CNS occurred in surviving mice. LXA4 protects mice against cytokine-mediated pathology primarily during the chronic phase of infection, and appears to affect CD11 cells. The down-modulatory effect of LXA4 is associated with an induction of SOCS-2 (suppressor of cytokine signaling). IL-10 has a down-modulatory effect associated with the induction of SOCS-1 and SOCS-3. T. gondii can generate LXA4 by using host lipid precursors. Extracts of the parasite have 15-lipoxygenase activity (Bannenberg et al., 2004), and 15-LO from plants induces LXA4 when inoculated into mice.

In other models, IL-12 appears to serve as a bridge between innate and adaptive immunity by promoting the development of Th1 effector cells (Blanco *et al.*, 2001). IL-10 appears to have a major down-modulatory effect on IFN- γ -mediated effector function when it is at a maximum during the acute phase of infection. IL-15 is needed for optimal priming of the CD4+ T cell response (Combe *et al.*, 2006).

In considering antigen presentation and processing by MHC molecules, recent work has also demonstrated that *T. gondii* may subvert the immune response by down-modulating MHC molecule expression as well as iNOS and NO production (Luder *et al.*, 1998, 2001, 2003a, 2003b; Rozenfeld *et al.*, 2003). This effect of *T. gondii* on MHC expression varied depending on the cell type and experimental model system under study (Schluter *et al.*, 1991, 1993; Bohne *et al.*, 1994; Goebel *et al.*, 2001; Luder *et al.*, 2001, 2003a, 2003b; Luder and Seeber 2001; Butcher *et al.*, 2005).

This subversion of the immune system may contribute to the persistence of *T. gondii* cysts in the central nervous system.

23.6.3 Effector cells

23.6.3.1 Importance of CD8+ T-cell response and CD4+ T-cell help

Early cell-transfer studies (Frenkel, 1967; Frenkel and Caldwell 1975; Brinkman et al., 1986), illness due to T. gondii infection in HIV patients who had low CD4+ T-cell counts (Remington et al., 2005), and the increased susceptibility of nude and SCID mice (Lindberg and Frenkel, 1977) compared with immunocompetent mice (Johnson, 1992b; Gazzinelli et al., 1993b, 1994c; Beaman et al., 1994; Hunter et al., 1994a) have indicated the importance of CD4+ T cells in protection against toxoplasmosis. CD4+ and CD8+ T cells are both important in limiting chronic CNS infection, and CD4+ T cells are needed for long-term protection. In acute infection, CD8+T cells are important and CD4+ T cells play a smaller role. CD4+ and CD8+ T cells both produce IFN-y in mice. Perforin and granzyme are more important in protection against chronic infections in mice, and are produced by CD8+ cells in mice. In humans, CD4+ and CD8+ T cells that produce INF-y, and CD4+ and CD8+ T cells that are cytolytic for infected cells, have been described (Curiel et al., 1993; Montoya et al., 1996). Interestingly, V_{β7} appears to be used preferentially by these cells (Montoya et al., 1996). Lymphokine-activated killer cells have also been described (Subauste et al., 1992). Th1 cytokines produced by effector and memory T cells that have expanded from naïve precursors are essential to prevent reactivation. CD45RAhi CD45 ROlo and αβT cells from naïve donors proliferate in response to T. gondii antigens (Subauste et al., 1998a). This depends on the increased expression of CD80, CD86, and IL-12 by monocytes, which requires CD40 in antigen-processing cells (APCs) and CD40 ligand on activated T cells (Subauste et al., 1998b).

CD8+ T cells play an important role in protective immunity against *T. gondii* infection (Khan *et al.*, 1988a, 1988b; Suzuki and Remington 1988; Brown and McLeod, 1990; Gazzinelli et al., 1991). This immunity is, in part, a result of production of hightiter IFN-γ by this T-cell population. CD8+ T cells form an important subset that modulates the level of IFN-y production against avirulent parasite challenge as well as adoptively transferring immunity (Gazzinelli et al., 1991; Khan et al., 1991). Studies in mice using mutant (ts-4) parasite and in vivo depletion of CD8+ T cells indicate that immune-mediated resistance is dependent upon CD8+ T-cell lymphocytes and IFN-y (Suzuki and Remington, 1990). Among its other functions during T. gondii infection, IFN-y also has been reported to be important for regulating CD8+ T cell response against the parasite. Mice deficient for the p40 chain of IL-12 heterodimer exhibit increased susceptibility and severely depressed CD8+T cell immunity to infection (Elv et al., 1999). However, treatment of these mutant animals with exogenous IFN-y restores their CD8+ T-cell response and ability to withstand Toxoplasma infection.

The specific parasite antigens responsible for induction of CD8+ T cell response have not been fully described. SAG 1, a major membrane antigen that comprises 3-5 percent of the total parasite protein, has been reported to evoke a strong CD8+ T cell response in immunized mice (Khan et al., 1991). This antigen is able to induce high-titer IFN- γ production and antigen-specific cytotoxic CD8+T cells that are directly parasiticidal to extracellular parasites (Khan et al., 1991). In combination with adjuvants like Quil-A or incorporation into liposomes, SAG1 is able to induce nearly 100 percent protection against acute challenge (Bulow and Boothroyd, 1991; Khan et al., 1991), with 100 percent protection against chronic infection measured as number of brain cysts in survivors (Kasper et al., 1992). T cell depletion studies followed by adoptive transfer into naïve mice support high IFN-γ titer producing CD8+ T cells as the primary mediators of this protection. In another study by the same group, a CD8+ T-cell clone generated against SAG 1 was able to induce almost 100 percent protection against T. gondii challenge in naïve recipients (Khan et al., 1994). A SAG 1 specific CD4+ T cell clone failed to transfer

significant protection in naïve animals in spite of its ability to produce quantities of IFN- γ similar to the CD8+ SAG 1-specific T cell clone. ROP2 has also been found to stimulate IFN production (Herion *et al.*, 1993; Saavedra *et al.*, 1996), and secreted proteins (ESA) have also been noted to induce protection (Mercier *et al.*, 1998; Zenner *et al.*, 1999).

The role of CD4+ T cells in the induction of CD8+ T cell immunity against the parasite has also recently been demonstrated (Casciotti *et al.*, 2002). Interestingly, *T. gondii* infection induced a normal antigen-specific CD8+ T cell immune response in

CD4^{-/-} mice. The frequency of the antigen-specific CD8+ T cell population in both knockout (CD4^{-/-}) and wild-type mice was measured at various time points after infection (e.g. on days 30, 90, and 180) by precursor cytolytic T lymphocyte (pCTL) assay. As shown in Figure 23.20, there was no significant difference in the generation of antigen-specific CD8+ T cell response between CD4^{-/-} and wildtype animals on days 30 and 90 after infection. However, the mutant mice were not able to sustain CD8+ T cell immunity. At 180 days after infection, the CD8+ T cell response in the gene-deficient mice was depressed, as determined by pCTL assay



FIGURE 23.20 Survival of $CD4^{-/-}(A)$ and wild-type (B) mice infected with different doses of T. gondii. Female CD4^{-/-} mice and parental C57BL/6 mice that were 5 to 6 weeks old were challenged perorally with 20, 50, or 100 cysts of T. gondii 76K. Survival of animals was monitored daily. There were six animals per group, and the experiment was performed twice with similar results. The data shown are the data from one of the two experiments. (Adapted from Casciotti et al. (2002), with permission.)

(Figure 23.20). This study demonstrated that although CD8+ T cell immunity can be induced in the absence of conventional CD4+ T cells, it cannot be maintained without such cells. In a subsequent study, the mechanism of induction of CD8+ T cell response against T. gondii in CD4+ T cell deficient mice, in the absence of CD4+ T cells T. gondii-infected mice exhibited an extended NK cell response, which was mediated by continued IL-12 secretion. This prolonged NK cell response was critical for priming parasite-specific CD8+T cell immunity. Depletion of NK cells inhibited the generation of CD8+ T cell immunity in CD4-/- mice. Similarly, neutralization of IL-12 reduced NK cell numbers in infected animals and led to the downregulation of CD8+ T cell immunity against T. gondii. Adoptive transfer of NK cells into the IL-12-depleted animals restored their CD8+T cell immune response, and animals exhibited reduced mortality. NK cell IFN-y was essential for cytotoxic T-lymphocyte priming. This report demonstrated that NK cells can play an important role in induction of primary CD8+ T cell immunity, in the absence of CD4+T cells, against an intracellular infection. These observations have therapeutic implications for immunocompromised individuals, including those with human immunodeficiency virus infection. In other systems, T-bet and eomesodermin induced enhanced expression of CD122, the receptor that specifies IL-15 responsiveness (Intlekofer et al., 2005).

Interestingly, there is also a proliferative response of T cells from unexposed individuals to high doses of *T. gondii* antigens (Subauste *et al.*, 1998a; McLeod *et al.*, unpublished data), but this does not appear to be similar in mechanism to the superantigen response observed by Denkers *et al.* (1996) in mice.

23.6.3.2 Macrophages and microbicidal mechanisms of other cell types

In vitro experiments have shown a crucial role for both IFN- γ and TNF- α in the induction of inducible nitric oxide synthase (iNOS). iNOS degrades L-arginine into citrulline and reactive nitrogen intermediates (RNIs) that have a potent

microbicidal activity in murine macrophages infected with T. gondii tachyzoites (Murray et al., 1979, 1980, 1985a; Nathan et al., 1984). In addition, intermediate RNI levels, while not killing the tachyzoites, appear to reduce parasite multiplication and favor stage conversion of T. gondii. Consistently, in vivo IFN-y-deficient mice and TNF- α -deficient mice, or wild-type mice treated with anti-IFN- γ or anti-TNF- α , express lower levels of iNOS when infected with T. gondii. However, RNI appears to be only one of the various IFN-yinducible mechanisms involved in the control of tachyzoite replication. Certain strains of mice (e.g. C57BL/6 background mice) treated with iNOS inhibitors (Gazzinelli et al., 1993b; Schluter et al., 1999; Roberts et al., 2000) or deficient in iNOS will succumb to infection only at 3-4 weeks post-infection, due to large number of cysts in the brain and intense toxoplasmic encephalitis. Mice deficient in iNOS function are relatively more resistant than the IFN-y-deficient, which die of a systemic infection in the first 2 weeks following infection with T. gondii (Gazzinelli et al., 1993a).

In mice, iNOS is needed for control of chronic infection, and iNOS is dependent on IFN-y and TNF- α (Adams *et al.*, 1990; Sibley *et al.*, 1991). The role iNOS plays in differing cell types is not clear (Wilson and Remington, 1979; Wilson et al., 1980a; Nathan et al., 1984; Murray et al., 1985a). Alternative IFN- γ -inducible mechanisms that are involved in T. gondii control have been identified. Indoleamine 2,3-dioxygenase (INDO) is an enzyme that catalyzes the initial rate-limiting step of tryptophan (Trp) catabolism to N-formylkynurenine and kynurenine (Pfefferkorn, 1984; Pfefferkorn et al., 1986). Different human cells express INDO upon stimulation of IFN-y; and INDO expression is regulated by IL-4 and IL-13 (Chaves et al., 2001). The restriction of the available essential amino acid, tryptophan, due to degradation by INDO, leads to the control of various intracellular pathogens, including T. gondii. The expression of INDO mRNA and activity is induced in mice infected with T. gondii in an IFN-γ- and IRF-1-dependent manner (Silva et al., 2002). However, the in vivo role of INDO in the control of T. gondii replication remains to be demonstrated.

More recently, a new mechanism involved in the control of T. gondii in vivo has been identified in mice. This mechanism involves a family of GTPases of 47 kDa (p47-GTPase) that are induced by IFN-γ (Collazo *et al.*, 2001; Butcher *et al.*, 2005). Mice deficient in either IGTP or Lrg47, two distinct members of the p47-GTPase family, are highly susceptible to T. gondii infection, as they fail to control T. gondii (ME49 strain) multiplication and succumb to infection at 9-11 days following infection. Importantly, both IGTP- and Lrg47-deficient mice showed a robust IL-12 and IFN-γ production, as well as regular expression of iNOS gene. Similarly, IGTP expression was normal in the Lrg47-deficient mice and vice versa. Importantly, IGTP is required for both hematopoietic and nonhematopoietic cell resistance to T. gondii (Collazo et al., 2002). Together, these findings suggest that the members of the p47-GTPase family are more important than iNOS for resistance during the early stages of infection with T. gondii in mice. Interestingly, IGTP is not essential for INF-γ-induced resistance in the chronic stage of infection, when RNI has been shown to have a more pronounced role.

23.6.3.3 Mononuclear phagocytes

IFN- γ produced by T lymphocytes stimulates macrophage microbicidal mechanisms, including NO- and iron-related mechanisms (Adams et al., 1990; Bogdan and Rollinghoff, 1999). In other cell types, INF-*γ*-induced tryptophan starvation results in reduction of intracellular parasite growth (Pfefferkorn, 1984; Pfefferkorn et al., 1986). CCL2 and CCL3 (MIP-1a) attract mononuclear phagocytes to an inflammatory focus (Butcher et al., 2005; Lewis and Wilson 2005). Infection of human monocytes with T. gondii induces CD80 and CD86 (Subauste et al., 1998b), and may enhance T-cell IFN-γ early, and Th1 differentiation (Catterall et al., 1986, 1987). Human monocytes have greater innate antimicrobial effect against T. gondii than do human monocyte-derived macrophages or tissue macrophages (McLeod and Remington, 1977b; Anderson and Remington 1979; McLeod et al., 1980, 1983, 1990; McLeod and Estes, 1982). Oxidative and non-oxidative mechanisms are involved (Murray et al., 1979, 1980, 1985a, 1985b; Wilson and Remington, 1979; Wilson et al., 1980a; Nathan et al., 1984; Wilson and Haas, 1984). Human alveolar macrophages and peritoneal macrophages have also been studied, and nonoxidative killing mechanisms found (Catterall et al., 1986). HIV reduces killing T. gondii by human monocytes. Mouse macrophages require TNF- α in addition to IFN-y for enhanced microbicidal capacity (Sibley et al., 1991). IFN-γ also stimulates non-oxidative mechanisms (Wilson and Remington, 1979; Wilson et al., 1980a; Wilson and Haas, 1984; Lewis and Wilson, 2005). Indoleamine 2, 3dioxygenase, which degrades tryptophan, deprives T. gondii of this essential nutrient and is one such non-oxidative mechanism found to be present in human fibroblasts (Pfefferkorn, 1984; Pfefferkorn et al., 1986). Lewis and Wilson reviewed findings concerning newborn monocytes (Lewis and Wilson, 2005): there appears to be reduced chemotaxis of newborn monocytes. MHC class II-mediated antigen presentation by monocytes appears to be intact. Blood monocytes from neonates are normal in number and similar to those of adults in microbicidal capacity, and are activated by IFN-y. There is a modestly lower production of cytokines and MyD88.

T. gondii (differently for different strains) (Saeij et al., 2005a, 2005b) commandeers the host's NF κ B-I- κ B signaling pathway (this is discussed in detail under host-pathogen interactions) (Adams et al., 1990; Langermans et al., 1992; Zhang et al., 1994; Lyons and Johnson 1995; Bellmann et al., 1996; Feinstein et al., 1996; Kim et al., 1997; Scharton-Kersten et al., 1997b; Miller et al., 1999a, 1999b, 2000; Torre et al., 1999; Arsenijevic et al., 2000; Butcher et al., 2001; Goebel et al., 2001; Dobbin et al., 2002; Molestina et al., 2003; Mason et al., 2004a, 2004b; Shapira et al., 2004; Molestina and Sinai, 2005; Saeij et al., 2005a). T. gondii can also block host-cell apoptosis (Curiel et al., 1993; Brown et al., 1995b). Antibodies (serum and secretory) may opsonize and interfere with or specify a different pathway for parasite entry into host cells (Chardes et al., 1990; Joiner and Dubremetz, 1993; Mineo et al., 1993). Heparin-binding proteins and lectins may play a role in initial interactions of *T. gondii* with somatic cells (Ortega-Barria and Boothroyd, 1999). Activated macrophages also down-modulates lymphocyte blastogenesis, at least in part by NO production (Wing *et al.*, 1977; Candolfi *et al.*, 1994; Gazzinelli *et al.*, 1996a), and partly by induction of apotosis (reviewed in Denkers and Gazinelli, 1998). *T. gondii* also alters leukotriene release by macrophages (Locksley *et al.*, 1985).

23.6.3.4 Gamma delta ($\gamma\delta$) T cells

 $\gamma \delta T$ cells are induced early in murine and human T. gondii infections (de Paoli et al., 1992; Scalise et al., 1992; Sayles et al., 1995; Subauste et al., 1995; Hara et al., 1996; Kasper et al., 1996). Vγ2Vδ2CR CD45RAlo CD45hi (activated in vivo) T cells are present in children with postnatally acutely acquired toxoplasmosis. They secrete cytokines (IL-2, IFN- γ , and TNF- α) and proliferate and lyse T. gondii-infected cells in an MHC-unrestricted manner. IFN-γ-producing γδT cells have conferred protection, but not in other studies against bradyzoites (de Paoli et al., 1992; Subauste et al., 1995; Hara et al., 1996; Egan et al., 2005). A 65-kDa heatshock protein that is induced in macrophages by γδT cells contributes to protection against T. gondii (Nagasawa et al., 1992, 1994; Hisaeda et al., 1995; Himeno and Hisaeda, 1996).

23.6.3.5 *Chemokines and chemokine receptors*

Chemokines (chemotactic cytokines) are small, heparin-binding proteins that couple the detection of pathogens with infiltration of tissues with neutrophils and monocytes. They guide circulating white blood cells to areas of inflammation and injury, thus participating in adaptive immunity and pathogenesis. There are approximately 50 human chemokines, which are considered to be in four families based on their structure and function. CC chemokines have their initial two cysteines situated adjacently, and include CCL2 (MCP-1), which attracts monocytes, dendritic cells, basophils, and memory T cells with the receptor CCR2; CCL3 (MIP–1 α), which attracts macrophages with the receptor CCR5; CCL4 (MIP-1 β), which attracts T cells

and monocytes with the receptor CCR5; and CCL5 (RANTES), which attracts T cells and monocytes with the receptors CCR1 or CCR5. A second family is CXC, members of which have an amino acid between the canonical adjacent cysteines. These attract polymorphonuclear leukocytes, activate monocytes and recruit them to vascular lesions, and mediate interactions of APCs and T cells. The CX3C has a mucin-like stalk with transmembrane and cytoplasmic regions. CX3 is cleaved from the cell membrane by TNF- α converting enzyme. The fourth family contains XCL1. Chemokines bind to 7-transmembrane domain-6-protein-coupled receptors activating signaling cascades, and alter movement of cell action. CCR7 and its ligands CCL19 and CCL21 link innate and adaptive immunity, and organize T cell zones in T cells (Denkers and Gazzinelli, 1998).

23.6.3.6 Other cell types

Platelets (Yong *et al.*, 1991), eosinophils, lymphokine activated killer cells (Subauste *et al.*, 1992), polymorphonuclear leukocytes (Marshall and Denkers, 1998; Bliss *et al.*, 2001; Butcher *et al.*, 2001; Del Rio *et al.*, 2001; Denkers *et al.*, 2003b), and mast cells have also been noted to have microbicidal activity for or play a role in the immune response to *T. gondii.*

23.6.3.7 Cytokines

Cytokines have been discussed throughout the sections above, and also considered in Table 23.2. IFN- γ , IL-12, IL-18, and TNF- α produced by NK cells, dentritic cells, and monocytes directly limit acute infection and lead to the differentiation of antigen-specific CD4 T cells into Th1 effector T cells that produce more IFN- γ . This IFN- γ and IL-12, IL-15, and IL-23 produced by non-T cells sustain the Th1 response. Dendritic cells infected with *T. gondii* produce little IL-12, but engagement of CD40 with CD40 ligand markedly upregulates IL-12 production. Cytokines may lead to evasion of initial activation of protective immunity or curtail a potentially lethal immune response (e.g. Neyer *et al.*, 1997). For example, in the absence of

IL-10, the inflammatory process is lethal. Presence of IL-4 early in infection inhibits protective Th1 responses, but later inhibits *Toxoplasma* replication within the CNS. Thus, the temporal sequence of cytokine production is important. A balance of Th1 and Th2 cytokines is critical in a protective immune response to *T. gondii* (see Table 23.2). Antibodies to TNF- α and steroids were reported to be associated with reactivation of human CNS toxoplasmosis (Maguire and Young, 2005).

23.6.3.8 Prostaglandins

As discussed above, there are prostaglandins that promote an inflammatory response and those that down modulate the inflammatory response. Prostaglandins appear to contribute significantly to the outcome of *T. gondii* infection. In other systems PGA1 supresses NF- κ B and Cox-2 gene expression, which is critical for proinflammatory prostaglandins (Mandal *et al.*, 2005).

23.6.3.9 Regulatory T cells

Regulatory T cells are very important in a variety of infections and autoimmune and neoplastic disease states. Their roles in human *Toxoplasma* infections have not been defined as yet.

23.6.3.10 NK cells and KIR

NK cells are important early after infection in mice (Hauser et al., 1982; Hauser and Tsai, 1986; Hughes et al., 1988; Dannemann et al., 1989; Hunter et al., 1997). NK cells also have been found in humans. KIR are modulating NK cell receptors that interact with T cells with certain MHC haplotypes (Stanley and Luzio 1998; Parham, 2005). They are important in a variety of different infections, but they have not been studied in human toxoplasmosis. In other systems, CPG stimulate TLR9 and thus NK cells. With loss of NKG2D receptor (also known as KLrk1) and its adapters Dap 10, DA12, cytotoxicity is lost and dentritic cell-like APC capacity is gained with upregulation of MHC II and co-stimulatory molecules. These cells kill target cells and present antigen linking innate and adaptive immunity.

Finally, ordered crosstalk occurs between NK, NKT, and DC. The relevance of these findings and KIR receptors (Bryceson *et al.*, 2005; Parham *et al.*, 2005; Rajagopalan and Long, 2005) and the specific ligands (Bottino *et al.*, 2005) to the function of these cells in adaptive immunity in *Toxoplasma* infection and toxoplasmosis remains to be determined.

23.6.3.11 NKT cells

NKT cells form a minor subset of T lymphocytes. They share receptors with NK cells and T cells. Murine NKT cells use a semi-variant Va14-Ja281 TCR β chain paired with V β 8-7 or -2 TCR β chain with NK cell receptors NKR-P1, Ly-49, and NK 1.1 in C57B1/6 mice. They are located primarily in the liver, spleen, thymus, and bone marrow, and are also found in the gastrointestinal tract. They recognize hydrophobic lipid antigens presented by CD1d, and CD1d and invariant TCRβ chain are essential for their development. NKT cells have been found to provide help for CD8+ T cell function by producing IL-2 (Denkers et al., 1996). They produce large amounts of IL-4 and IFN-y when activated, are cytotoxic, help in antibody production, regulate Th1/Th2 differentiation, and participate in parasite clearance by shifting the cytokine profile toward a Th1 pattern. They contribute to an immunopathologic gastrointestinal response when the Th1 response remains uncontrolled (Ronet et al., 2005). SAG1 has elicited this gastrointestinal immunopathology (Rachinel et al., 2004). The T. gondii ligand for NKT cells remains to be defined. Mice with CD4+ T cells but without CD8 $\alpha\beta$ T cells and NKT+ cells cannot develop a vaccine-induced protective immune response (Denkers and Sher, 1997).

23.7 IMMUNOLOGICAL CONTROL IN ANIMAL MODELS

23.7.1 Systemic disease and harmful effects of immune response

Toxoplasma infection rapidly overcomes hosts with impaired T-cell function and diminished ability

to produce type 1 cytokines (i.e. IL-12, IFN- γ , and TNF- α). The parasite is itself a strong stimulus of this type of immunity during acute toxoplasmosis, reflecting the need to keep the host alive during the early stages of infection. However, in many instances this early type 1 cytokine response is so intense that it can result in pathologic changes associated with acute toxoplasmosis. Therefore, regulatory mechanisms, in particular the production of IL-10, are critical to control tissue damage and lethality due to excessive type 1 cytokine production during the early stages of infection with *T. gondii*.

The most frequent clinical symptoms associated with acute toxoplasmosis are lymphadenopathy and fever, which occur simultaneously with parasite-induced activation of the immune system and concurrent release of proinflammatory cytokines. However, it may be difficult to differentiate the pathologic changes caused by the parasite by direct tissue destruction from systemic damage caused by parasite-induced cytokines. A recent study has suggested that, in large part, the pathology and lethality caused by the highly virulent RH strain is caused by the excessive production of proinflammatory cytokines, specially IL-1ß (Mordue et al., 2001). Further, pretreatment with β-galactosamine, a model to study mechanisms induced by low doses of endotoxin, makes the injection of tachyzoite extracts lethal. This work indicates that granulocytes contribute to the inflammatory pathologic changes triggered by Toxoplasma infection (Marshall and Denkers, 1998).

Additional evidence that T-cell-derived cytokines promote some of the pathologic change during the early stages of infection with *T. gondii* is described below and in other chapters. During oral infection, the susceptible C57BL/6 mouse strain develops a severe intestinal inflammatory reaction, characterized by necrosis of the villi and mucosal cells of the small intestine (see, for example, Buzoni-Gatel *et al.*, 1997, 2001; Liesenfeld *et al.*, 1999). The pathologic changes are largely reversed by administration of anti-CD4 or anti-IFN- γ mAb, indicating the critical role of Th1 lymphocytes in the intestinal lesions during peroral infection of this strain of mouse with *T. gondii*. However, it has also been noted that IL-4- and IL-5- deficient mice do not develop lethal necrosis in their intestines (see, for example, Nickdel *et al.*, 2001). In addition, in a model of ocular infection, parasites and T cells both contribute to the pathologic process (Hu *et al.*, 1999a, 1999b; Lu *et al.*, 2003). *T. gondii* also can make atherosclerotic lesions in large vessels in a mouse model worse (Portugal *et al.*, 2004).

IL-10 is a cytokine that was first identified by its ability to inhibit IFN-y production by Th1 lymphocytes (Mosman and Moore in Gazzinelli et al., 1993b), mainly through the inhibition of IL-12 production by accessory/antigen-presenting cells. During acute toxoplasmosis in mice, the immunoregulatory role of IL-10 appears to be critical to avoid excessive production of IFN-y by Th1 cells and systemic pathology. IL-10-deficient mice on a BL6 background have enhanced susceptibility to infection, with all such deficient mice dying by day 14 post-infection. Mortality of these mice is delayed by depletion of CD4+ T cells, indicating that these cells have a role in promoting early death. Increased mortality is associated with elevated levels of IL-12 and IFN- γ in sera from infected IL-10-deficient mice, relative to those from uninfected controls, or infected wild-type mice. In addition, the co-stimulatory molecules CD28/B7 and CD40/CD40 ligand have been shown to play a critical role in the excessive Th1 lymphocyte stimulation and pathology observed during acute infection with Toxoplasma in IL-10deficient mice (Hunter et al., 1997). Importantly, parasite burden is similar or diminished in tissues of IL-10-deficient mice. Further, BALB/c mice are resistant to the intestinal pathology observed in the C57BL/6 mice perorally infected with the ME49 strain of T. gondii. Nevertheless, IL-10-deficient mice on a BALB/c background become susceptible and die of the Th1 lymphocyte-mediated intestinal pathology when they are perorally infected with T. gondii (Liesenfeld et al., 1999). Taken together, these studies indicate the critical role of IL-10 as a physiological regulator of production of type 1 cytokines and systemic disease during acute toxoplasmosis (Gazzinelli et al., 1996b).

23.7.2 Toxoplasmic encephalitis

In contrast to acute disease, most of the pathologic findings associated with chronic toxoplasmosis are thought to be caused by lack of appropriate T cell immunity rather than an excessively vigorous response. In general, toxoplasmic encephalitis (TE) positively correlates with the number of cysts in the central nervous system (CNS) of mice experimentally infected with T. gondii. As discussed previously, immunogenetic studies in mice have demonstrated a major role for MHC loci, more specifically the L^d gene, in the development of TE of mice infected with the ME49 strain, implicating T cell involvement in resistance of cyst formation in the central nervous system (Suzuki et al., 1991c; Brown et al., 1995b). These studies suggest the role of CD8+ T-cell responses restricted by this MHC class I molecule as a critical element in protective immunity to TE (Brown et al., 1995b). MHC class IIrestricted CD4+T cells appear to be equally important as animals depleted of CD4+T cells (Gazzinelli, 1991, 1992a; Brown et al., 1995a; McLeod et al., 1995, 1996) as well as mice carrying mutations in the class II $\alpha\beta$ locus also display increased cyst numbers in their CNS.

Infection with T. gondii induces a strong and polarized Th1 response characterized by the production of high levels of IFN-y in addition to IL-2 and TNF-α. The levels of IL-4, IL-13, and IL-5 produced by CD4+ T cells from infected mice are rather low, and their role in host resistance and control of TE is more limited. During T. gondii infection, CD8+ T cells are also an important source of IFN- γ and their function appears to be in part dependent on IL-2 produced by CD4+ T cells. Importantly, IFN- γ produced by a specific subset of CD8+ T lymphocytes that express V $\alpha\beta$ chain in the TCR appears to have a primary role in preventing TE in BALB/c resistant mice. The most critical cytokine involved in the control of TE appears to be IFN- γ . Mice treated with anti-IFN- γ develop severe TE characterized by a dramatic increase in cyst numbers and the appearance of tachyzoites in the brain. This leads to death in 100 percent of mice 7-10 days after the beginning of treatment that neutralizes endogenous IFN-y. Once the chronic infection is established, simultaneous treatment with anti-CD4 and anti-CD8 monoclonal antibodies (mAbs) is necessary to promote increased numbers of cysts, severe TE, and lethality. Thus, both CD4+ T and CD8+ T cells contribute IFN- γ , which controls parasite replication in the brain. Other cytokines, e.g. IL-6 and TNF- α , are also produced by T cells and contribute to host resistance to TE. C57BL/6 mice treated with neutralizing anti-TNF- α mAbs also display a dramatic increase in cyst numbers, severe TE, and die. Similarly, mice lacking the functional receptor(s) for TNF- α have an increased number of parasites in their brains and increased severity of TE (Gazzinelli et al., 1993a). Additionally, mice deficient in IL-6 also have an increase in the cyst numbers, areas of necrosis, and tachyzoite-associated inflammation in the CNS.

The effector mechanisms elicited by these cytokines that are involved in host resistance to cyst formation, release of tachyzoite replication, and development of TE are not completely understood. Nevertheless, it is clear that, in some strains of mice, expression with inducible nitric oxide synthase (iNOS) and production of reactive nitrogen intermediates (RNI) are involved in this process. Mice treated with specific inhibitors of iNOS and those lacking the functional iNOS gene develop a large number of cysts in the brain and severe TE, and succumb 3-4 weeks following infection. In contrast, perforin, a molecule which is critical for the cytotoxic activity of CD8+T cells, appears not to be important in the control of parasite replication and development of TE in mice infected with T. gondii (Denkers et al., 1997a).

23.7.3 Intestinal immunity and toxoplasmic enteritis

The gut represents the first line of defense for most naturally acquired *T. gondii* infections, but is often bypassed in experimental infections that use intraperitoneal or subcutaneous routes. Infection by the oral route can be initiated by *T. gondii* cysts or oocysts. These are susceptible to acid pepsin digestion in the stomach of the host, and release

bradyzoites or sporozoites, respectively, which are pepsin resistant. The bradyzoites or sporozoites are assumed to invade the gut epithelial cells and ultimately disseminate through the blood and lymphatic systems. Immunity in the gut could in theory prevent systemic infection, and is therefore worthy of study for the purpose of vaccine design alone.

Mice fall into two categories regarding susceptibility to orally induced T. gondii infection. While most mouse strains examined to date can tolerate an orally induced infection, at least one, the C57BL/6 strain, is exquisitely susceptible. C57BL/6 mice infected orally with either the ME49 (Liesenfeld et al., 1996) or the Beverley strains of T. gondii (Nickdel et al., 2001) develop severe intestinal necrosis that normally results in death within 8-10 days following infection. The mechanism of this intestinal pathology in C57BL/6 mice is complex, and would appear to involve aspects of the immune response normally associated with Th1 and Th2 cells. Initial studies demonstrated that necrosis and death are dependent on CD4+ T cells, as their depletion at strategic time points could reduce necrosis and promote survival. Furthermore, neutralization of IFN- γ has similar effects, implying that Th1 cells are responsible for the production of IFN- γ and intestinal necrosis (Liesenfeld et al., 1996). IL-10 gene deficient C57BL/6 mice have increased susceptibility to oral infection compared with wild-type mice, and pathology is reduced by neutralizing IL-12. IL-10 gene deficient BALB/c mice were found to be susceptible to intestinal necrosis. Pathology in the intestine of these mice could be prevented, and the time to death prolonged, by neutralization of IFN-γ (Liesenfeld et al., 1996, 1999).

In contrast with these studies implying a detrimental role for Th1 cells and their products, other studies have implicated Th2 cells and their products in the disease process. IL-5 gene deficient C57BL6 mice developed significantly less severe intestinal pathology than wild-type control mice following oral inoculation of *T. gondii* (Beverley strain) (Nickdel *et al.*, 2001). A similar decrease in susceptibility was evident in IL-4-deficient mice compared with wild-type mice infected with *T. gondii* (Beverley strain) (Nickdel *et al.*, 2004). Administration of L-NAME to these mice increased susceptibility, indicating that susceptibility is not associated with excess NO production (Nickdel *et al.*, 2004). IL-4-deficient mice had increased levels of transcripts for IL-10 compared with wild-type mice, consistent with a protective role for this cytokine at this anatomical site (Nickdel *et al.*, 2004).

Studying the immunological events in mouse strains that are largely resistant or strains that are susceptible to intestinal necrosis has provided valuable information on what constitutes a protective or pathological response in the intestine. Intraepithelial cells (IELs) from mice infected 11 days previously are able to transfer immunity to naïve syngeneic animals of the CBA, BALB/c, or C57BL/6 strain. As C57BL/6 mice are susceptible to intestinal necrosis and BALB/c are resistant, these data would indicate that IELs are important in a protective immune response and as regulators of the pathological immune response that can occur in C57BL/6 mice (Buzoni-Gatel et al., 1997). Isolated CD8 $\alpha\beta$ + IELs could again confer protection, but this was abrogated by co-administration of neutralizing IFN- γ . The anti-*T. gondii* action of IFN- γ in the intestine would appear to be independent of arginine and not due to tryptophan starvation, but dependent on iron (Dimier and Bout, 1998). IELs were found to produce IL-10 and TGF-B, both of which are known to inhibit many aspects of inflammation. Adoptive transfer of primed IELs from IL-10-deficient mice was able to prevent pathology, indicating that a molecule other than IL-10 was responsible for protection (Mennechet et al., 2004). However, neutralization of TGF-B can ablate the protective effects of IELs, indicating that this molecule is critical to their protective effects (Buzoni-Gatel et al., 2001).

Overall, these observations indicate that the adaptive immune response plays an important role in control of parasite replication and in prevention of immune pathology at this anatomical site. T cell-derived IFN- γ is not only important in parasite killing, but also contributes to pathology. However, there is also a clear role for the Th2-associated cytokines IL-4 and IL-5, as mice deficient in these cytokines are resistant to intestinal necrosis.

23.7.4 Ocular toxoplasmosis

In addition to congenital toxoplasmosis, there is strong evidence that acquired toxoplasmosis can also be a relatively common cause of uveitis (Glasner et al., 1992; Couvreur and Thulliez, 1996; Burnett et al., 1998; Bosch-Dreissen et al., 2000; Silveira et al., 2001). Pregnancy has been reported to be associated with reactivation of T. gondii infection in humans (Garweg et al., 2005), but how often this occurs has not been defined. In a mouse model of ocular toxoplasmosis, based mainly on studies of mice with acquired infection with T. gondii, both CD4+T and CD8+T cells are observed in the inflammatory process in the eye and are associated with the presence of the parasite (Khan et al., 1997; Roberts et al., 2000). Similarly to toxoplasmic encephalitis, IFN- γ , TNF- α , IL-6, and iNOS appear to be critical elements in the control of parasite replication in the eye. Specifically, neutralization of endogenous IFN- γ or TNF- α as well as inhibition of iNOS with specific inhibitors results in enhancement of parasite replication in the eye tissue, and development of severe involvement (Gazzinelli, 1994a; Hayashi et al., 1996b; Hu et al., 1999a, 1999b; Roberts et al., 2000; Lu et al., 2003). Consistently, IFN-ymRNA was expressed in high levels in the eyes of animals infected with T. gondii, and IFN-y-deficient mice were highly susceptible to toxoplasmic uveitis. IL-6-deficient mice also developed more severe toxoplasmic uveitis. Employing a particular model of ocular toxoplasmosis in which ocular infection developed after mice were immunized with temperature-sensitive mutant Toxoplasma and then received an intraocular inoculation of RH tachyzoites, IL-10-deficient mice were more susceptible and IL-10 transgenic mice more resistant to toxoplasmic uveitis (Lu et al., 2003). Similarly, Fas-FasL interaction and apoptosis of T lymphocytes were important in the control of cellular infiltrates and intensity of eye inflammation elicited by T. gondii antigens (Hu et al., 1999b). Parasite proliferation and CD4+ and CD8+ T cells were important in pathogenesis, and the latter were important in protection as well (Hu et al., 1999a; Lu et al., 2003).

Whether the mechanisms for congenital and acquired ocular disease in humans infected with *T. gondii* are similar or not remains to be defined.

Nevertheless, congenital infection with *T. gondii* is associated with decreased responsiveness of T cells to parasite antigens (McLeod *et al.*, 1990), and episodes of recrudescence of the parasite in the eye. It is likely that congenital ocular toxoplasmosis is the result of a less effective immune response against the parasite. The critical roles of IFN- γ , TNF- α , IL-6, and iNOS in control of parasite replication in the eye in mice may be relevant to the pathogenesis of congenital ocular toxoplasmosis in humans.

23.7.5 Influence of co-infection with other pathogens

In addition to inducing an effective parasite-specific T cell-mediated immune response, T. gondii infection elicits strong cell-mediated immunity and host resistance to non-related pathogens and tumor cells (Ruskin and Remington, 1968; Hibbs et al., 1971). These 'non-specific' immune responses are thought to be dependent on activation of innate immunity, which plays an important role in the development of pathogen-specific Th1 lymphocytes. The Th1 lymphocytes will then mediate activation of cellular effector mechanisms involved in the control of unrelated pathogens. More specifically, T. gondii infection protects the host against pathology elicited during infections with Schistosoma mansoni and Leishmania major and the murine leukemia viruses (Mahmoud et al., 1976, 1977; Gazzinelli et al., 1992b; Santiago et al., 1999) in which the pathogenesis is mediated by Th2 lymphocytes. In addition, infection with T. gondii protects the host against Listeria monocytogenes and certain tumors, where host resistance is largely mediated by T-cell-mediated immunity. HIV interferes with macrophage killing of T. gondii (Biggs et al., 1995), and also alters Th1 cytokines and IL-12, but not monokine, production (Gazzinelli et al., 1995).

Timing of infection with *T. gondii* appears to be critical in terms of specific effects on infections with other parasites. In the case of infection with *L. major*, acute but not chronic infection with *T. gondii* promoted resistance to lesion development and parasite growth, by promoting a *Leishmania*-specific Th1 response in the susceptible BALB/c

mice. In the case of Schistosoma mansoni, chronic infection with T. gondii resulted in resistance to the trematode infection with a remarkable modulatory effect on granulomas, probably through the control of Th2 responses in co-infected animals. In contrast, acute Toxoplasma infection associated with S. mansoni infection resulted in more intense hepatic lesions and lethality that was associated with high TNF- α levels (Mahmoud, et al., 1976, 1977; Araujo et al., 2001). This effect was dependent on endogenous IL-12 levels (Mahmoud et al., 1976, 1977; Araujo et al., 2001). The interaction of T. gondii and Nippostrongylus braziliensis was evaluated in mice, where T. gondii infection largely inhibited the Th2 responses (i.e. IL-4 and IL-5) elicited by this intestinal worm (Liesenfeld et al., 2004). In contrast, there was no major change in Th1 responses (i.e. IFN- γ) elicited by T. gondii in coinfected mice. No change in the course of diseases caused by either of these pathogens was observed. In summary, these studies suggest that infection with T. gondii in mice is highly efficient in suppressing Th2 responses elicited by other parasites (such as L. major, S. mansoni, and N. braziliensis). However, the opposite is not true, since Th1 responses elicited by T. gondii are not controlled when mice are co-infected with infectious agents that preferentially stimulate Th2 responses.

23.7.6 Summary and generalized scheme

Figures 23.1–23.6 summarize the interactions of innate and adaptive immunity, antigen presentation to CD8+ T cells and CD4+ T cells, effector mechanisms elicited, and the initiation and regulation of effector functions.

23.8 IMMUNOLOGICAL CONTROL IN HUMANS

23.8.1 Immunologically normal older children and adults

Anderson first described lymphocyte blastogenesis in response to *Toxoplasma* antigens in individuals with chronic infection, and occasional patients who had little or no response early on after infection (Anderson et al., 1976). In patients with a prolonged lymphadenopathy syndrome, there was an elevation in suppressor (Leu-2 positive) T cells. In patients with asymptomatic lymphadenopathy, a decrease in the number of T helper cells, or an increase in the number of suppressor cells, or both, was noted. Patients who were completely asymptomatic with only serological evidence of acute infections did not have abnormalities in their T cell populations (Luft et al., 1984a). Others have reported similar subset modifications (de Waele, 1985). Vy2V82 T cells are increased in the circulation of children who have acquired toxoplasmosis postnatally (Hara et al., 1996). These are predominantly CD45RAlo-CD45 ROhi, suggesting that they are activated cells. Lysis by such cells is not MHC restricted. yoT cells can lyse infected targets when obtained from individuals without prior infection.

23.8.2 Immunocompromised older children and adults

This clinical problem is considered elsewhere in this book (see Chapter 4) and will not be reviewed here. One interesting recent report of recrudescent toxoplasmosis in conjunction with steroid treatment augmented by antibody to TNF- α was recently noted (Maguire and Young, 2005). Disease due to immunological reconstitution in patients with AIDS is another recently described problem (Petrof and McLeod, 2002).

23.8.3 Protecting the fetus by immunity in the mother, and the implications for vaccine development

Mothers who are chronically infected with *T. gondii* do not appear to transmit *T. gondii* infection to their fetus if they are immunologically normal, once an established immune response has developed. This finding makes it likely that it would be possible to elicit a protective immune response that would allow a woman to be immunized before becoming pregnant. This approach has been used for vaccines for animals (Buxton and Innes, 1995),

and in experimental murine models (McLeod *et al.*, 1988; Roberts *et al.*, 1994).

23.8.4 The fetus and infant

Many components of the immune system of the newborn infant function less robustly than in the older child. This has been reviewed by McLeod and Dowell (2000); Lewis and Wilson (2005), and others (Blackman et al., 1990; Marodi et al., 1994; Barakonvi et al., 1999; Marchant et al., 2003; Arnold et al., 2005); and is summarized in Figure 23.6 and Table 23.7. In newborn infants infected congenitally with T. gondii, there have been studies describing percentages of lymphocyte subpopulations (McLeod et al., 1985b, 1990; Lecolier et al., 1989; Hohlfeld et al., 1990) and their immune responses to T. gondii antigens. Wilson and Remington (1979) initially described a robust blastogenic response to Toxoplasma lysate antigens. In a larger series, McLeod et al. (1985b, 1990) found that the responses were not present or not robust in most of the children in the newborn period. This was especially true of infants who had severe disease and were infected early in gestation, and those infected late in gestation. By 1 year of age most children had developed some lymphocyte blastogenic responses to T. gondii antigens, once medicines were discontinued, but the responses were often less than those in their mothers. This was the case for prolonged periods of time, and a similar diminished response was also noted in a Brazilian cohort of congenitally infected children. Fatoohi et al. (2003) noted the response of most congenitally infected children to soluble Toxoplasma antigen with IL-2 production and CD25 expression. This occurred in about 7 percent of apparently uninfected children as well (Fatoohi et al., 2003). Many of these children had been treated in utero and had much milder disease than those children in the US cohort (Fatoohi et al., 2003). Differences in the results in these studies may be due to differences in methodology, or to the major differences in the clinical status of the patients studied. Yamamoto et al. (2000) also described lower lymphocyte blastogenic responses in children they believed to be congenitally infected, relative to those they believed to have acute acquired infection. Fetuses and infants with congenital toxoplasmosis produce IFN- γ (Lewis and Wilson, 2005).

In an interesting study of $\gamma\delta T$ cell function in infants with congenital toxoplasmosis, some infants had increased expansion of V γ 2V δ 2-bearing $\gamma\delta T$ cells expressing a CD45RAlo–DC45RO hi surface phenotype. These cells had a poor proliferative response when cultured with peripheral blood mononuclear cells infected with live unirradiated or irradiated *T. gondii* tachyzoites.

23.8.5 Fetal and neonatal defenses

Fetal and neonatal defenses (Berman and Johnson, 1978) that have been described include monocyte-derived and placental macrophages activated by IFN- γ that kill *T. gondii* (Wilson and Remington, 1979). T cell numbers and the repetoire for antigen recognition are limited in the first half of gestation (Lewis and Wilson, 2005). Sequelae occur regardless of the time during gestation that infection is acquired (Eichenwald, 1960; Wilson *et al.*, 1980b; Koppe *et al.*, 1986; Remington *et al.*, 2005), suggesting that protective mechanisms are immature even late in gestation.

Congenitally infected infants generally have less lymphocyte blastogenesis, and less IL-2 and IFN-y production in response to T. gondii (Figure 23.21) (McLeod et al., 1990). Fatoohi et al. (2003) found CD25+ CD4+ T cells response measured as IL-2 production in response to a soluble T. gondii antigen preparation (STAg), suggesting that perhaps these might be regulatory T cells. The children studied by Fatoohi et al. (2003) were often born to mothers treated in gestation and had mild or no manifestations of infection, whereas McLeod et al. (1990) studied children with severe as well as milder manifestations. The most profound deficit in the McLeod et al. (1990) series was present in children with the most severe disease (Figure 23.21). No data are available regarding CD8+T-cell responses in congenitally infected newborns. Some of the possible mechanisms to explain absent or less robust Text continued on p. 694

	Gw ¹	Fetus	Newborn
MHC molecule expression in the fetus and neonate	12	Expression of MHC class I and MHC class II molecules by fetal tissues. All APCs, including mononuclear phagocytes, B cells, and DCs are present.	MHC class I expression lower than on adult cells. The amount of MHC class II expressed by neonatal monocytes or B cells is similar to or greater than that expressed by adult cells. Most of the neonatal monocytes lack HLA-DR surface expression.
Circulating neonatal dendritic cells			Most DCs found in the tissues, small numbers in the blood, including immature DC1 and pre-DC2 (0.5% of circulating mononuclear cells). Predominance of pre-DC2s.
Fetal tissue blood	16	Epidermal Langerhans cells and dermal DCs	Expression of MHC class II (HLA-DR) and co-stimulatory molecules on neonatal and adult blood DCL and pro DC2 is similar
dendrific cells	19–21	Cells with the features of pre-DC2 cells in fetal lymph nodes.	Diminished ability of neonatal DCs to produce type I IFN and IL-12.
Neonate-derived dendritic cells			Neonate dendritic cells from cord blood express less HLA-DR, CD1a, and co-stimulatory molecules (CD40 and CD80); they also have decreased allostimulatory activity for T cells. Limitations in $T_{\rm H}1$ immunity, such as delayed-type hyper- sensitivity skin reactions and antigen-specific CD4+ T-cell IFN- γ .
			APC function of monocytes and B cells from human neonates appears to be intact.
			Functional pre-immune T-cell receptor repertoire is fully formed at birth.
Neonatal T-cell proliferation and IL-2 production			IL-12 production by circulating neonatal T cells and by adult T cells is similar. Decreased responsiveness of neonatal T cells that are generally antigenically naïve.

 TABLE 23.7 Immune function in the fetus and newborn infant

Continued

	Gw ¹	Fetus	Newborn
T-cell development and function in a	6–8	Fetal liver contains CD34+ lymphoid cells and appears to include prothymocytes.	The production of most other cytokines and their mRNAs by unfractionated neonatal T cells or the
fetus and neonate	8–9	Initial colonization of the fetal thymus by prothymocytes followed by expression of proteins that are characteristic of T-lineage cells, including CD4, CD8, CD38, and the β-TCR- CD3 complex.	CD4+ T cell subset is reduced in response to different stimuli (e.g. anti-CD3 mAb mitogen). For most cytokines (IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IFN- γ , GM-CSF) this is an important reduction; for a few, such as TNF- α , the reduction
	12	Pattern of expression of other proteins such as CD2, CD5, CD38, and the CD45 isoforms matches that in the postnatal thymus. Clear separation between the thymic cortex	is modest. The low capacity of neonatal T cells to produce IFN- γ and IFN-4 is due to an almost complete absence of IFN- γ and IL-4 mRNA-expressing cells.
	14	and medulla. Major human thymocyte subsets present.	more IL-13 than adult cells.
	17	ICOS co-stimulatory molecule expressed by thymus.	
	18–23	Fetal thymocytes express chemokine receptors CXCR4 and CCR5 (co-receptors for entry of the HIV-1). Medullary CDs in the fetus express high levels of CD80.	
Fetal and neonatal T-cell receptors	8–9	Thymic involution begins at the end of the third trimester.	
		Generation of the $\alpha\beta$ -TCRs.	
	11-13	Increased diversity of the use of D and J segments in the rearrangement of the TCR β chain gene in the thymus.	
Ontogeny of T-cell surface phenotype	12-13	The CDR3 region of the TCR β chain transcripts is reduced in length and sequence and increases by the second trimester.	
	13-15	Circulation T cells detectable.	
	14	CD4+ and CD8+ cells are found in the fetal liver and spleen and CD4+ T cells are detectable in lymph nodes.	

 TABLE 23.7 Immune function in the fetus and newborn infant—cont'd

		The percentage of the T cells in the fetal circulation gradually increases during the 2nd and 3rd trimesters through 6 months of age, then gradually declines.	
		The ratio of CD4+ to CD8+ T cells in the circulation is relatively high during fetal life (about 3.5) and gradually declines with age.	
Fetal T-cell function	14–20	All peripheral fetal and neonatal T cells express the CD38 molecule. A significant fraction of T cells in the fetal spleen lack CD38 expression.	
		Circulating T cells in the 2nd and 3rd trimester fetus and term and preterm neonate predominantly express a CD45RAhi–CD45RAlo surface phenotype.	
		A substantial proportion of T cells in the second trimester fetal spleen are CD45RAhi–CD45RAlo, a T-cell population that is absent from the spleen of young infants. Fetal CD45RAhi express high levels of CD25 and proliferate with IL-12; however, they are not fully functional. Their $\alpha\beta$ -TCR repertoire is diverse.	
	15-16	Mucosal T cells with the capacity to secrete substantial amounts of INF- γ after stimulation with anti-CD3 with exogenous IFN- α , are present in the fetal intestine.	
Fetal and neona T-cell expression of TNF ligand family membe	ntal 19-31 on rs	Substantial proportion of circulating fetal T cells express CD40 ligand <i>in vitro</i> in response to polyclonal activation.	Decreased CD40 ligand production by neonatal cells. T cells have decreased Fas ligand expression after anti-CD28 mAb stimulation compared with adults.
Neonatal T-cell co-stimulation and anergy	ı		Neonatal T cells have a greater tendency to become anergic, particularly under conditions in which production of inflammatory mediators or co-stimulation (e.g. by CD40, CD80, or CD 86 on the APC) may be limited.

Continued

 TABLE 23.7 Immune function in the fetus and newborn infant—cont'd

	Gw^1	Fetus	Newborn
Fetal and neonatal T-cell chemokine receptor expression			Neonatal naïve T cells lack CCR1 surface expression, they do not increase CXCR3 expression, and they do not decrease CCR7 expression, after activation by anti-CD3 and anti-CD28 mAbs. The CCR7 expressed on neonatal T cells is functional and mediates chemotaxis of these cells in response to CCL19 and CCL21.
			Neonatal T cells can increase their surface expression of CCR5 by treatment with IL2.
			Neonatal naïve CD4+ T cells also have the capacity to acquire expression of chemokines characteristic of Th1 or TH2 effectors following their differentiation in presence of IL-12 and anti-IL-4 for Th1 and IL-4 and anti-IL-12 for TH2.
Fetal and neonatal T-cell-mediated cytotoxicity		Capacity to generate a functional CD8+ T cell effector population <i>in vivo</i> during chronic stimulation.	Neonatal T cells are moderately less effective than adult T cells as cytotoxic effector cells.
Neonatal T-cell apoptosis			Circulating mononuclear cells from cord blood, including naïve CD4+ T cells, are more prone than those from adults to undergo spontaneous apoptosis <i>in vitro</i> . Fas levels are undetectable on neonatal lymphocytes, including CD4+ and CD8+ T cells.
			Increased tendency of neonatal naïve CD4 and unfractioned T cells to undergo apoptosis. Expression of lower ratio of Bcl-2 to Bax protein compared with adults.
			Treatment of neonatal naïve CD4+ T cells with IL-7 can block spontaneous apoptosis.
			The circulating levels of soluble Fas, TNF, and p55 TNFR increase in the first days of life.
Regulatory T cells of the neonate			Neonatal mononuclear cells are also more prone than in adults to undergo apoptosis after engagement of MHC class I achieved by mAb treatment.

			About 5% of circulating CD4+ T cells in neonates but also infants and young children express high levels of CD45RA and CD25 and contain CD45RO transcripts.
Natural killer T cells in the neonate			NKT cells represent less than 1% of circulating T cells in the neonatal circulation. Neonatal NKT cells are similar to adult NKT cells in having a memory/effector-like cell surface phenotype, including expression of CD25, the CD45RO isoform, and the low level of expression of L-selectin. Neonatal NKT cells produce only limited amounts of IL-4 or INF-γ on primary stimulation (functional immaturity).
			Neonatal CD56+ T cells express less perforin than do adult cells. Because CD56+ T-cell population is highly enriched in CD1d-restricted NKT cells, NKT cell cytotoxicity probably is limited at birth.
T-cell reactivity to environmental antigens	20	Fetal T cells become primed to environmental and dietary protein allergens as a result of maternal exposure and transfer to the fetus.	
		Regulatory (IL-10-dominant) response. Production of IFN- γ 100-fold higher than IL-4, however the ratio is still reduced compared to adults. Protein allergen-specific T-cell proliferation detected at birth is more common when allergen exposure occurs in the first or second trimester rather that in the third trimester.	
Fetal T-cell sensitization to maternally administered vaccines and maternally derived antigens		Fetal T-cell sensitization can occur in cases of antigen exposure due to chronic infection of the mother with parasites or viruses.Fetal exposure to parasitic antigens without infection can downregulate subsequent postnatal Th1 responses to unrelated antigens.	

IMMUNOLOGICAL CONTROL IN HUMANS

Continued

 TABLE 23.7 Immune function in the fetus and newborn infant—cont'd

	Gw ¹	Fetus	Newborn
Maternal transfer of T-cell immunity to the fetus		Maternal-to-fetal transfer of leukocytes occurs, however their number in the fetus is very low(< 0.1%).	Neonatal T-cell responses as a result of transfer of maternal immunity should remain suspect unless the T-cell population is identified and antigen specificity and MHC restriction are demonstrated.
T-cell response to congenital infection CD4+ T cells		Reduced CD4+ T cells responses, particularly in first- or second- trimester infections, are not absolute and dual Th1- and Th2-type immune responses can develop after some congenital infections or fetal exposure to pathogenic antigens from the mother. They may be the result of antigen-specific unresponsiveness (anergy, deletion, or the failure of the CD4+ T cell to be initially activated by antigen).	Pathogen-specific T-cell proliferative responses and cytokine responses (IL-2 and IFN- γ) in infants and children with congenital infection are markedly decreased or absent compared with postnatal infection. T cells from children with <i>Toxoplasma</i> infection retain the ability to respond to alloantigen and mitogen.
CD8+ T cells		CD8+ T cell responses to congenital infections appear to be relatively robust.	The alterations in CD45RO expression by T cells in congenital infection may persist at least through early infancy.
		Congenital infection with <i>Toxoplasma</i> or viruses during the second and the third trimesters may result in appearance of CD45ROhi memory cells and an inverse ratio of CD4+ to CD8+ cells, findings that also suggest that the fetal CD8+ T cells are activated and expanded in response to serious infection.	
B cells and Immunoglobulins	8 10	Pre-B cells detected in the fetal liver and omentum. B cells express surface IgM but not IgD. Those IgM+ IgD- B cells are transitory stage and express CD21 surface molecule.	 Pre-B cells are developed solely in bone marrow. Declining concentration of B cells until adulthood. High levels of CD34+ CD38- progenitor cells in
Ontogenesis of B cells and	8–11	Transcripts for IgA and IgG present in the fetal liver.	circulation which are capable of differentiating into B cells in bone marrow of children and
Immunoglobulins	13	Pre-B cells detected in the bone marrow, which becomes the predominant site in the mid-gestation period.	adults.B cells have increases surface levels of IgM compared to the adult.
B-cell maturation and pre-immune	16	Fetal bone marrow B cells expressing sIg of all heavy chain isotypes are detectable.	 High but gradually decreasing level of CD5+ B cells.
selection	18–22	Pro-B cells and Pre-B cells in smaller numbers in the liver, lung, and kidney.	• B1 is the major source of the low amounts of circulating natural IgM at birth.

	22	 The proportion of B cells in spleen, blood, and bone marrow is similar to that in adult; further increase of B-cell concentration in third trimester. B-1 cells predominate during early fetal development; high frequency of CD5 expression 	
		 (> 40% in mid-gestation) indicates predominance of B-1a subset. CXCL12 (SDF-1) is a critical for B cells expressing CXCR4 chemokine receptor. The first recognized pre-B cells contain cytoplasmic IgM heavy chains but no light chains or sIg 	
Development of Igs		 In fetal bone marrow light chain rearrangement can occur in the absence of productive heavy chain rearrangement. Pre-immune immunoglobulin repertoire is limited and significantly shaped by self antigens. There are differences in the usage of particular heavy chain D and J segments between first, second, and third trimesters. Over-representation of certain fragments f.ex. DH7-27. CDR3 region of the immunoglobulin heavy chain is relatively short at the beginning of the third trimester and increases until birth. 	 Small numbers of CD10 are found at birth and then decline during infancy. Increased expression of CD38 on neonatal B cells. Lower levels of chemokine receptor CCR7 than
B-cell surface genotype		 Most fetal bone marrow and spleen B cells express CD10. Absence of significant differences in MHC class II expression by circulating fetal B cells and in adults. 	 in adults. Reduced CD21 and CD32 expression on neonatal B cells. Neonatal CD5 cells have reduced expression of: CD11a, CD44, CD54 (ICAM-1), and L-selectin. Circulating neonatal cells have lower levels of MHC class II and inability to use intracellular calcium after engagement of MHC class II by mAb. B cells (90% B-1a) express CD28 (typically found on T cells) and more SD27 and CD80 than in adults.
Response to T-dependent antigens			The capacity of the neonate to respond to T-dependent antigens is well established at birth and is modestly reduced in comparison with the response in the adult.
			Continued

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 TABLE 23.7 Immune function in the fetus and newborn infant—cont'd

	Gw1	Fetus	Newborn
Response to T-independent antigens			Antibody production by human neonatal B cells to TI type antigen <i>in vitro</i> is modestly reduced. The response to TI type II antigens is the last to appear chronologically.
			Decreased expression of CD21 on neonatal B cells has been proposed as a possible mechanism for limitations in TI type II response in a neonate.
			Human neonatal B cells demonstrate a marked decrease in CD22 expression following engagement of IgM, a stimulus to mimic a TI type II antigen.
Specific antibody response by the fetus to maternal immunization and congenital infection		Antibody response by the human fetus may occur following maternal immunization during the third trimester.	
		Specific antibody may be present at birth to agents of intrauterine infection.	
		T-cell dependent isotype switching and immunoglobulin production occurs during fetal life, at least for certain pathogens.	
Maternally derived IgG		The mechanism of the transmission of maternal IgG to the fetus depends on the recognition of maternal IgG through its Fc domain.	
		Maternal IgG and interacellular receptor FcRn expression can be detected in placental syncytiotrophoblasts during the first trimester but transport does not occur.	
	17	IgG detectable in the fetus.	
	30	Half of the serum concentration at term.	
	38	IgG concentration equal that of the mother or higher.	

Inhibition of neonatal antibody responses by maternal antibodies		Maternal antibody may inhibit the production by the fetus or newborn of antibodies of the same specificity. The inhibition varies with the maternal antibody titer and with the type and amount of antigen.	
Fetal and neonatal T-cell-dependent immunoglobulin production	12 20-30	IgG and IgM synthesis in fetal organ cultures. IgG and IgA secreting plasma cells detectable. Pre-B cells have capacity for isotype switching during fetal ontogeny.	Neonatal plasma cells can differentiate into IgM- secreting plasma cells as efficiently as adult cells. Production of IgM, IgG, and IgE by neonatal activated B cells is similar to that in antigenically naïve B cells.
Immunoglobulin synthesis by the fetus and neonate		Passively transmitted maternal IgG is the source of all subclasses detected in a fetus and neonate.	
IgG		Maternal IgG may inhibit certain postnatal antibody responses by binding to FcyRII receptors and by rapidly clearing or masking potential antigens.	
IgM		Does not cross the placenta. Natural IgM, which is not the result of a B-cell response to foreign antigens, plays an important role in innate defense against infection, allowing time for the initiation of antigen-specific B-cell response; it also enhances antigen-specific B-cell responses.	Some of the human neonatal IgM is monomeric and therefore non-functional. Elevated IgM concentrations in cord blood suggest possible intrauterine infections, but many congenitally infected infants have normal IgM cord blood levels. At birth, the frequency of IgA ₁ and IgA ₂ bearing
IgA IgD		IgA does not cross the placenta.	cells is equivalent. Functions of IgM and IgD are largely redundant.
IgE	11	Synthesis detectable.	
NK cells NK development and surface phenotype	6 2nd trim 3rd trim.	 Fetal liver is the main site of gradually increasing fetal NK cells production, probably due to CD34+ CD38+ Lin- cell development. NK are present in the greatest numbers in mid-gestation. From late gestation onwards the bone marrow is a site of NK production. The development of NK cells precedes that of αβ- thymic independence. Commitment to NK lineage is made prior to acquisition of inhibitory receptors. IL-15 is critical for directing some lymphocyte precursors into NK lineage and survival of mature NK cells 	 The number of NK cells is the same or greater than in adults. 50% of NK cells at birth are CD56– cells.

Continued

 TABLE 23.7 Immune function in the fetus and newborn infant—cont'd

	Gw ¹	Fetus	Newborn
NK-mediated cytotoxicity		 Most fetal liver NK cells express the CD3-ε and CD3-γ components associated with TCR and CD16 surface expression. All fetal and neonatal NK cells lack expression of CD57 and have reduced expression of CD2 and CD56 of about 30–50%. Fetal NK cells commonly express CD28. Cytolytic function of NK cells increases progressively in fetal life and reaches approximately 50% at term of those in adult life. Preterm neon function compatible and IFN-γ in r those of adult Neonatal NK cadults to active /li>	 Preterm neonates have reduced cytolytic NK function compared to those of term. Cytolytic activity depends on target–pathogen-related differences. Neonatal NK cells express on their surface IL-2 and IFN-γ in numbers equal to or greater than those of adult NK cells. Neonatal NK cells are less responsive that in adults to activation by the combination of U. 10 cm du 10 fm in the combination of U. 10 cm du 10 cm du 15 cm decimation of U. 10 cm du 15 cm d
Cytokine production			 II-12 and IL-13 by induction of CD09 surface expression. II-12-induced production of INF-γ by neonatal mononuclear cells (mostly NK cells) may be reduced in contrast with adults. These cells produce, however, more INF-γ after stimulation with combination of IL-12 and IL-18 Neonatal lymphokine-activated killer cells (LAK) have normal capacity to be primed by exogenous cytokines. Neonatal CD56– NK population comprises phenotypical and functionally immature NK cells to a mature CD56 computation
Congenital infection and NK cells		 Congenital viral or <i>Toxoplasma</i> infection may increase the number of circulating NK cells. NK cell numbers remain elevated until birth accompanied by decreased NK expression of the CD45RO isoform of CD45 tyrosine phosphatase. 	cens that give rise to a mature CD56+ population

ADAPTIVE IMMUNITY AND GENETICS OF THE HOST IMMUNE RESPONSE

Neutrophils in the fetus and neonate	Yolk sac 14–16 Mid- gestation	 Neutrophil precursors detected. In the liver, spleen, and bone marrow they appear later than macrophage precursors. Mature neutrophils. The numbers of postmitotic neutrophils in the fetal liver and bone marrow are markedly lower than in term newborns and adults. Neutrophils constitute less than 10% of circulation leukocytes, rising to values of 50–60% at term. The number of circulating neutrophil precursors (CFU-GMs) are 10–20-fold higher in a fetus and neonate than in an adult. When compared with mononuclear cells and monocytes from adults, those cells from mid- gestation fetuses and premature neonates generally produce less granulocyte-CSF and granulocyte-monocyte (GM)-CSF after stimulation <i>in vitro</i>. The most critical deficiency in phagocyte defenses in the fetus and premature neonate is their limited ability to accelerate neutrophil production in response to infection. 	 Bone marrow contains an abundance of neutrophil precursors. Maximal rate of CFU-GMs. Within hours of birth the number of CFU-GMs increases sharply in term and preterm neonates. 15% of immature neutrophils in healthy neonates. 	IMMUNOLOGICAL CO
Migration to sites of infection				NTROL IN
Eosinophilic granulocytes	18–30			HUMAI
Mononuclear phagocytes in the fetus and neonate				SN

Continued

	Gw ¹	Fetus	Newborn
Humoral mediators of inflammation and opsonization			
Complement	6–14	• Little if any maternal complement is transferred to the fetus.	 Substantial inter-individual variability in term neonates but alternative pathway components are more often decreased than is the classic pathway activity. Decreased abundance of some terminal components. Preterm infants have more often components of both pathways decreased. Small for gestational age neonates have values similar to those of healthy neonates. Serum levels in healthy neonates are higher than in adults.
C-reactive protein		• Does not cross placenta.	
Mannan- binding- lectin (MBL)			• Concentration of MBL in preterm neonates is approximately 50% lower than in term neonates.
Fibronectin			 Plasma fibronectin concentrations are low in neonates, particularly in prematures; they are further reduced in infections; they reach normal ranges at 1 year of age
Opsonic activity			 Efficiency with which neonatal sera opsonize organisms is quite variable but they are less able to opsonize organisms in the absence of antibody
Chemotactic factor generation			 Sera from term neonates generate less chemotactic activity than adult sera and this diminished activity reflects a defect in complement activation rather than antibody.

 TABLE 23.7 Immune function in the fetus and newborn infant—cont'd

Concepts adapted from Lewis and Wilson (2005), with permission.

¹GW = gestational week in which response appears.

ADAPTIVE IMMUNITY AND GENETICS OF THE HOST IMMUNE RESPONSE



FIGURE 23.21 Lymphocyte blastogenic responses in infants with congenital toxoplasmosis.

(A) *Toxoplasma* lysate antigens (top), concanavalin A (center), and mixed leukocyte culture(bottom). The dashed lines demarcate positive and negative responses.

- (B) Lymphocyte response of all study children to *Toxoplasma* lysate antigens. Infants younger than 13.5 months are represented by solid bars and those children older than 15 months by open bars. The horizontal dashed line at S.I. = 2.5 demarcates positive and negative responses. The horizontal dashed and dotted line at S.I. = 8 indicates the lowest responses of lymphocytes from infected adults.
- (C) Correlation of lymphocyte blastogenic response to *Toxoplasma* lysate antigen and severity score. Correlation between diminished transformation and severity was significant (P = 0.002). Patient numbers are those used in publications from the US National Collaborative Study Group (NCCTS). (Adapted from McLeod *et al.* (1990), with permission.)

lymphocyte blastogenesis in response to *T. gondii* antigens in congenitally infected infants include the following:

- 1. Limitations of antigen presentation
- 2. Down-modulation of responding T cells, for example by regulatory T cells
- 3. Sequestration of responding T cells
- 4. Deletion of responder T cells
- 5. Immaturity of responding T cells or co-stimulatory molecules that are not present; different; or in different amounts.

No antibodies specific to *T. gondii* were detectable in approximately 50 percent of infected newborn infants. Newborns lack DTH, have IgG similar to their mother, and have little or no circulating IgM or IgA (Suzuki *et al.*, 1991a; Lewis and Wilson, 2005).

23.8.6 Pregnancy and recrudescent eye disease

Recrudescent eye disease occurs in some pregnant women in successive pregnancies, but the pathogenesis of this process is not understood.

23.8.7 Chronic infection

Congenital toxoplasmosis in humans is a recrudescent, recurring disease (Koppe et al., 1974, 1986; Koppe and Kloosterman, 1982; Desmonts and Couvreur, 1984; Koppe and Rothova, 1989; Binquet et al., 2003). Rarely, chronically infected older children or adults with postnatally acquired infection have had unusual, severe or protracted neurologic signs, some with persistent CSF local antibody production (Desmonts and Couvreur 1984; Couvreur and Thulliez, 1996; Dardé, 1996; Bossi et al., 1998; Dardé et al., 1998; Ajzenberg et al., 2002, 2004; Carme et al., 2002). Other that these isolated cases, the consequences of chronic T. gondii infection in the brains of 2 billion people worldwide remains to be determined. In humans, remote chronic infection is not transmitted to the fetus unless there is immunocompromise (Remington et al., 2005), but in mice such vertical transmission does sometimes occur (Beverley, 1959).

23.8.8 Guillain-Barré syndrome

There have been isolated cases of Guillain-Barré syndrome in individuals with postnatally acquired toxoplasmosis (Couvreur and Thulliez, 1996; Ajzenberg *et al.*, 2002, 2004), including a case due to an atypical strain of *T. gondii* in a patient in Guyana (Carme *et al.*, 2002). The pathogenic mechanism(s) were not determined in these cases.

23.8.9 Recurrent signs and symptoms in congenitally infected persons and those with acute acquired infection and eye disease

The pathogenesis of this clinically important problem is unknown. It appears that recrude-scenses are more frequent in adolescence, and slightly more frequent around the age that children enter grammar school (Hogan *et al.*, 1956, 1957, 1964; Holland *et al.*, 1988; Holland 1999, 2003; Binquet *et al.*, 2003; McLeod *et al.*, in preparation).

23.8.10 Repeated infections with different clonal types of parasites

In mice, a first infection protects against a second infection (McLeod *et al.*, 1988). The relevance of this observation and the natural history of repeated *T. gondii* infections with the same or different clonal types of parasites is not known. It is of interest that a seropositive recipient of a transplanted heart from an acutely infected individual develops IgM-specific antibody and an increase in *T. gondii*-specific IgG antibody, but does not become clinically ill. In contrast, a seronegative recipient may develop life-threatening infection on organ transplantation from a seropositive donor (Ryning *et al.*, 1979).

23.9 INFLUENCE OF CO-INFECTION WITH OTHER PARASITES

The effect of HIV infection on depletion of CD4+ T lymphocytes and the results of this immunosuppression predisposing to *T. gondii* encephalitis and

disseminated infection are commonly recognized. Cytomegalovirus modified the pattern of intracellular replication of *T. gondii* (Ghatak *et al.*, in Remington *et al.*, 2005), and such conjoint congenital infections have been reported. Little is known about the influences of these two pathogens on the host when acquired together.

23.10 PREGNANCY AND CONGENITAL DISEASE

During pregnancy there are number of immunological changes that facilitate implantation of the placenta and survival of the fetus (Morrell, 1995). It is perhaps not surprising that susceptibility to a number of pathogens is noted during pregnancy. Modulation of the immune response is mediated by changes in a number of sex- and pregnancyassociated hormones, including estrogen, testosterone, and progesterone, all of which are raised during pregnancy compared with normal physiological levels. Most notably, progesterone, which cycles between 1.8 ± 0.34 and 43 ± 13 mmol/l in a non-pregnant woman, is raised incrementally as pregnancy proceeds, to over 500 nmol/l in the late stages of pregnancy. This hormonally induced regulation of immune cell function means that in spite of the presence of macrophages, NK cells, mast cells, eosinophils, neutrophils, and T cells in the decidua, the trophoblast is not rejected (reviewed in Roberts et al., 1996b, 2001).

Local T-cell function is tightly regulated in fetoplacental tissues and a Th2 phenotype is favored with the production of IL-4, IL-5, and IL-10 (Lin *et al.*, 1993; Piccinni *et al.*, 2000a, 2000b). Notably, disruption of this by administration of Th1-associated cytokines such as IFN- γ or IL-2 can mediate abortion in pregnant mice (Lin *et al.*, 1993; Raghupathy, 1997). The actions of these cytokines are most probably mediated downstream by TNF- α and/or reactive nitrogen intermediates (RNI), as administration of LPS, for example, can also induce similar effects (Gendron, 1990). The importance of progesterone in polarizing the developing T-cell response towards Th2 has been demonstrated, and this hormone has even been demonstrated to induce IL-4 and IL-5 production by established Th1 clones (Piccinni *et al.*, 1995). Progesterone and IL-4 have been shown to induce LIF (leukemia inhibitory factor), which is necessary for embryo implantation (Piccinni *et al.*, 2000a, 2001a, 2001b). The expression of CCR5 and CXCR4 is downregulated on activated T cells in the presence of progesterone (Vassiliadou *et al.*, 1999).

Progesterone also modulates NK cell function in a number of different ways. For example, progesterone induces lymphocyte production of PIBF (pregnancy-induced blocking factor), which binds NK cells and inhibits their degranulation (Faust et al., 1999). PIBF is produced by lymphocytes during normal pregnancy, and its production positively correlates with successful conception (Check et al., 1997). The gene has recently been cloned from humans and the expressed recombinant protein demonstrated to downregulate NK cell function (Polgar et al., 2003). Administration of PIBF to mice has also been demonstrated to prevent abortion in mice injected with the progesterone antagonist RU486 (Polgar et al., 2003). Progesterone has also been shown to be responsible for the expansion of γ 1.4 δ 1 T cells that produce IL-10 throughout pregnancy and inhibit NK cell function and IL-12 production (Barakonyi et al., 1999; Polgar et al., 1999).

The need for tight regulation of inflammatory products and a local Th2 response for successful pregnancy is opposed to with what is required for control of *T. gondii* infection. Conversely, a strong immune response to *T. gondii* involving NK stimulation and Th1 cell activation is incompatible with successful pregnancy.

23.10.1 Pregnancy increases susceptibility to *T. gondii* infection

Mice have increased susceptibility to *T. gondii* when infected during pregnancy (Shirahata *et al.*, 1992). In keeping with the ability of pregnancy to polarize the immune response towards Th2, pregnant mice infected with *T. gondii* produce less IFN- γ than similarly infected non-pregnant female mice (Shirahata *et al.*, 1992). This reduced ability to produce IFN- γ correlates with increased mortality,

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but can be ablated by administration of exogenous IFN- γ or IL-2 (Shirahata *et al.*, 1993).

Early observations suggested that a chronic T. gondii infection in humans presented no risk of congenital transmission. While this is generally true, there are now a number of cases reported in the literature that demonstrate congenital transmission from a chronic infection (see, for example, Kodjikian *et al.*, 2004). This implies that there has been a degree of reactivation of disease. Indeed, in a recent study that followed pregnancy in 18 females with chronic T. gondii infection, 7 cases of reactivation in the ocular site were observed (Garweg et al., 2005). The incidence of disease reactivation is likely to be higher, as it may go unobserved in other anatomical sites. This has implications for the health of the pregnant mother and the fetus. Similarly, it was largely believed that sheep were only at risk of congenital transmission if infected during pregnancy. Furthermore, chronic infection was thought to prevent congenital transmission if the sheep were exposed to a further infection. However, a recent study has found consistently high levels of congenital transmission in pedigree flocks of sheep, implying that this is not always the case (Morley et al., 2005).

In mice, congenital transmission of T. gondii through successive generations of mice was reported as early as 1959 (Beverley, 1959), but has since been demonstrated to be mouse-strain dependent (Roberts and Alexander, 1992). Thus, chronically infected BALB/c mice do not transmit disease to their offspring even if re-challenged with T. gondii during pregnancy (Roberts and Alexander, 1992). This demonstrates that the ability of mice to prevent congenital transmission has a genetic component. The BALB/c model has been used by a number of workers to determine the functional correlates of this protection both for prevention of congenital transmission following infection during pregnancy, and in pregnant mice with chronic infections (Roberts et al., 1994; Abou-Bacar et al., 2004a, 2004b). RAG-/- mice have reduced levels of congenital transmission compared with BALB/c mice when infected during pregnancy (Abou-Bacar et al., 2004a). This indicates an important role for innate immunity, which was confirmed by depletion

of NK cells in RAG-/- mice. Interestingly, similar NK-cell-depletion experiments had no effect on the rate of transmission in BALB/c mice, suggesting that T or B cells play a role in even at these early stages in immunocompetent mice (Abou-Bacar et al., 2004a). A surprising finding is that neutralizing IFN-y increases the rate of congenital transmission, which would suggest that transmission is enhanced by an inflammatory response (Abou-Bacar *et al.*, 2004a). A role for CD8+ cells and IFN- γ has been demonstrated in prevention of congenital transmission from chronically infected mice. In contrast, depletion of CD4+ cells did not cause disease reactivation or disease transmission (Abou-Bacar et al., 2004b). The role of IL-4 in prevention of congenital transmission is complex, and is likely to be mouse-strain-dependent. A study using BALB/c IL-4-deficient mice found these to have reduced rates of congenital transmission compared with wild-type mice (Thouvenin et al., 1997), whereas a separate study using B6/129 mice found no difference in congenital transmission rates between IL-4-deficient mice and wild types (Alexander and Hunter, 1998).

The role of various components of the immune response in preventing congenital disease is difficult to study. In keeping with the studies that found no role for CD4+ cells in preventing congenital transmission from chronically infected mice (Abou-Bacar *et al.*, 2004b), rates of congenital *T. gondii* infection in HIV-infected mothers are low (European Collaborative Study, 1996). There are some cases reported where congenital transmission has been recorded from chronically or recently infected mothers with HIV infection (see, for example, Vogel *et al.*, 1996; Bachmeyer *et al.*, 2005; Remington *et al.*, 2005).

23.10.2 *T. gondii* infection can have an adverse effect on pregnancy

In apparently healthy pregnant women that become infected with *T. gondii* during pregnancy, the rate of transmission and consequence var for the fetus vary by trimester. Thus, infection during the first trimester has a relatively higher risk of spontaneous abortion, although this still appears relatively small compared with the second or third trimester. In contrast, the likelihood of congenital infection increases by trimester in which the infection occurs - first trimester 25 percent, second trimester 54 percent, and third trimester 65 percent (for review, see McCabe et al., 1987). In spite of the relatively short pregnancy in mice, a number of similarities have been observed in the BALB/c model. Infection during the first trimester normally results in abortion or fetal resorption, whereas infection in the second trimester normally results in approximately 50 percent of the pups becoming infected (Roberts and Alexander, 1992). The ability of T. gondii to induce a Th1 response may be sufficient to induce abortion during the first trimester, when pregnancy-induced Th2 bias is low. In contrast, in the third semester, by which time pregnancy has induced a strong Th2 polarization, this may counteract the T. gondii-induced Th1 response and prevent abortion. As a consequence, parasite survival may be favored in the third semester and account for the increased rates of transmission.

23.10.3 Immune response in congenital infection is abrogated

Congenitally infected humans develop a reduced immune response to T. gondii compared with that observed in adults. Specifically, lymphocytes from congenitally infected infants have been found to have poorer blastogenic responses to T. gondii antigen extracts than those of recently or chronically infected adults (McLeod et al., 1985b). Poor blastogenic responses correlated with more severe disease symptoms in congenitally infected infants. Furthermore, IFN- γ and IL-2 production was impaired in congenitally infected infants (McLeod et al., 1990). A further study found that both $\alpha\beta$ T cells and $\gamma\delta$ T cells are an ergized in congenitally infected infants less than 1 year of age. However, whereas $\gamma\delta$ cells were no longer anergic to T. gondii antigen after 1 year, and produced IFN-y, the αβ cells were still unresponsive to T. gondii antigen (Hara et al., 1996). A degree of impaired immune function to T. gondii is likely to be present in all congenitally infected individuals for the remainder of their lives. Recurrent ocular disease is common in congenitally infected people. Although it is now recognized that not all cases of ocular disease are due to congenital infection, recurrent ocular disease in people with adult acquired *T. gondii* disease is considerably rarer.

23.11 SUMMARY AND CONCLUSIONS

Adaptive immunity and the genes that specify this response and the innate immune response, and their interplay, are critical to the outcome of *T. gondii* infection. Understanding these immune responses and other genetic factors that influence outcome is central to understanding pathogenesis and protection in this infection, and will provide the foundation for development of protective preparations. This infection causes significant morbidity, costs for care and loss of productivity, and suffering (Roberts and Frenkel, 1990). Understanding pathogenesis and protective mechanisms in this infection will lead to strategies to prevent this disease.

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Vaccination Against Toxoplasmosis: Current Status and Future Prospects

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24.1 INTRODUCTION

Toxoplasma gondii is a zoonotic disease and, as such, a successful vaccine would have beneficial impacts in both medical and veterinary fields. An effective vaccine for use in humans, while serving in the first instance to reduce mortality and morbidity associated with infection, would also have economic benefits, as it would reduce the financial burden of lifelong care needed for those with severe chronic disease. The ideal vaccine for veterinary use would have the dual advantages of increasing livestock productivity while reducing the public health risk associated with eating meat.

24.2 SCOPE OF PROBLEM AND POTENTIAL BENEFITS OF VACCINATION

24.2.1 Toxoplasmosis in animals and the potential benefits of vaccination

T. gondii is a widely disseminated parasite which is capable of infecting all warm-blooded vertebrates to different degrees of severity, depending on the species infected. Prior to a discussion on the benefits of vaccination, it is important to define the problems associated with toxoplasmosis in different economically important livestock animals, 722

such as sheep, goats, pigs, cattle, and chickens, and in the definitive host species, the cat.

24.2.1.1 Toxoplasmosis in sheep and goats

The main problem associated with T. gondii infections in sheep and goats is fetal death. In countries with a high prevalence of T. gondii, such as the UK and Spain, the parasite is responsible for 25 percent of all abortions (Buxton, 1998; Pereira-Bueno et al., 2004). In general, sheep become infected with oocysts derived from contaminated feed, pasture, or water. Since most sheep and goats are kept outdoors (with the exception of milk goats), they are all at risk. In non-pregnant sheep a T. gondii infection normally goes undetected and is associated with mild flu-like symptoms, which coincides with the presence of tachyzoites in the circulation. The induction of protective immunity results in reduced parasitemia and conversion of tachyzoites to bradyzoites, followed by a persistent lifelong infection with tissue cysts. Most evidence indicates that after a primary infection sheep become immune to re-infection, and this immunity prevents fetal infections during subsequent pregnancies (for review, see Munday, 1972; McColgan et al., 1988; Frenkel, 1990; Buxton and Innes, 1995). However, a recent paper from Williams et al. (2005) would suggest that there is a high rate (54 percent) of transplacental transmission of Toxoplasma in sheep, proposed to be due to reactivation of chronic infections. These data conflicted with older reports (Hartley, 1966; Munday, 1972) and led to much discussion, which quickly resulted in another independent study that could not confirm the results from Williams (Rodger et al., 2005). Thus, although pregnant immune ewes may vertically transmit Toxoplasma to their offspring, this seems to remain more the exception than the rule.

Sheep and goats that contract a primary infection during gestation have a high risk (> 80 percent) that tachyzoites will infect the placenta and traverse to the fetus, leading to resorption, abortion, or stillbirth (Buxton, 1998). The clinical outcome depends on when during the 145-day gestation period a pregnant ewe becomes infected. Infection early in pregnancy is likely to cause fetal resorption, whereas infection late in pregnancy (after day 120) usually results in the birth of an apparently normal lamb, which may in fact be infected. Such infected newly born lambs usually have developed a protective immune response to *T. gondii*, and therefore few congenital defects are observed (Buxton, 1998). Infection in mid-term (days 50–120) will cause fetal death, mummification, and abortion, the time from infection to abortion being about 40 days. *T. gondii*-specific antibodies may be detected in the fetal circulation 30 days after initial infection of the ewe (for review, see Buxton, 1998).

24.2.1.2 Toxoplasmosis in pigs

Fetal *T. gondii* infections in pigs can lead to abortion and stillbirth, in a similar manner to sheep (Dubey and Urban, 1990). However, transplacental infections in pigs appear to be less common than postnatal infections (for review, see Dubey, 1986). In particular, young nursing pigs are susceptible to toxoplasmosis, showing fever, coughing, weakness, and wasting. In adults toxoplasmosis is mostly subclinical, but infection is persistent, with tissue cysts being present in many different tissues (see Dubey, 1986). As such, the contamination of pork meat, for human consumption, with tissue cysts defines the major problem in pigs.

The prevalence of T. gondii-positive pigs is complicated by the different farm facilities and farm managements that are used, and furthermore depends on the age of the animal. There is an extremely high incidence of toxoplasmosis in pigs reared outdoors. In the late 1960s, when pigs were kept outdoors, 75 percent of pigs were infected with T. gondii (Tenter et al., 2000). The introduction of indoor farming facilities has dramatically reduced infection rates, to as low as 1 percent (Davies et al., 1998). In Argentina seroprevalence was only 4 percent in indoor-reared pigs, whereas in some farms outdoor-reared sows were 100 percent positive (Venturini et al., 2004). In fact, a recent study in the Netherlands showed that pigs reared indoors were completely free of T. gondii infections, while 3 percent of animal welfarefriendly reared pigs were seropositive for T. gondii (Kijlstra *et al.*, 2004). Thus *T. gondii* infections at present are mainly an issue for the minority of pigs that are reared outdoors. However, currently within the EU there is broad concern about animal welfare and an increasing trend to purchase animal welfare-friendly products (Kyprianou, 2005). This will undoubtedly lead to more free-ranging pigs within the EU, which is likely to coincide with a re-emergence of infected pigs and pork meat.

24.2.1.3 Toxoplasmosis in cattle

Cattle do not get clinically ill from T. gondii infection, and the only substantive concern is whether or not beef can be infective to consumers. Various surveys could not identify cattle that had been infected with T. gondii in the field, and controlled infections with T. gondii displayed only transient infections, which were quickly eliminated (Dubey, 1990). Experimental infection followed by feeding of edible tissue to mice and cats demonstrated that T. gondii could not be isolated in significant quantities from these tissues. Mice fed with homogenized organs remained negative, whereas cats shed oocysts after feeding on heart and tongue (Dubey et al., 1993). Abortions in cattle reported as being due to T. gondii in the past were probably a result of Neospora caninum infection, which was first recognized in 1989 (Dubey and Lindsay, 1996). Thus, although beef cattle can theoretically be transiently T. gondii positive, this poses only a small risk for human health.

24.2.1.4 Toxoplasmosis in chickens

Chickens can be infected with *T. gondii*, which results in the development of tissue cysts in multiple organs, and generation of specific antibodies, but not clinical toxoplasmosis (Dubey *et al.*, 1993). The parasite is present in free-ranging chickens, as shown in a study from the USA, where roughly 10 percent of 118 birds analyzed contained infectious tissue cysts (Dubey *et al.*, 2003). However, chicken meat is not considered a risk factor for humans, because most chickens are reared indoors, and poultry products are usually frozen

for storage and are thoroughly cooked to avoid infections with other pathogens.

24.2.1.5 Toxoplasmosis in cats

Cats, as the definitive hosts, normally become infected by ingestion of tissue cysts, and although adult cats usually remain clinically healthy (apart from in a few isolated reports - see Henriksen et al., 1994; Dubey, 1995), kittens sometimes die of acute toxoplasmosis (Dubey and Frenkel, 1972). A large proportion of all cats are seropositive, as evidenced in a recent study from Ohio, where 48 percent of analyzed cats were seropositive (Dubey et al., 2002). Cats that become infected shed around 20 million oocysts over a short period of about 2 weeks, before the development of a strong protective immune response which limits further oocyst shedding (Dubey, 1995). However, some immune cats can re-shed oocysts if they are challenged after a long period (6 years) with a heterologous strain (Dubey, 1995).

The shedding of highly infectious oocysts, which remain viable for more than 1 year, into the environment by cats poses a major risk for both humans and livestock. Oocysts can contaminate food and thereby create an effective route of infection for both humans and livestock. Feces from cats and wild felids can contaminate drinking water, thereby causing outbreaks of toxoplasmosis in humans – as shown in Panama, the USA, and Brazil (Benenson *et al.*, 1982; Bowie *et al.*, 1997; Bahia-Oliveira *et al.*, 2003). Finally, in contrast to contact with cat litter, petting of cats that had recently shed oocysts was shown to pose a minor risk of infection for humans (Dubey, 1995).

24.2.2 Benefits of animal vaccination

In the case of the domestic cat, successful vaccination would limit oocyst shedding and ultimately reduce the incidence of toxoplasmosis generally. Otherwise, vaccination should improve livestock productivity by reducing fetal damage and hopefully also the incidence of human disease by limiting contamination of meat products with tissue cysts. As discussed above, clinical toxoplasmosis resulting in fetal damage occurs mostly in sheep and goats. For these animals, a commercial live tachyzoite vaccine already exists – Toxovax (derived from the S48 'incomplete' strain). Toxovax does efficiently reduce fetal deaths, and as a non-persistent strain has a good safety record (further discussed in section 24.3.2.1). It is unknown whether Toxovax also aids in the reduction of contaminated lamb meat with tissue cysts, and future vaccine studies on sheep and goats are needed to address this question.

While the vast majority of pigs are reared indoors, toxoplasmosis in this species remains a minor problem and the market for a vaccine is perhaps too limited to be of commercial importance. However, there is an increasing public perception in the developed world that 'organic' farming and 'free-range' produce is superior to that intensely reared indoors, both in terms of quality and public health, not to mention the improved animal-welfare considerations. Consequently, freeranging pigs are becoming more common, with the subsequent increasing human health risk associated with an undoubted rise in the incidence of toxoplasmosis in pigs. Therefore, vaccination to protect young nursing pigs as well as to block cyst formation is likely to become necessary for a free-ranging population that will increase in the future.

Since cattle display no clinical signs of toxoplasmosis, and as *Toxoplasma* infections do not persist in these animals, they do not require vaccination. Also chickens, although they can have persistent infection with tissue cysts, do not require a vaccine, as they display no clinical manifestations and chicken meat is processed in such a way that tissue cysts are unlikely to survive.

Finally, as the definitive hosts, cats are a major risk responsible for contaminating food, pastures, and drinking water with oocysts. Cats are a particular risk around farms, and vaccinating farm cats with an experimental live bradyzoite vaccine (T-263) not only demonstrated that it was possible to neutralize oocyst shedding, but also within a few years local mice were found to be *Toxoplasma* seronegative and the incidence of seropositivity in finishing pigs had decreased (Mateus-Pinilla *et al.*, 1999). This trial clearly demonstrates the feasibility and the potential benefits of vaccinating cats. Reduced oocyst shedding from farm cats would limit the incidence of toxoplasmosis not only in domestic livestock, but also in man via contaminated food and water (Bahia-Oliveira *et al.*, 2003). Similarly, vaccinating household cats would also reduce the likelihood of oocyst-initiated infection to humans, and in particular to pregnant women.

In conclusion, a veterinary vaccine against toxoplasmosis already exists for sheep and goats that limits the incidence of abortion. The question that remains to be addressed is whether it also limits cyst burdens. Obviously, an ideal vaccine would also have this outcome. If new veterinary *Toxoplasma* vaccines are developed, successful vaccination of cats would be expected to have the biggest impact on reducing infection for both livestock and humans.

24.2.3 Toxoplasmosis in humans and the potential benefits of vaccination

Essentially, all humans (approximately 6500 million) are at risk of T. gondii infection, and all could arguably benefit from vaccination against this parasite. Current treatments are inadequate, as they only control the proliferative tachyzoite stage of the life cycle but do not eliminate the cyst stages associated with chronic infection (reviewed in Roberts et al., 2002). There are several groups where the consequences of infection could be particularly severe and where the potential of vaccination would be great. T. gondii is a major cause of congenital disease with potentially severe sequelae. Pregnant women are more likely to acquire the infection (Gilbert and Gras, 2003; Avelino et al., 2004). Consequently, vaccination of women before they reach childbearing age may be a reasonable strategy to reduce or eliminate this risk. The benefits of such a program would vary according to country, and clearly would have the greatest impact where the incidence of congenital infection is highest. For example, the incidence of congenital toxoplasmosis is estimated to be 10 per 1000 births in Paris, France (Desmonts and Couvreur, 1974), 0.5 per 1000 births in the UK (Williams *et al.*, 1981), and 1–10 per 10 000 births in the USA (Lopez *et al.*, 2000). The financial cost of such a program would be considerable, but would be offset by a reduction in the cost of caring for those congenitally infected. In the USA, the estimated total medical costs and loss of productivity as a consequence of human toxoplasmosis, excluding AIDS patients, was \$2 628 000 000 per annum ('Charting the cost of Food Safety', Food Review, US Department of Agriculture, May– August, 1994).

At one time it was a widely held assumption that ocular toxoplasmosis only occurred in the immune competent following congenital infection, but recent evidence has found that it occurs in a significant number of adult acquired infections (see Roberts and McLeod, 1999). It has been estimated to occur in 2-3 percent of adult acquired infections (Perkins, 1973), and 49 cases were identified over a 13-year study in France (Couvreur and Thulliez, 1996). The incidence of adult acquired ocular disease varies considerably by geographical region. In certain populations in Brazil, seropositivity was found to be over 80 percent in adults over 25 years of age. The incidence of ocular disease within this population was as high as 14 percent, although the contribution of congenital infections to this figure is difficult to estimate (Petersen et al., 2001). Certain atypical strains of T. gondii have increased association with ocular disease, and may account for geographical differences in incidence of ocular disease (Grigg et al., 2001). Consequently, the whole human population is at risk of adult acquired ocular disease, and there is a good argument for vaccinating the entire population. This may be especially important in regions where atypical strains are more abundant.

The vast majority of congenitally infected children appear asymptomatic at birth. However, in one USA-based study around 20 percent of apparently asymptomatic children had ocular involvement at birth. Ocular disease had risen to over 80 percent in the subjects of this study by adolescence (see Roberts and McLeod, 1999). It has been suggested that therapeutic vaccination in childhood may be useful in reducing the incidence of ocular disease amongst asymptomatic congenitally infected individuals (Wilson *et al.*, 1980; Koppe *et al.*, 1986). Such a vaccine would have to overcome the mechanism that prevents this patient group from developing the solid immunity that is normally exhibited by those who have adult acquired infection. Although immunological tolerance could play a role, the precise mechanism is currently unknown. Therefore, rational design of such a vaccine may prove challenging.

Other possible groups that might significantly benefit from a therapeutic vaccine are those with active disease. This would include those with active adult acquired disease, or the immunosuppressed. There would be inherent problems in vaccinating people with ongoing disease, as the immune response to the vaccine may be influenced in a detrimental manner by the natural infection. Successful vaccination of immunosuppressed people as an alternative to antimicrobial therapy would also prove challenging, due to the very fact that they have poorly functioning immune systems.

24.3 CURRENT STATUS OF VACCINES FOR INTERMEDIATE HOSTS

24.3.1 Vaccination using extracts or killed parasites

The earliest of numerous studies to test the vaccine potential of killed or crude antigen extracts against toxoplasmosis were carried out in 1956 (Cutchins and Warren, 1956; Jacobs, 1956). A summary of more recent studies carried out since the early 1970s is listed in Table 24.1. The vaccine potential of whole fixed tachyzoites has been examined, as well as whole tachyzoite lysates, soluble fractions, particulate fractions, excretory/secretory products, detergent extracts, cysts and soluble cyst fractions, and crude whole rhoptry extracts. Numerous adjuvants have also been employed as part of the vaccine formulation, including Freund's Complete Adjuvant

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 TABLE 24.1 Killed and crude antigen vaccine studies and their outcomes in animal model

Reference	Antigen	Adjuvants or carrier	Route of vaccination	Animal model	Immunology	Challenge	Survival	Parasite burden	Other
Krahenbuhl <i>et al.</i> , 1972	Formalin fixed tachyzoites, total lysate, soluble and particulate fractions	w/wo; FIA or FCA	s.c. and i.p.	Swiss- Webster mice	Antibodies	C56 strain tachyzoites (i.p.)	++		
Araujo and Remington, 1974	Tachyzoite soluble and particulate fractions and RNA	w/wo; FIA	i.p.	Swiss- Webster mice		C56 tachyzoites (i.p.)	++		
Beverley <i>et al.</i> , 1971	Tachyzoite lysate		S.C.	Sheep	Antibodies	Cysts (s.c.)			++fetal death – maternofetal transmission
Waldeland and Frenkel, 1983	Tachyzoite lysate	FCA; liposomes, fatty acid anhydrides	S.C.	Outbred CF-1 mice	Antibodies	Tachyzoites, bradyzoites, M-7741 strain (s.c.)	+/++ FCA > liposomes		
McLeod <i>et al.</i> , 1985	Tachyzoite lysate	Liposomes	i.m., oral	Swiss- Webster mice	Antibodies	Me49 cysts (oral)		-	
Duquesne et al., 1990	Tachyzoite excretory/ secretory	FCA	s.c.	Fischer/ nude rats	Antibodies, lymphopro- liferation	RH tachyzoites (i.p.)	++		
Overnes <i>et al.</i> , 1991	Detergent extract; plasma membrane tachyzoite	ISCOM	S.C.	Outbred white mice	Antibodies, lymphopro- liferation	M-7441 tachyzoites (s.c.)	+/-		

Lunden <i>et al.</i> , 1993	Detergent extract tachyzoites	ISCOM	S.C.	Swiss- Webster mice	Antibodies, DTH	C56 tachyzoites (i.p.)	+	-	
						C56 cysts (oral)	+	-	
						Me49	++	-	
						(oral)			
Roberts et al., 1994	Soluble tachyzoites	w/wo; non-ionic surfactant vesicles (NISV)	s.c.	BALB/c	Antibodies, lymphoprol- iferation, IFN-γ	Beverley cysts (oral)			+++ Fetal death Ag w NISV Fetal death Ag wo
									NISV ++ Maternofetal transmission Ag w NISV
Garcia <i>et al.</i> , 2005	Rhoptry proteins	ISCOM	s.c.	Pigs	Antibodies	VEG oocysts (oral)		+	– Febrile response

Survival key (compared with control group): --- decreased survival; -- no difference in survival; +-- moderate (\leq 50% increased survival); +++ significant (\geq 50% increased survival); +++ highly significant (\geq 90% increased survival).

Parasite burden key (compared with control group): -- Increased parasite burden; - no difference in parasite burden; + moderate (\leq 50% decrease in parasite burden); ++ significant (\geq 50% decrease in parasite burden); ++ highly significant (\geq 90% decrease in parasite burden).

(FCA) and Freund's Incomplete Adjuvant (FIA), lipid vesicles, ISCOMs (Immuno-Stimulating Complexes), cholera toxin, PLG microspheres, and CpG for vaccination. Guinea pigs, mice, rats, sheep, and pigs have all been used as models, and challenge infections have utilized both virulent and avirulent tachyzoites, cysts, and bradyzoites.

Overall, while some vaccines increased survival following challenge infection (Krahenbuhl et al., 1972), others did little (Waldeland and Frenkel, 1983; Saavedra et al., 2004). The fact that the immune system tends to recognize life cycle stagespecific antigens (Kasper, 1989) in some respect may account for some discrepancies. In addition, although not a hard and fast rule, lipid vesicles such as liposomes and non-ionic surfactant vesicles and ISCOMs may be better adjuvants than FCA and FIA, and excretory/secretory antigens may induce stronger protection than tachyzoite lysates, while the addition of cyst antigens to tachyzoite preparations has improved efficacy (Elsaid et al., 2001). Again, while some vaccines reduced cyst burden following challenge (Alexander et al., 1996; El-Malky et al., 2005), others failed to do so (McLeod et al., 1985; Lunden et al., 1993); however, in murine and rodent models of congenital toxoplasmosis, vaccination with crude tachyzoite lysate (Roberts et al., 1994; Elsaid et al., 2001) or tachyzoite and cyst lysate (Elsaid et al., 2001) or excretory secretory factors (Zenner et al., 1999) proved extremely effective at limiting both maternofetal transmission and fetal death. Nevertheless, killed whole tachyzoites alone (Beverley et al., 1971) or in FIA (Wilkins and O'Connell, 1992) could not protect sheep from aborting.

More recently, tachyzoite sonicates were formulated in QuilA-containing vesicles 'ISCOMs' and used to vaccinate pregnant sheep. One vaccination given 4 weeks prior to mating was followed by two injections in the first 10 weeks of gestation. A challenge was given at day 91 of pregnancy, using oocysts of the M1 strain (Buxton *et al.*, 1989). Ewes were not protected against the acute phase of the infection, as was apparent from their febrile response comparable to non-vaccinated controls. However, fewer abortions were induced in the vaccinated ewes, and the gestational time was comparable to non-infected controls although, probably due to low numbers, these differences were not significant. Fetal infection could not be prevented using this vaccine. Using a similar vaccine formulation, vaccination of pigs with rhoptry proteins in ISCOMs had little effect on the febrile response following oral oocyst infection, although the cyst burden was slightly reduced (Garcia *et al.*, 2005).

A similar approach was applied more recently by Stanley et al. (2004), using tachyzoite extracts formulated in cholera-toxin containing microspheres. Vaccination of sheep was performed intranasally, and induced mucosal and serum IgA. Since the oral route is the natural route of infection in sheep, intestinal mucosal immunity may contribute to reducing the numbers of infectious parasites, as was shown previously in mice (Bourguin et al., 1993; reviewed in Kasper et al., 2004). Stanley and co-workers vaccinated non-pregnant sheep three times, but the effect on the acute infection measured as a febrile response was only marginal. Antigenic differences between sporozoites, the stages invading the mucosal lining, and the later-developing tachyzoites which cause the febrile response may account for these observations.

Thus, while vaccines based on killed or lysed tachyzoite antigens could induce protective immunity in mice and limit maternofetal transmission, results from a more practical model employing outbred sheep were less promising and could not compare with the effects induced by the incomplete S48 strain. However, relatively few studies have been documented in sheep, and further improvement could be achieved by applying different immunization schedules or better adjuvants. The success of the killed *Neospora* vaccine which protects against abortion in cattle warrants further studies using antigen preparations to enhance protective immunity against *Toxoplasma*-induced abortion in sheep.

Although no studies are documented on vaccination of humans, it is known that chronically infected women can protect their fetus from congenital infection. From the studies described above, it is clear that sterile immunity cannot be achieved using killed or subunit vaccines in any of the infection models used. Since tachyzoites are very efficient in reaching the fetus, and since prevention of congenital infection is a prime target for vaccination of humans, a killed vaccine will probably not be sufficiently efficacious.

24.3.2 Vaccination using live, attenuated parasites

The ubiquitous RH strain is a type I strain, and infection with only a few tachyzoites is sufficient to kill a mouse. This strain is, however, significantly less pathogenic in other animals, such as pigs. Moreover, inoculated tachyzoites did not cause a persistent infection in these animals, making this strain useful as a vaccine strain. Pigs, which can harbor a huge cyst burden, are considered a major source of infection for humans. Vaccination of pigs using the RH strain protected against challenge with a persistent strain, and reduced the presence of tissue cysts in the meat. When challenge occurred at 220 days post-vaccination, all vaccinated pigs were negative for tissue cysts in a mouse bio-assay, whereas all control challenged animals were positive (Dubey et al., 1994). Attenuated lines were derived from this RH strain by chemical mutation using N-methyl-N'-nitro-N-nitrosoguanidine. Temperature-sensitive mutants were isolated, of which the TS-4 strain demonstrated the most favorable phenotype of retarded growth at 37°C while maintaining immunogenicity in mammals (Pfefferkorn and Pfefferkorn, 1976). Mice vaccinated with tachyzoites of TS-4 were protected against a lethal challenge infection with RH. While vaccination could also reduce congenital transmission in a pregnancy model, tissue cyst formation was not prevented after vaccination with TS-4 and challenge with an avirulent strain (McLeod et al., 1988).

Frenkel *et al.* (1991) used similar mutation methodology and selected a strain deficient in the coccidial cycle of the parasite (the sexual replication in the intestine of the cat). In contrast to RH and TS-4, this T-263 strain did produce tissue cysts in the intermediate host. However, it lost the

capacity to form oocysts in cats that had ingested tissue cysts. The deficiency was associated with infertile microgametes. Vaccinating young cats with T-263 bradyzoites could prevent oocyst shedding in 80 percent of recipients when challenged with an oocyst-forming strain of *T. gondii*. Such effects could not be induced by vaccination with live tachyzoites only (Freyre *et al.*, 1993). These data confirm that bradyzoites and tachyzoites carry different antigens, and immunity differs between intermediate and definitive hosts, relating to the interfaces where the infection is occurring. Although commercialization of the T-263 strain as a vaccine for cats was considered, a product was never released.

Attenuation, while preserving immunogenicity, can also be achieved by gamma irradiation, as has been shown for many different organisms, including parasites (for example, in a commercial lungworm vaccine; McKeand, 2000). Dosages of less than 1000 Gy resulted in tachyzoites that could invade cells, and although they could not replicate they were still able to induce cell-mediated immune responses and some protection (Seah and Hucal, 1975). However, the effectiveness of different irradiation doses differed from study to study (summarized in Dubey, 1996). Oocysts of the VEG strain failed to induce persistent infections when irradiated with doses higher than 200 Gy (Dubey, 1996). Oocysts irradiated with 200 Gy could induce partial protection in mice against oral challenge as measured by extended survival and fewer brain cysts. This was once again confirmed in a more recent experiment (Hiramoto et al., 2002). Tachyzoites (107) irradiated with 200 Gy extended the survival time of mice by roughly 4 days when challenged with 1000 RH tachyzoites. More importantly, it reduced tissue cyst development in the brain by more than 10-fold when mice were challenged orally with 25 ME49 tissue cysts. Since irradiated tachyzoites do not persist and are able to reduce the number of tissue cysts, such an approach may have implications in the design of a therapeutic vaccine.

In addition to inducing an attenuated phenotype by chemical mutation or irradiation, serial passage of a type II strain has also been shown to evoke changes that lead to an attenuated phenotype. This methodology was used to develop the S48 strain, which is now commercially applied as an effective vaccine against abortion in sheep (Buxton, 1993). This strain was originally isolated from the cotyledon of an aborted lamb and passaged twice weekly thereafter through mice for more than 30 years. The strain consequently lost its capacity to form tissue cysts in any animal challenged, but kept its immunogenic characteristics (S48 is described in greater detail below).

In conclusion, a T. gondii infection is generally able to induce a status of lifelong protection, which would be ideal if the primary infection did not have the potential to damage neural tissues or interfere with the outcome of pregnancy. Attenuated vaccines may have the potential to induce similar protection to natural primary infection in the absence of such pathology. The preferred attenuation should be stable, and thus irradiation is not a particularly good option. Selection of sufficiently attenuated strains after mutagenic actions may have the potential to deliver stable vaccine strains. However, the use of such strains in immunocompromised animals or humans should be restricted. New reverse-genetic techniques will allow specific genes to be deleted such that the reversion to virulence can be excluded (discussed below).

24.3.2.1 A commercial vaccine against toxoplasmosis in sheep and goats

T. gondii normally causes disease when infection occurs for the first time while the animal is in gestation, allowing the parasite to invade the nonimmune fetus. However, an animal that is already immune prior to gestation is normally sufficiently protected to prevent fetal infection (McColgan *et al.*, 1988; Frenkel, 1990). Indeed, while a natural infection with *T. gondii* in sheep induced protective immunity (McColgan *et al.*, 1988), vaccination with inactivated *Toxoplasma* tachyzoites could not protect pregnant sheep from infection (Beverley, 1971; Wilkins *et al.*, 1987). This illustrated that vaccination with live *T. gondii*, but perhaps not a killed preparation, prior to gestation could prevent subsequent fetal death. Accordingly, the 'incomplete' S48 strain was developed into a safe live vaccine (Wilkins et al., 1988). S48 was isolated in 1956 from an aborted lamb in New Zealand. Since this time pre-dates the era of cryopreservation and tissue culturing, it was maintained by passaging the tachyzoites through mice, twice weekly for a period of 30 years. During this time S48 lost its pathogenicity for sheep as well as its ability to form tissue cysts (Wilkins and O'Connell, 1992). Not only has S48 lost its ability to form tissue cysts, it also does not generate oocysts in cats (Intervet, unpublished observations). The safety of the strain was proven by its absence in any animal from 4 weeks post-vaccination (Buxton, 1993). In 1988 it was introduced as Toxovax, a live vaccine, in New Zealand. Subsequently, it was registered for use in the UK and Eire in 1992, and is now sold by Intervet as OvilisTM Toxovax in many European countries.

Ovilis[™] Toxovax is a live tissue culture grown vaccine, which is supplied as a frozen product and is distributed to end users at 4°C. The vaccine is applied either intramuscularly or subcutaneously, no later than 3 weeks prior to mating. To demonstrate the efficacy of the vaccine, the Moredun Research Institute together with Intervet performed a series of vaccination-challenge experiments (Buxton et al., 1991; Buxton and Innes, 1995). Using 2000 sporulated oocysts of the M3 strain, they induced a severe infection in pregnant S48-vaccinated and control sheep. While less than 18 percent of the lambs from control sheep were born alive and viable, 80 percent of lambs survived in the vaccinated group and their weights were comparable with lambs from non-challenged ewes. Toxovax induced an IgG antibody response, although this response waned by 20 months after vaccination, in contrast to persistent infections that maintain high antibody titers in the blood throughout life. S48 vaccination also induced specific CD4+ and CD8+ T cells that produced IFN- γ as documented for normal live tachyzoite infections in sheep (Buxton and Innes, 1995). Further studies demonstrated that animals were still immune 18 months after vaccination, despite their antibody levels having waned, highlighting

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the ability of S48 to induce a potent adaptive type-1 cell mediated response (Buxton, 1993). Field trials in the UK have shown the economic profitability of vaccination against *T. gondii*-induced abortion in sheep (Bos and Smith, 1993). Vaccination with S48 has also shown moderate efficacy in goats (Chartier and Mallereau, 2001).

24.3.3 Vaccination using gene deletion attenuated parasites

Increased knowledge of T. gondii at the molecular level, greatly facilitated by the completion of the genome project, in combination with rapid progress in the development of genetic tools to manipulate the parasite, has generated opportunities to create new attenuated vaccines. Such targeted approaches have been used to create parasites with incomplete life-cycle stages, with reduced proliferative capacity, or with reduced virulence. Mutant parasites have been generated either with an irreversible gene deletion or, more recently, by conditionally inhibiting or activating expression of an essential gene. Genetically 'crippled' parasites have been analyzed in vitro and in vivo to characterize their mutant phenotypes and to determine whether a gene is possibly redundant. At present, the infectivity of all Toxoplasma mutants that have been generated by reverse genetics has only been analyzed in mice and not in larger animals. Whether vaccine potential or its absence demonstrated in mice can be translated into success and failure in larger animals is a matter of some conjecture. For example, while both the RH strain and the incomplete S48 strain are highly lethal to mice, RH is not persistent in pigs and induces protective immunity upon challenge with oocysts (Dubey et al., 1994). Similarly, the incomplete S48 strain does not persist in any other animal so far examined, and is commercially used as a live vaccine. Thus, the potential of a live mutant parasite cannot be determined in mice only, but will ultimately have to be established in a larger animal.

In the past 7 years, various *Toxoplasma* gene deletion mutants have been generated – although the objective has usually been to gain further

insight into the function of a particular gene rather than to generate a vaccine. However, it is likely that any biologically important gene will contribute to the fitness and/or virulence of the parasite, and consequently the vaccine potential of these mutants has been of significant interest (see Table 24.2):

- By far the most promising vaccine mutant that has been generated is RH strain with a disruption of carbamovl phosphate synthetase II (CPSII) (Fox and Bzik, 2002). CPSII is the first enzyme in the metabolic pathway for de novo pyrimidine synthesis (generating the building blocks for RNA and DNA), and disrupting this enzyme made Toxoplasma dependent on externally supplied uracil, which it can salvage. Disruption of CPSII thus created a uracil auxotroph, which only grew in vitro when host cells were supplemented with uracil. In the absence of uracil, CPSII knockout parasites invaded host cells normally but failed to replicate. No growth was observed (without added uracil) in vitro and in vivo. Injection of mice with CPSII knockout parasites did not kill BALB/c mice, and mice previously infected with CPSII knockout parasites 40 days previously were resistant to a lethal challenge with 200 pfu of RH strain tachyzoites.
- Surface antigens (SAGs) of Toxoplasma have ٠ also been targeted for deletion. SAGs are thought to be involved in host-cell attachment and the activation of a host immune response. The major tachyzoite surface antigen is SAG1, and two types of SAG1 mutants have been generated. One was made by chemical mutagenesis and the other was recently genetically engineered (Δ SAG1). Both attach to, enter, and proliferate at approximately normal rates within host cells (Kasper and Khan, 1993; Mineo and Kasper, 1994; Rachinel et al., 2004). ΔSAG1 tachyzoites were lethal in susceptible C57BL/6 mice (although survival was slightly prolonged compared with wild-type infected mice), but deletion of SAG1 prevented an acute ileitis when tachyzoites were directly injected into the intestine.

Reference	Targeted gene	Parental strain	Dosage	Mouse strain	Survival and/or protective effect	
Fox and Bzik, 2002	CPSII RH		Up to 10 ⁷ tachyzoites	BALB/c	No proliferation, all survive	
					Protects against lethal challenge	
Rachinel et al., 2004	SAG1	RH	10 ⁴ tachyzoites*	C57BL/6	30% reduced mortality	
Dzierszinski <i>et al.</i> , 2000	SAG3	RH	20 tachyzoites	BALB/c	85% reduced mortality	
Soldati <i>et al</i> ., 1995	ROP1	RH	50 tachyzoites	Swiss	Lethal	
Mercier <i>et al.</i> , 1998 Bohne <i>et al.</i> , 1998	GRA2 BAG1	RH PLK**	10 tachyzoites 104 tachyzoites	Swiss (CD1) C57BL/6	50% reduced mortality No reduced mortality	
Zhang <i>et al.</i> , 1999	BAG1	PLK**	10 ⁵ tachyzoites	Swiss (CD1)	Five-fold reduced cyst burden	

 TABLE 24.2
 Toxoplasma knockout strains and induced immunity in mice

*applied via surgical injection into the intestines.

** PLK is a clonal derivative of ME49.

C57BL/6 is a susceptible mouse, BALB/c is a relatively resistant mouse, and CD1 is an outbred, relatively resistant line.

- SAG3 deletion mutants showed more pronounced effects than ΔSAG1 tachyzoites; these had significantly reduced adherence to host cells *in vitro*, and mortality was reduced 80 percent upon infection in BALB/c mice compared with wild-type organisms (Dzierszinski *et al.*, 2000).
- Recently, a genomic cluster containing four bradyzoite-specific SAGs (SAG2c, SAG2d, SAG2x, and SAG2y) was deleted in one knockout. Deleting these four SAGs (Δ SAG2cdxy) yielded viable tachyzoites that could still differentiate into bradyzoites *in vitro*. In contrast, preliminary studies *in vivo* showed that 9 out of 10 Δ SAG2cdxy-infected mice were negative for brain cysts when assayed 3 months after infection (J. Saeij and J. Boothroyd, personal communication). Consequently, of the SAG knockouts Δ SAG2cdxy appears the most promising as a vaccine candidate, because although it is still infective and can transform into bradyzoites it persists poorly if at all.
- Two secretory vesicle protein (ROP1 and GRA2) gene-deletion mutants have also been generated. Disruption of either ROP1 or GRA2 resulted in no difference in growth rates or

host-cell invasiveness *in vitro*, although Δ GRA2 was less virulent in mice (Soldati *et al.*, 1995; Mercier *et al.*, 1998).

Disruption of BAG1, a bradyzoite-specific heat-shock protein, was thought to interfere with the formation or viability of tissue cysts. However, in one study (Bohne et al., 1998) disruption of BAG1 had no effect on tissue-cyst formation, while in a second study (Zhang et al., 1999) disruption of BAG1 could only reduce the number of tissue cysts in mouse brains by roughly five-fold. In the latter study, the lethal dose with Δ BAG1 did increase from 2×10^6 to 5×10^7 , compared with the parental PLK strain (a clonal line derived from ME49). Nevertheless, tissue cysts were still being formed and were completely normal, proving that BAG1 is not essential for bradyzoites. The authors suggested that BAG1 homologous genes may exist in Toxoplasma, generating some redundancy.

Attenuated parasites can also be generated by targeting expression of essential genes. Since deletion of such genes will immediately result in non-viable parasites, targeting the expression of essential genes should occur in a conditional way. For the generation of such conditional knockout parasites, an inducible expression system that provides stringent regulation of gene expression would be optimal. Ideally, switching on the expression of the targeted gene will result in a normal phenotype of the parasite, whereas reducing the expression of the targeted gene will result in an attenuated (lethal) phenotype. We and others developed a tetracycline-inducible expression system to allow conditional expression of essential genes. At Intervet Parasitology, Nicole van Poppel showed that efficient transcriptional control of T. gondii by tetracycline-inducible expression system could be obtained using YFPtetR, a repressor consisting of yellow fluorescent protein (YFP) fused to the N-terminus of wild-type tet repressor (tetR). YFPtetR was shown to be a functional repressor with the capacity of 88-fold repression of transcription when expressed in T. gondii (van Poppel et al., 2006). As shown in Figure 24.1, in the absence of tetracycline YFPtetR will block the expression of the reporter beta-galactosidase, whereas when tetracycline is present YFPtetR will be released from its operator elements, relieving the block on transcription and thus yielding normal expression of beta-galactosidase.

YFPtetR was 10 times more sensitive to tetracycline compared with wild-type tetR, and as a result YFPtetR could be regulated with low non-toxic concentrations of tetracycline. During these studies we noted that T. gondii promoters often have multiple transcriptional start sites, which will reduce the level of regulation with tet-repressors. Therefore, the promoter from TgRPS13 gene (encoding small subunit ribosomal protein 13) was selected in our laboratory as a strong promoter containing only one major transcriptional start site, which upon integration of 4 repeated tetoperator elements showed a 100-fold regulation with YFPtetR. Today, these are the highest inducible levels of expression achieved in T. gondii. Currently, we are studying to what extent this inducible system can regulate the expression of an endogenous and essential ribosomal protein, TgRPS13, by integration of multiple tetO sites in its gene promoter region. Such conditional lethal parasites require that they are propagated in the presence of tetracycline. In the absence of tetracycline, such parasites are depleted of TgRPS13 and are expected to die if the concentration of TgRPS13 is too low. Such parasites are useful as invasive but non-persistent vaccine strains in animals or humans. Using conditional mutants as live vaccines



- (3) Release of YFPTetR upon tetracycline binding followed by beta-galactosidase expression
- (4) Depletion of tetracycline will once again create the inhibitory status

FIGURE 24.1 Tetracycline-inducible expression with YFPtetR in *T. gondii*. A schematic figure demonstrates how genomically integrated YFPtetR regulates tetO-containing promoters in a tetracycline-dependent manner (see text for further details).
requires that these parasites are safe – i.e. incapable of reversion to a virulent phenotype due to mutations. At the moment it is too early to determine the safety of such parasites, but conditionallive HIV strains containing tetracycline-regulated transcription are being considered as human vaccines, illustrating that this may have clinical potential (Verhoef *et al.*, 2001).

Soldati and co-workers generated a similar regulation system, but they used a codon-adapted tetR named tetR^s to obtain expression in T. gondii, and used the anhydrotetracyline derivative to overcome the toxic effects of tetracycline (Meissner et al., 2001). In their system, TetR^s exerted a 15-fold regulation. During these studies they also showed that a fusion of tetR^s with the transactivator VP16 was inactive, probably because VP16 does not connect with the Toxoplasma transcriptional machinery. In a subsequent study (Meissner et al., 2002a) an artificial transactivator was generated, where tetR^s was fused to a 26 amino-acid long hydrophobic sequence, creating an anhydrotetracycline-dependent transactivator (named TATi-1) with a 15-20-fold level of regulation. TATi-1 was used to regulate expression of myosin A, when placed under control of a promoter containing 7 repeated tet-operator elements. Myosin A is thought to power the gliding motility of Toxoplasma, thereby being crucial for parasite dispersion and/or invasion. Thus parasites were generated with myosin A expression being dependent on TATi-1. In the presence of anhydrotetracycline, TATi-1 no longer transactivates and myosin A expression was stopped, leading to parasites that were unable to glide and were only 20 percent invasive compared to wild-type parasites, but that could grow at a normal rate once inside the host. These parasites were injected into BALB/c mice and were shown to be non-lethal when maintained in the presence of anhydrotetracycline (supplied via drinking water). Interestingly, after 11 days the anhydrotetracycline supplement was stopped, and the mice remained viable and were protected from a normally lethal challenge with 150 RH-strain tachyzoites. The same TATi-1 system was recently used to regulate TgAMA1 expression (Mital et al., 2005). AMA1 is a leading vaccine candidate in *Plasmodium* research, and is a secreted micronemal protein. A conditional expressor of TgAMA1 was generated, the expression of which was regulated with TATi-1. Studies with these mutants showed that tachyzoites not expressing TgAMA1 were no longer invasive and could not secrete rhoptries. No *in vivo* studies were reported with these mutants, but it is possible to envisage a vaccine application with this conditional TgAMA1 strain.

In summary, reverse genetic techniques have enabled the creation of mutant attenuated *Toxoplasma* strains with vaccine potential. The uracil auxotrophic mutant generated by disruption of CPSII is particularly promising. In addition, conditionally lethal mutant parasites have been generated that are infective and can induce a protective immune response without apparent detrimental effects to the host. It will be particularly important to demonstrate the safety of the mutants before such genetically modified organisms (GMOs) can be tested and used in the field.

24.3.4 Vaccination using viral vectors

Viral delivery of immunogenic antigens has been tested widely against various cancers and infectious diseases, including malaria, although only a few studies have used this technique to express Toxoplasma antigens. Multiple viral vectors are available that are considered safe and are being tested in animals and humans. Poxviruses, adenoviruses, and herpesviruses are mostly used as vectors. Some poxviruses, such as vaccinia, can be a minor human pathogen, whereas others, such as fowlpox and MVA (modified vaccinia Ankara), cannot replicate in mammalian cells and are non-pathogenic (Paoletti, 1996). Likewise, replication-defective adenoviruses, such as Ad5, are being used (Graham et al., 1977). Due to their safety, replication-defective viruses may also be considered for a human Toxoplasma vaccine.

If we consider the use of viral vectors as vaccine carriers for parasitic diseases, then most work has focused on malaria. For example, vaccination of mice with a *Plasmodium yoelii* circumsporozoite

protein, expressed by either a replication-defective Ad5 adenovirus or a combination of Ad5 with a vaccinia, induced sterile and long-lasting immunity (Rodrigues *et al.*, 1997; Bruna-Romero *et al.*, 2001). In another study, the multi-epitope vaccine fused to TRAP (ME-TRAP) induce sterile immunity in some human subjects (Webster *et al.*, 2005). In this latter study ME-TRAP was delivered with a prime and boost combination of a recombinant fowlpox vector followed by an MVA vector. These examples merely illustrate the potential of such vectors.

A few infectious viral vaccine vectors have been tested against T. gondii, including a feline herpesvirus (FHV1) tested in cats (Mishima et al., 2002). FHV1 was selected as carrier because it spreads among cats contagiously, thereby hopefully disseminating the vaccine to neighboring domestic and stray cats. An attenuated strain was generated by deleting thymidine kinase and inserting Toxoplasma ROP2. FHV-ROP2 induced specific antibodies, and upon bradyzoite challenge of cats the number of brain tissue cysts was reduced. However, it did not reduce oocyst secretion - an essential prerequisite of a cat vaccine. In another study, the vaccine potential of ROP2 was tested with MVA in mice (Roque-Resendiz et al., 2004). High dosages of MVA-ROP2 did induce specific antibody titers and delayed the time of death (by 2 days), but could not protect mice against a challenge with 300 RH tachyzoites. Vaccination with MVA-ROP2 also failed to reduce the formation of brain cysts if mice were orally challenged with 20 ME49 cysts. Gazzinelli recently reported preliminary studies with three recombinant adenoviruses, expressing SAG1, SAG2, or SAG3 (Gazzinelli et al., 2005). Immunization of BALB/c mice with these viruses induced both antibodies and IFN-y responses. However, upon challenge with a lethal dose of RH parasites these mice were not protected. Conversely, challenge with a P-BR strain did show a reduction of tissue cysts in the brain.

In conclusion, vaccinations with viral vectors carrying *Toxoplasma* antigens have met with limited success. Lack of protection is either due to an improper immune response, and/or because non-protective antigens have been expressed. In this respect it is interesting to note that in a *Neospora* study a vaccinia vector expressing NcSRS2 (but not NcSAG1) was able to prevent vertical transmission of *Neospora* in a mouse model (Nishikawa *et al.*, 2001). Thus, recombinant viruses do have potential as vaccine vectors, but undoubtedly the right mix of *Toxoplasma* antigens expressed to yield protective immunity has yet to be found.

24.3.5 Vaccination using bacterial vectors

Live bacterial vaccine vectors have been extensively used to deliver and express heterologous vaccine antigens to protect against cancer and various infectious agents, including AIDS (see Drabner and Guzman, 2001, for review). Live bacterial vaccines have the advantage that they can express multiple antigens, are easily mass produced, can be orally or intranasally applied, and induce strong immune responses. However, relatively few studies have tested whether heterologous expression of parasitic antigens with bacterial vaccine vector strains can lead to protective immunity.

Invasive bacteria such as Salmonella, Listeria, Yersinia, Shigella, and Mycobacterium bovis BCG have been used as vaccine vectors, capable of mounting potent humoral and cellular immune responses. Since these are pathogenic bacteria, they were attenuated to generate suitable non-pathogenic vaccine strains. Many attenuated strains have been reported that are nonpathogenic and have limited proliferative capacity in vivo. Attenuation can, however, lead to reduced immune stimulation. Moreover, overexpression of heterologous genes can result in a rapid selection for low or non-expressors. Experiments in our own laboratory demonstrated that overexpression of GFP in a Salmonella typhimurium vaccine strain leads to rapid selection of non-expressors or low expressors (see Figure 24.2) (Schaap and Vermeulen, unpublished results). High T7 polymerase-driven expression of GFP could not be obtained on a stable basis, with GFP being either present on a plasmid or on the genome. Fluorescence was



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FIGURE 24.2 Rapid selection of non- or low-expressors of GFP upon (over)expression in a Salmonella typhimurium vaccine strain. GFP was constitutive (over)expressed with T7 RNA polymerase, and expression in individual cells was analyzed over time by FACS. GFP-expressing clones were selected at low temperatures to reduce expression of GFP. A starter culture was produced at 28°C and subsequently followed for 2 days at 41°C. Samples were taken at regular intervals and analyzed by FACS. Time points are indicated on the top. Cultures were twice diluted, as indicated, to maintain growth.

- (A) S. typhimurium: T7pol with a GFP expression plasmid (cultured in the presence of ampicillin).
- (B) S. typhimurium: T7pol with a genomically integrated GFP expression cassette.

(C) S. typhimurium: mT7pol (with a T7 RNA polymerase being point mutated to reduce its activity

20-30-fold) with a genomically integrated GFP expression cassette.

reduced from a high fluorescent population (1000 units) to either a non-fluorescent population or a low fluorescent population (10 units) for plasmid- or genomic-expressed GFP respectively (see Figures 24.2A, 24.2B). However, genomic integration did extend the time period over which low expressors were selected. If expression of genomically integrated GFP was reduced by a point mutation in the T7 polymerase (leading to a 20–30-fold reduced activity), such a strain expressed GFP stably for a prolonged time, with reduced fluorescence (30 units; Figure 24.2C). These results demonstrate that GFP overexpression cannot be maintained if expressing bacteria are cultured for prolonged times.

Different approaches have been used to obtain stable expression with bacterial vaccine strains for in vivo use. For example, inducible promoters were used, such as the Salmonella nirB promoter, which becomes activated in vivo under anaerobic conditions (Chatfield et al., 1992). Alternatively, a mixed population approach was tested whereby expressing bacteria are constantly derived from non-expressing carrier cells (Yan and Meyer, 1996). Finally, bacterial vaccine strains have been successfully used to deliver eukaryotic expression plasmids. In one convincing example, eukaryotic expression plasmids containing Listeria antigens were successfully delivered with Salmonella typhimurium, protecting mice from a lethal challenge with Listeria monocytogenes (Darji et al., 1997).

Recently, oral immunization with a live attenuated Salmonella typhimurium vaccine strain was used to protect mice against T. gondii (Cong et al., 2005). SAG1 and SAG2 were delivered with a eukaryotic expression plasmid which also contained cholera toxin subunits A2 and B. Cholera toxin (CT) is known to have an adjuvant effect, and indeed the addition of CT subunits A2 and B induced a strong cellular immune response, as measured by induced specific IgG2a titers, splenocyte proliferation, and IFN-y production. Upon challenge with 1000 RH tachyzoites the vaccinated mice survived longer, and 40 percent of the mice survived the whole trial period. Whether this vaccine could prevent tissue cyst formation and reduce cyst burden is unknown.

In conclusion, vaccination with live bacterial vectors can induce both strong humoral and cellular immunity, and in a single study where *Toxoplasma* antigens were expressed such bacterial vectors demonstrated potential as live vaccine.

24.3.6 DNA vaccines

It is the general consensus of opinion that a type-1 response, particularly associated with CD8+T cells producing IFN- γ , is the major mediator of immunity against T. gondii infection. Nevertheless, numerous vaccine and immunological studies have also demonstrated that a broad-spectrum immune response requiring elements of type-2 immunity with antibodies provides the best overall protection against infection. Consequently, as DNA vaccines are known to induce CD8+ T-cell responses in addition to broad-spectrum immunity, there has been in recent years a substantial effort to determine their effectiveness against toxoplasmosis. While the majority of studies have concentrated on SAG1 (Table 24.3), the vaccine potentials of GRA1, GRA4, ROP1, ROP2, HSP70, HSP30, MIC1, MIC2, MIC3, MIC4, M2AP, and AMA1 have also been investigated, either alone or in combination, with varying degrees of success. Vaccination with SAG1 has been found to be particularly effective at limiting mortality against both virulent and avirulent challenge (Nielsen et al., 1999; Angus et al., 2000; Chen et al., 2002; Couper et al., 2003). In addition, the effectiveness of SAG1 DNA vaccines was generally enhanced by utilizing cocktail vaccines comprising other antigens such as ROP2 (Fachado et al., 2003b) and GRA4 (Mevelec et al., 2005). The incorporation of adjuvants into the vaccines, particularly pGM-CSF (Desolme et al., 2000; Ismael et al., 2003; Mevelec et al., 2005), also enhanced general efficacy irrespective of the antigen under investigation. Consequently, while a SAG1 DNA vaccine failed to limit maternofetal transmission (Couper et al., 2003), a SAG1/GRA4 vaccine adjuvanted with pGM-CSF did increase pup survival if dams were infected during pregnancy, but again without preventing vertical transmission (Mevelec et al., 2005). However, the former study utilized BALB/c

Reference	Antigen (route)	Adjuvant or carrier	Route of vaccination	Animal model	Immunology	Challenge (route)	Survival	Parasite burden
Nielsen <i>et al.</i> , 1999	SAG1		i.m.	BALB/c	Antibodies, CD8+ T cells	RH (i.p.)	+++	
Angus <i>et al.</i> , 2000	SAG1		i.m.	C57BL/6	Antibodies, splenocyte IFN-γ and IL-2 production	Me49 cysts (oral)	+++	++
				Rats	Antibodies	VEG oocysts (oral)		++
Vercammen et al., 2000	GRA1		i.m.	C57BL/6, BALB/c, C3H	Antibodies, T-cell proliferation, IFN-γ	IPB-G or 76K cysts (oral)	++ C3H, – BALB/c, – C57 BL/6	++ C3H, – BALB/c
Desolme et al., 2000	GRA4	w/wo pGM- CSF, w/wo pIL-12	i.m.	C57BL/6	Antibodies, splenocyte proliferation, IFN-γ, IL-2, IL-10	76K cysts (oral)	++ GRA4 and GRA4 & pGM- CSF	
Leyva <i>et al.</i> , 2001	ROP2		i.m.	BALB/c, C57BL/6, CBA/J	Antibodies	RH (s.c.)	+ BALB/c, – C57BL/6, – CBA/J	
Chen <i>et al.,</i> 2001	ROP1		i.m.	BALB/c	Antibodies			
Chen <i>et al.</i> , 2002	SAG1	pIL-2	i.m.	BALB/c	Antibodies, IFN-γ	RH	+	
Chen <i>et al.</i> , 2003	SAG1	Liposomes	i.m.	BALB/c	Antibodies, IFN-γ, IL-2			
Couper <i>et al.</i> , 2003*	SAG1		i.m.	BALB/c	Antibodies, IFN-γ	Beverley cysts (oral)	++	+++
Bivas-Benita <i>et al.</i> , 2003	GRA1	Chitosan microparticles	oral, i.m.	C3H/HeN	Antibodies			
Mohamed <i>et al.</i> , 2003	HSP70,		i.d.	C57BL/6, BALB/c	IFN-γ	Fukaya cysts (oral)		+++/+, HSP70 > HSP30 & SAG1, i.d. > i.m. & i.p.

 TABLE 24.3 DNA vaccine studies and their outcomes in animal models

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	HSP30, SAG1		i.m. i.p.					
Fachado <i>et al.,</i> 2003a	SAG1, ROP2, SAG1 & ROP2		i.m.	BALB/c	Antibodies, T-cell proliferation, IFN-γ	RH (i.p.)	++ SAG1 & ROP2	
Fachado <i>et al.,</i> 2003b	Genomic library		i.m.	BALB/c	Antibodies, T-cell proliferation, CD4+ & CD8+ activation, IFN-γ	RH (i.p.)	++	
Ismael <i>et al</i> ., 2003	MIC3	± pGM-CSF	i.m.	CBA/J	Antibodies, lymphocyte proliferation, IFN-γ, IL-2	76K cysts (oral)		++, pMIC3 & pGM- CSF > MIC3
Scorza <i>et al.,</i> 2003	GRA1		i.m.	C3H/HeN	Antibodies, CD4, cytolytic CD8, IFN-γ	IPB-G cysts (i.p.)	++	++
Martin <i>et al.,</i> 2004	GRA4		i.m.	СЗН	Antibodies	Me49 cysts (oral)		++
Mevelec et al., 2005**	SAG1, GRA4, SAG1 & GRA4	pGM-CSF	i.m.	C57BL/6, Swiss OF1**	Antibodies, splenocyte proliferation, IFN-γ	76K cysts (oral)	++++/++, SAG1 & GRA4 & pGM-SCF >SAG1 & GRA4	++, SAG1 & GRA4 & pGM-CSF
Beghetto <i>et al.</i> , 2005	MIC 1, 2, 3, 4; M2AP; AMA1		i.m.	BALB/c	Antibodies	SS1119 cysts (oral)		++

*Vaccination did not prevent congenital toxoplasmosis in BALB/c mice.

**Vaccination did not limit congenital transmission in SWISS OF1 mice but increased survival.

Survival key (compared with control groups): -- decreased survival; - no difference in survival; + moderate (< 50% increased survival); ++ significant (> 50% increased survival); +++ highly significant (\geq 90% increased survival).

Parasite burden key (compared with control group): - - Increased parasite burden; - no difference in parasite burden; + moderate (≤ 50% decrease in parasite burden); ++ significant (≥ 50% decrease in parasite burden); +++ highly significant (≥ 90% decrease in parasite burden).

mice while the latter used Swiss OF1 mice, which could also provide an explanation for relative success and failure. Indeed, it has been shown that the degree of protection afforded by a particular DNA vaccine can be dependent on the animal model used, and whereas GRA1 protected C3H mice with regard to both survival and cyst burden, it did not protect BALB/c or C57BL/6 mice (Vercammen et al., 2000). Conversely, ROP2 improved survival of BALB/c mice following infection with RH tachyzoites, but not survival of C57BL/6 or CBA/J mice (Leyva et al., 2001). In addition to adjuvanting the vaccines, efficacy may also be enhanced by changing the route of vaccination, as in one study intradermal inoculation proved more effective than the intramuscular or intraperitoneal routes (Mohamed et al., 2003).

The normal portal of entry of T. gondii is via the gut mucosa. Furthermore, intraepithelial IFN-γ producing CD8+ T cells cytolytic for parasitized enterocytes have been shown to be generated following infection (Chardes and Bout, 1993; Chardes et al., 1994), while IgA may protect mucosal surfaces from parasite invasion (Mineo et al., 1993). Unfortunately, conventional parenterally administered vaccines do not generally induce mucosal immune responses, and the most successful method to induce this type of response in addition to systemic immunity has been to administer vaccines orally (Gallichan and Rosenthal, 1996). Current evidence would suggest that entrapment of such vaccines in lipid vesicles would enhance the immune response generated by both protecting the DNA from degradation and targeting the DNA directly to APC (Gregoriadis et al., 2002). Furthermore, it has been demonstrated that DNA vaccines can influence both mucosal and systemic immunity by the oral route if suitably encapsulated (Chen and Langer, 1998). However, only one study to date has tested this route, using chitosan microparticle entrapped pGRA1 (Bivas-Benita et al., 2003). Unfortunately, mucosal immunity was not measured and no challenge infections were undertaken in this study. Nevertheless, a very recent study that employed RNA vaccinated intranasally has highlighted the potential of specifically targeting the mucosal immune system (Dimier-Poisson *et al.*, 2005). C57BL/6 mice receiving three intranasal doses of tachyzoite mRNA developed systemic and mucosal humoral immunity, as well as systemic and mucosal-cell-mediated immunity. Furthermore, survival rates were significantly improved and a partial reduction in brain-cyst burdens was noted following normally lethal or sublethal oral challenge with brain cysts of the 76K strain (Dimier-Poisson *et al.*, 2005).

While, overall, DNA vaccine formulations based on the above antigens have resulted in reduced mortality, in those studies where brain-cyst burdens have also been quantified only a reduction has been achieved with no sterile immunity observed. What may be significant is that the antigens are either tachyzoite-specific (e.g. SAG1) or shared (e.g. GRA1, -2, and -4), while none is bradyzoite-specific. Similar incomplete protection has also resulted when attenuated or 'crude' tachyzoite antigen preparations have constituted the vaccine (Buxton, 1993; Roberts et al., 1994). What could be of significance in this respect is that serological studies in T. gondii-infected humans (Kasper, 1989; Lunden et al., 1993; Zhang et al., 1995) as well as mice have indicated that recognition is generally of immunodominant stagespecific antigens, with little recognition of shared antigens. This would imply a requirement for additional antigens to those expressed in tachyzoites for complete protection following vaccination, which can be concluded from some experimental studies (Freyre et al., 1993; Alexander et al., 1996). Thus the ideal vaccine to induce complete immunity against T. gondii is one that would promote protection against more than one lifecycle stage, induce mucosal as well as systemic immunity, and in addition induce a strong CD8 response. A DNA vaccine delivered by the oral or other appropriate mucosal route would offer a rational solution.

24.3.7 Subunit vaccines

While various crude antigen preparations have been tested for their vaccine potential against toxoplasmosis for almost 50 years, studies on the efficacy of purified subunits or recombinant

products are relatively new, with the earliest investigations taking place around 20 and 10 years ago respectively (Table 24.4). Subunit vaccines have the advantage that specific immunogenic antigens are presented without adding antigens that are at best irrelevant and at worst capable of inducing febrile or disease-exacerbating immune responses. In addition, antigens that induce little immunity in the context of the parasite can be boosted to be more immunogenic if applied in a non-natural context. Thus subunit vaccines are focused in their immune objectives and safe, but tend to lack immunological potency and therefore require formulation with appropriate adjuvants to enhance their effectiveness. Typically, SAG1, the immunodominant tachyzoite life cycle specific surface antigen, has been the most extensively studied product, both as a purified subunit and as a recombinant antigen (Table 24.4). Additional vaccine candidates that have been investigated include ROP1, ROP2, GRA1, GRA2, GRA4, GRA5, HSP70, SAG2, SAG3, SRS1, P54, and P24, as well as uncharacterized antigens recognized by monoclonal antibodies such as F3G3. Collectively, these have been studied both individually and as 'cocktail' vaccines administered by a variety of routes and using a variety of adjuvants. Recombinant subunit vaccines have also utilized live infectious expression vectors such as BCG, Salmonella, feline herpes virus, and vaccinia virus (Supply et al., 1999; Mishima et al., 2002; Roque-Resendiz et al., 2004; Cong et al., 2005). In addition to utilizing tachyzoite life-cycle stage-specific antigens and antigens shared between life-cycle stages, a bradyzoite life-cycle stage-specific antigen, MAG1, has also been studied (Parmley et al., 2002).

The choice of adjuvant can be crucial and profoundly influence vaccine efficacy. Thus a purified subunit vaccine which is protective as measured by vaccinee survival (Bulow and Boothroyd, 1991; Khan *et al.*, 1991) and cyst burden following infection when entrapped in liposomes or formulated with saponin Quil A is disease exacerbatory if adjuvanted with FCA (Kasper *et al.*, 1985). Adjuvants so far utilized for subcutaneous administration include FCA, FIA, liposomes, saponin Quil A, ISCOMs, SBAS1, and IL-12; for intraperitoneal administration, FCA, FIA, liposomes, and VetL-10; for intramuscular administration, aluminum hydroxide (ALUM); for oral administration, cholera toxin, chitosan microparticles; and for intranasal administration, salbutamol, cholera toxin, and enterotoxin.

Overall, SAG1, adjuvant withstanding, has induced comparatively good protection in terms of decreasing mortality (see, for example, Bulow and Boothroyd, 1991) and cyst burden (Khan et al., 1991), and limiting maternofetal transmission (Letscher-Bru et al., 2003). Variation in outcomes following vaccination, while in part dependent on the adjuvant employed and undoubtedly route of administration, is often also dependent on the strain and life-cycle stage of the parasite used and the route of challenge infection. The animal model (species/strain) utilized is also crucial to success. Thus, subcutaneous SAG1 vaccination protected guinea pigs (Haumont et al., 2000) and BALB/c mice (Letscher-Bru et al., 2003) against maternofetal transmission, but failed to protect CBA/J mice (Letscher-Bru et al., 2003). Subcutaneous vaccination of Fischer rats with GRA2 and GRA5 adjuvanted with FIA also inhibited maternofetal transmission (Zenner et al., 1999), but whether these subunits would also be effective in other murine models or elsewhere requires further investigation. Interestingly, vaccination with the Th2-inducing adjuvant ALUM incorporating SAG1 (Petersen et al., 1998) or various combinations of ROP2 and GRA4 (Martin et al., 2004) was able to promote survival of BALB/c mice infected with virulent parasites (Petersen et al., 1998), and reduce parasite burdens of C57BL/6 and C3H mice infected with Me49 cysts (Martin et al., 2004). In these studies mixed Th1/Th2 responses were induced, although the bias was antigendependent: SAG1 and ROP2 induced primarily a Th2 response, and GRA4 primarily a Th1 response. ALUM (as one of a few licensed human adjuvants) has been used in humans for over 60 years and, although a type 2 adjuvant, if it is formulated with type 1 inducers such as CpG (Stacey and Blackwell, 1999) or IL-12 (Pollock et al., 2003) a strong type 1 response can also be induced. In this context, Toxoplasma immunogens such as cyclophilin

Reference	Antigen	Adjuvant or carrier	Route of vaccination	Animal model	Immunology	Challenge (route)	Survival	Parasite burden	Other
Kasper <i>et al.</i> , 1985	SAG1	FCA	i.p./s.c.	BALB/c CD1 mice	Antibodies	Tachyzoites C strain (i.p.)			
Khan <i>et al.</i> , 1991	SAG1	Saponin Q and A	s.c.	Outbred A/J mice, CD1 mice, C57BL/6 mice	CD8+ cytolytic T cells, IFN-γ, IL-2	P strain (Me49) cysts (i.p.)	+++	+++	
Bulow and Boothroyd, 1991	SAG1	Liposomes	i.p.	Female Swiss- Webster mice	Antibodies	Tachyzoites C strain (i.p.)	++++/++ SAG1 liposomes > SAG1		
Duquesne <i>et al.</i> , 1991	P24	Vaccinia	s.c. or i.p.	Fischer/ Nude rat	T cells	Tachyzoites (i.p.)	++		
Darcy <i>et al.,</i> 1992	SAG1 monomeric peptide	IFA	s.c.	Mice OF1, Fischer/ Nude rat	T cells, antibodies	76K cysts (oral)	– SAG1 MP, ++SAG1 MAP, +SAG1 MAP		
	SAG1 multiple antigenic peptide					RH tachyzoites (i.p.)			
Brinkmann <i>et al.</i> , 1993	F3G3 antigen 2G11-AG 1E11	IFA	s.c. and i.p.	Outbred Swiss- Webster mice	Antibodies, CD4+ T cells, IL-2	C56 tachyzoites (i.p.), Me49 cysts (i.p.)	+++F3G3 antigen		
Debard <i>et al.</i> , 1996	SAG1	Cholera toxin	i.n.	CBA/J mice	Antibodies, IgG and IgA, T cells, IL-2, IL-5	76K cysts (oral)		++	
Velge-Roussel <i>et al.</i> , 1997	SAG2	FCA/IFA	s.c.	C57BL/6 CBA/J	Antibodies, T cells	76K cysts (oral)		- +	
Lunden <i>et al.</i> , 1997	SAG2	ISCOM, GST	s.c.	Swiss- Webster	Antibodies	Oocysts Me49, Cysts C56 (oral)			

TABLE 24.4 Subunit vaccine studies and their outcomes in animal models

Mevelec <i>et al.</i> , 1998	GRA4	Cholera toxin, GST	Oral	C57BL/6	Antibodies, IgA, IgG	76K cysts (oral)	+	++	
Letscher- Bru <i>et al.,</i> 2003	SAG1	IL-12	s.c.	CBA/J	Antibodies, IFN-γ	PRU cysts (oral)		+	
Petersen <i>et al.</i> , 1998	SAG1	ALUM	i.m.	Outbred NMRI	Antibodies	RH tachyzoites (i.p.), SS1119 cysts (s.c.)	+	-	
Fermin <i>et al.</i> , 1999	SAG1	Salbutamol	i.n.	CBA	T-cell proliferation	Cysts		++	
Mun <i>et al.,</i> 1999	HSP70, HSP 70/ bag1			C57B/6, BALB/c		Fukaya strain cysts (oral), RH tachyzoites (i.p.)	+ HSP70/bag1, HSP70	+ HSP70/ bag1, HSP70	(RENT STALOS
Supply <i>et al.</i> , 1999	GRA1	BCG	i.p., s.c, i.v.	OF1 outbred mice, sheep	Antibodies, lymphocytes, IFN-γ	Virulent, oocysts (oral)	_		+ temp
Aosai <i>et al.,</i> 1999	SAG1	RNAs (lymphoma)				RH tachyzoites (i.p.)			NES FOR
Bonenfant <i>et al.</i> , 2001	SAG1	Cholera toxin, heat- labile enterotoxin	i.n.	CBA/J	IgG and IgA, lymphocytes, IFN-γ, IL-2	76K cysts (oral)		++	IN I EKMEDI
Haumont <i>et al.</i> , 2000	SAG1	SBAS1	s.c.	Dunkin Harley guinea pigs	Antibodies	C57 tachyzoites (i.d.)			++, congenital trans- mission
Mishima <i>et al.</i> , 2001	SAG1, SAG2, SAG3, SRS1, P54	FCA/FIA	i.p.	BALB/c	Antibodies	Beverley bradyzoites (i.p)	+ SAG2, + SRS1, + P54		

Continued

		Adjuvant or	Route of	Animal		Challenge		Parasite	
Reference	Antigen	carrier	vaccination	model	Immunology	(route)	Survival	burden	Other
Parmley <i>et al.</i> , 2002	MAG-1	Quil A/GST	s.c.	Swiss- Webster mice	Antibodies	Me49 cysts (oral)	+	++	+ inflamma- tion
Mishima <i>et al</i> ., 2002	ROP2	Feline herpes virus type 1		Cats	Antibodies			+	
Letscher- Bru <i>et al.</i> , 2003	SAG1		s.c.	BALB/c CBA/J	Antibodies, IFN-γ, IL-10 Antibodies, IL-10	Me49 cysts (oral)			++ BALB/c, CBA/J, congenital transmission
Bivas- Benita <i>et al.</i> , 2003	GRA1	Chitosan microparticles	Oral	C3H/HeN	Antibodies				
Martin <i>et al.,</i> 2004	GRA4, ROP2, GRA4 + , ROP2	ALUM	i.m.	C57BL/6, C3H	Antibodies, lymphocytes, IFN-γ, IL-4	Me49 cysts (oral)		+/++	
Yang <i>et al</i> ., 2004	SAG1/2	Vet L-10	i.p.	BALB/c	Antibodies, lymphocytes IFN-γ, IL-4	RH tachyzoites (s.c.)	+++		
Roque- Resendiz <i>et al.</i> , 2004	ROP2	MVA vaccine			Antibodies	RH tachyzoites	+++		
Cong <i>et al.</i> , 2005	SAG1/SAG2	Salmonella typhimurium, cholera toxin A2/B	Oral	BALB/c	Antibodies, lymphopro- liferation, IFN-γ, IL-4	RH tachyzoites (i.p.)	++++/++, SAG1-2 & CT A2/B > SAG1-2		

 TABLE 24.4
 Subunit vaccine studies and their outcomes in animal models—cont'd

Survival key (compared with control groups): -- decreased survival; - no difference in survival; + moderate (\leq 50% increased survival); ++ significant (\geq 50% increased survival); +++ highly significant (\geq 90% increased survival).

Parasite burden key (compared with control group): -- Increased parasite burden; - no difference in parasite burden; + moderate (\leq 50% decrease in parasite burden); ++ significant (\geq 50% decrease in parasite burden); +++ highly significant (\geq 90% decrease in parasite burden).

(Aliberti *et al.*, 2003) may be ideal co-stimulators in an ALUM-formulated vaccine to enhance a potent type 1 response.

It has also been suggested that multivalent vaccines may be more successful than those comprising a single antigen (Martin et al., 2004). More importantly, targeting more than one lifecycle stage immunodominant antigen may further enhance vaccine efficacy. Only one bradyzoitespecific subunit antigen has been studied to date in this respect: MAG1 (Parmley et al., 2002). Significantly, vaccination with MAG1 adjuvanted with QuilA not only reduced the cyst burden in the brain but also reduced inflammation. As SAG1 is also effective by this route using QuilA, the outcome of vaccination with a combined SAG1/MAG1 vaccine would be extremely interesting. Indeed, as bradyzoites initiate infection in the intestine, the outcome of mucosal immunization with this combination linked to, say, cholera toxin would be intriguing.

24.4 THE RODENT AS A MODEL TO STUDY CONGENITAL DISEASE AND VACCINATION

The success of an attenuated vaccine for preventing Toxoplasma-induced abortion in sheep is reported in detail above. However, experimental studies are extremely expensive and comparatively difficult to perform and coordinate in domestic animals. Consequently, much effort has been expended in elucidating the immunology of congenital toxoplasmosis in the rodent model and exploring the efficacy of prophylactic therapies. Very early studies on the mouse model indicated that vertical transmission through successive generations was the normal situation in mice (Beverley, 1959), unlike either humans (Cook, 1990) or ovids (Beverley and Watson, 1971), where only a primary infection during pregnancy resulted in congenital infection. These differences would suggest that mice would make poor substitutes for studying immune prophylaxis of human or ovine congenital disease. More recently, vertical disease transmission has also been observed to

occur occasionally in humans in the apparent absence of re-infection (Vogel et al., 1996; Kodjikian et al., 2004). Conversely, we and others have now demonstrated that vertical disease transmission in mice in the absence of primary infection during pregnancy is in fact mouse-strain dependent, and that the BALB/c mouse is an excellent model of the human disease (Roberts and Alexander, 1992; Roberts et al., 1994; Thouvenin et al., 1997; Alexander et al., 1998; Elsaid et al., 2001; Couper et al., 2003; Letscher-Bru et al., 2003; Abou-Bacar et al., 2004a, 2004b). BALB/c dams previously infected with and recovered from cyst-forming strains of T. gondii, unlike mice infected for the first time during pregnancy, produced healthy litters even if infected for a second time by the oral route during pregnancy. Thus the mouse, and the BALB/c mouse in particular, provides an appropriate model to study the immunology of vertical disease transmission and design appropriate vaccine strategies.

The physiological/immunological environment associated with pregnancy, which favors a type 2 immune response (for review, see Roberts et al., 1996, 2001), probably promotes a permissive environment for vertical transmission to occur. Indeed a type 2 response bias in the murine placenta has been demonstrated to facilitate successful implantation, maintenance of early pregnancy, and suppression of inflammation: a switch towards a type 1 response can result in fetal death (Krishnan et al., 1996a, 1996b). It is well recorded that humans, as well as mice, develop more severe primary infections during pregnancy (Luft and Remington, 1982; Shirahata et al., 1992, 1993). This is associated with reduced IFN-y levels (Shirahata et al., 1992), and administration of recombinant IFN- γ promotes the resistance of pregnant mice against toxoplasmosis. Similarly, the Th1 cytokine IL-2 also promotes resistance against lethal challenge in pregnant mice (Shirahata et al., 1993). Furthermore, IL-4 deficient-mice that should have a type 1 bias are more resistant to T. gondii infection during gestation than their wild-type counterparts (Thouvenin et al., 1997; Alexander et al., 1998). In addition, although vertical disease transmission from infected dams to pups was not

impaired in IL-4-deficient B6/129 (Alexander *et al.*, 1998), there was a 50 percent decrease in congenital infections in BALB/c IL-4-deficient mice compared with wild-type controls (Thouvenin *et al.*, 1997). Neutralization of IFN- γ and depletion of CD8+ T cells increased the incidence of maternofetal transmission in BALB/c mice (Abou-Bacar *et al.*, 2004a), although studies using RAG2^{-/-} mice which lack B and T cells also indicated a protective role for NK cells. Paradoxically, in this study neutralizing IFN- γ inhibited transmission to the fetus in this model (Abou-Bacar *et al.*, 2004b).

As previously infected BALB/c dams are able to totally prevent vertical disease transmission to their pups even when re-infected during pregnancy, this model offers a gold standard for testing putative vaccines. Consequently, vaccination of dams with crude soluble tachyzoite antigens, with or without cyst antigens, entrapped in lipid vesicles prior to pregnancy has been demonstrated to limit vertical disease transmission and pup mortality following infection with cysts orally on day 11 of pregnancy, associated with enhanced IFN-y production (Roberts et al., 1994; Elsaid et al., 2001). Indeed, a recombinant SAG1 vaccine, although not a SAG1 DNA vaccine (Couper et al., 2003), has been demonstrated to be sufficient to inhibit vertical transmission in BALB/c mice associated with CD8+ T cells and IFN-y production (Letscher-Bru et al., 2003). While rSAG1, though adjuvanted with a type 1 promoter, was also protective in a congenital guinea pig infection model (Haumont et al., 2000), rSAG1 actually promoted vertical disease transmission in CBA/J mice (Letscher-Bru et al., 2003). Thus the success of a vaccine may be mouse-strain or species dependent, but how the vaccine is adjuvanted is also crucial to success. For example, while lipid vesicle-entrapped STAg (Roberts et al., 1994) successfully vaccinated against maternofetal transmission, unadjuvanted STAg and FCA adjuvanted STAg increased rates of fetal death, abortion, and vertical transmission. FCA, reputably the gold standard type 1 adjuvant, had previously been shown to be counter-protective in a SAG1 vaccine (Kasper et al., 1985), promoting increased death and parasite burdens following challenge infection. However, while vesicular adjuvants such as lipid vesicles and ISCOMs have been demonstrated to induce CD8+ T cell responses (Debrick et al., 1991; Zhou et al., 1992), there is little evidence that emulsion systems such as FCA are capable of inducing a similar response (Roberts et al., 1994). These observations, however, do not explain why rSAG1, but not a SAG1 DNA vaccine, induces sufficient protective immunity to prevent maternofetal transmission in BALB/c mice, as both vaccines induce or should induce CD8+ T cell responses and IFN-y production (Couper et al., 2003; Letscher-Bru et al., 2003). This could perhaps be a result of the utilization of the different parasite strains, Beverley (Couper et al., 2003) and Me49 (Letscher-Bru et al., 2003), used for challenge, although both these are type II strains. The protection afforded by SAG1 in the guinea pig study required inclusion of a type 1inducing immune response adjuvant (Haumont et al., 2000), SABS1, and the challenge involved a type III strain, which is less virulent than type II strains and not often associated with the human disease. However, using the Beverley strain, Roberts et al. (1994) did demonstrate significant protection against maternofetal transmission with a vaccine comprising a cocktail of soluble tachyzoite antigens, suggesting that an approach utilizing more than one vaccine candidate may be a more effective one. This has been confirmed by Mevelec et al. (2005), who found that a combined SAG1, GRA4 DNA vaccine adjuvanted with plasmid GM-CSF was more effective than vaccines expressing single antigens. Consequently, vaccination of outbred Swiss OF1 dams with this 'cocktail' reduced parasite-induced fetal death during pregnancy, although it did not limit maternofetal transfer. Overall, these reports demonstrate that a type 1 response induced by an appropriately adjuvanted multivalent vaccine would provide the most effective protective immunity against murine congenital toxoplasmosis. The protective activity of vaccines may also be enhanced, first by introducing bradyzoite antigens to the cocktail (Elsaid et al., 2001) and secondly by inducing mucosal as well as systemic responses. Thus McLeod et al. (1988) found that intraintestinal immunization of mice with the temperature-sensitive mutant

TS-4 but not subcutaneous immunization was effective in reducing the incidence of congenital disease.

The need to immunize using different life-cycle stage-specific antigens of Toxoplasma gondii has been recently highlighted in the rat model of congenital transmission (Freyre et al., 2006). Previous studies using Fischer (Zenner et al., 1993), Wistar, and Holtzman (Paulino and Vitor, 1999) rats had indicated that rats recovered from a primary infection with T. gondii and produced healthy non-infected pups even if dams were re-infected during the gestation period. However, Freyre and colleagues (2006), using Sprague-Dawley rats, have demonstrated that immunization with RH tachyzoites induces only low rates of protection against cyst or oocyst challenge. Furthermore, immunization with cysts provided incomplete protection against oocyst challenge even if the same parasite strain was used. Indeed, complete protection was only demonstrated in cyst-immunized rats challenged with cysts of the same strain, and complete protection was rarely achieved following challenge with different Toxoplasma strains. Conversely, Zenner et al. (1999) found that full protection against vertical transmission was irrespective of which Toxoplasma strain was used for immunization and which for challenge. Differences between these two studies perhaps reflect the different rat strains used in each. However, in the latter study vaccinating dams with a vaccine comprising excretory/ secretory tachyzoite antigens did significantly protect against congenital infection, highlighting the potential importance of the rat model.

24.5 REVIEW OF VACCINES FOR THE DEFINITIVE HOST – CATS

Whether or not a cat vaccine is required is a matter of debate. Although cats are the definitive host for *Toxoplasma*, they seldom develop disease. A future cat vaccine should therefore not be aimed at protecting the cat from illness, but rather at preventing oocyst shedding, thereby reducing oocyst contamination of the environment and risk to livestock and/or humans. Cats shed high numbers of oocysts after a first infection and most (but not all) cats remain immune afterwards (Dubey, 1995). Kittens frequently become infected soon after weaning, when they start eating prey. Vaccinations should thus be applied as early as possible in kittens of outdoor-roaming cats. Ideally, domestic and stray cats should be vaccinated in order to prevent oocyst contamination of food, water, and soil. It is clear that this can only be accomplished if nationwide vaccination campaigns are organized, including the vaccination of stray cats. Vaccination of stray cats could be accomplished by a similar approach as with rabies. Foxes were orally immunized by distributing vaccine baits using a vaccinia-rabies glycoprotein recombinant virus, which successfully controlled the disease in Europe and the USA (Pastoret, 2002). Clearly, such a radical vaccination approach for Toxoplasma would be most beneficial to areas with high incidences of toxoplasmosis, being mainly countries with poor hygiene conditions - as exemplified in Brazil, where it was shown that contaminated drinking water caused a high incidence of human toxoplasmosis (Bahia-Oliveira et al., 2003). Although postnatal acquired infection is mostly asymptomatic, it has been demonstrated to cause ocular disease (Burnett et al., 1998; Vallochi et al., 2002), making a strong case to control Toxoplasma. Apart from extensive nationwide vaccination campaigns, a cat vaccine can be envisaged that will allow domestic cat owners to prevent or reduce the incidence of Toxoplasma infections during pregnancy consequently preventing congenital infections.

A cat vaccine should thus prevent oocyst shedding, which can be induced in cats upon infection with each of the three infectious forms of *Toxoplasma* – tachyzoites, bradyzoites, and sporozoites (Dubey, 1998). The natural and most efficient route of infection of cats is via tissue cysts that are present in prey. Released bradyzoites can generate tachyzoites, but also directly initiate the enteroepithelial life cycle (Dubey and Frenkel, 1972). A vaccine should be focused on inhibiting this enteroepithelial cycle to prevent the formation of oocysts, and should not be limited to tachyzoites. Currently no enteroepithelial antigens have been defined that are specific to schizonts, gametocytes or zygotes. A few Toxoplasma antigens (including HXGPRT, HSP70, and a 14-3-3 homolog) have been cloned from enteroepithelial stages, but these are not specific to these stages (Koyama et al., 2000). No antigens from enteroepithelial stages have currently been tested as vaccine candidates. However, it may be questioned whether a subunit vaccine will induce strong enough immunity to prevent or dramatically reduce oocyst shedding. Eimeria can serve as an example, where various subunit vaccines against coccidiosis in chickens were tested and were shown to reduce oocyst shedding by only 50 percent (Vermeulen, 1998). This demonstrates the difficulty of making an effective subunit vaccine to significantly reduce oocyst shedding. Eimeria is a related coccidial parasite with only enteroepithelial stages, and currently all commercial Eimeria vaccines contain live sporulated oocysts. These vaccines can reduce oocyst shedding by more than 90 percent, and frequently even 100 percent (Vermeulen et al., 2001). It is therefore most likely that a live attenuated Toxoplasma vaccine will be required to prevent oocvst shedding.

The T-263 mutant Toxoplasma strain has been extensively tested as a live vaccine for cats (Frenkel et al., 1991). The T-263 strain is unable to produce oocysts in cats (due to a defect in the sexual stages), but can be propagated as tachyzoites and bradyzoites. Oral vaccination with T-263 bradyzoites prevented oocyst shedding in 84 percent of kittens following a single dose, and was 100 percent effective following a secondary vaccination (Freyre et al., 1993). Importantly, live tachyzoites from the T-263 strain could not protect cats from oocyst shedding upon challenge (Freyre et al., 1993), indicating that live tachyzoite vaccines are not an option for cats. The effectiveness of the T-263 strain as a cat vaccine was subsequently tested in a field trial on eight commercial swine farms in the USA (Mateus-Pinilla et al., 1999). Over a 3-year period during which time cats around the farms were trapped and vaccinated, the number of oocyst-shedding cats was reduced and the number of seropositive pigs was reduced. Trapped mice were also found to be seronegative for Toxoplasma. Despite this success, T-263 has never been commercialized. A serious drawback for such a vaccine is the production and shelf-life. Bradyzoites or tissue cysts are most efficiently produced in vivo, and cannot be frozen without considerable loss of viability. On the other hand, it is not known how long tissue cysts remain viable at room temperature and/or at 4°C. Currently, no alternatives to T-263 are available, since attenuated strains such as S48 do not develop into bradyzoites, and the TS-4 mutant may be too limited to develop in vivo (apart from the safety issue for TS-4). Interestingly, a T-263 vaccine may be easily integrated into bait for wild animals by simply infecting bait animals prior to their deployment. As a precaution, it should be realized that the use of bait with live Toxoplasma parasites can be lethal to some highly sensitive animal species, such as New World monkeys and Australian marsupials (Hill et al., 2005).

In conclusion, the impact of reduced oocyst burden on reducing the risk of transmission to animals and humans justifies the vaccination of the feline definitive host. Although such a target is technically achievable, this will require large investments from authorities and health organizations in making vaccination of cats compulsory. It is, as yet, not likely that this will be given such priority. Until such time as it is, hygiene measures remain the only tools to reduce the risk of *Toxoplasma* infection.

24.6 INSIGHTS FROM OTHER COCCIDIAL PARASITES

Among the coccidian parasites, effective commercial vaccines have already been developed against members of the genera *Eimeria*, *Toxoplasma*, *Neospora*, and *Sarcocystis*. The latter three genera are heteroxenous, meaning that they use an intermediate host and a definitive host (the last to complete the coccidial part of the life cycle). This sexual reproduction occurs at the intestinal lining and results in the shedding of oocysts with the feces.

Commercial vaccines against each of these parasites target their intermediate hosts, since the economical problems associated with that part of the life cycle are far greater than those associated with the definitive host. Apart from this important issue, the difficulty of achieving mucosal immunity can hamper the development of protective vaccines for the definitive host.

24.6.1 Neospora caninum vaccines

Neospora caninum was discovered in 1984 and has been recognized as the most commonly diagnosed cause of abortion in cattle (Dubey and Lindsay, 1996). The life cycle is very similar to that of *T. gondii* having asexual multiplication through tachyzoites and bradyzoites in different mammals, though mainly ruminants, and sexual multiplication in canids such as the dog, but also coyotes, as part of a sylvatic cycle (Gondim *et al.*, 2004). Although extensively studied, there is no indication that human infections occur on any significant scale. Very low seroprevalence is detected in man, and no relation to any pregnancy problems has been found (Dubey and Lindsay, 1996).

The negative effects of *N. caninum* infection include not only embryo mortality and abortion, but also reduction in milk production, a higher culling rate, and calves with congenital abnormalities and decreased growth rate (Trees *et al.*, 1999). The consequences of *N. caninum* infection depend on when during gestation parasitemia occurs (Innes *et al.*, 2002). Infection in late gestation seldom results in abortion, but in the birth of calves congenitally infected or calves with congenital abnormalities.

Cattle do mount an effective immune response protecting the fetus from aborting, although repeated abortions may occur in 5 percent of animals. Previously infected animals have a greater chance of abortion than seronegative animals due to recrudescence of existing infection during pregnancy, whereas exogenous infections from dog-spread oocysts are relatively rare (Davison *et al.*, 1999). The efficiency of transplacental transmission is over 90 percent, which makes this disease hard to control, and although drugs are available against the acute phase they are not effective against the tissue-cyst stage.

In line with the use of live vaccines against Toxoplasma-induced abortions, it was demonstrated that experimental infection of naïve animals prior to pregnancy could reduce or even prevent abortion when they were challenged at 10 weeks' gestation (Williams et al., 2003), and could even reduce vertical transmission when animals were challenged at around 130 days' gestation (Innes et al., 2001). However, no effect was seen on the recrudescing infection during mid-gestation in previously infected animals (Williams et al., 2003). This indicates that vaccination with live tachyzoites is feasible in seronegative animals; however, since no attenuated strain of N. caninum is available that would not induce a chronic infection, no such vaccine has been developed. Some naturally low-pathogenic strains may be used for this purpose, as reported for the Nowra strain in Australia (Miller et al., 2005). The main target of vaccination would be to develop a vaccine that prevents vertical transmission of the parasite in previously infected animals, and not only in naïve animals.

Vaccination of cattle has been performed using killed tachyzoites and found to be effective in reducing the incidence of abortion. The commercial vaccine resulting from these studies (Bovilis® Neoguard or, in the USA, NeoGuard, Intervet) consists of killed tachyzoites with Havlogen adjuvant in an oil-in-water emulsion given subcutaneously to cattle that are 1-3 months pregnant (Schetters, 2004). Romero et al. (2004) applied this vaccine on farms in Costa Rica, and found a reduction of 50 percent in the risk of abortion in vaccinated animals (total n = 876). Most abortions occurred at 5-6 months' gestation, which is in line with earlier observations. The same vaccine has been used in New Zealand with similar efficacy (Schetters, 2004). Killed tachyzoite preparations were also efficacious in reducing transplacental transmission in pregnant mouse studies (Jenkins, 2001), although there are no data as yet to suggest that this will also happen in cattle.

Rodent studies showed that recombinant antigens could be effective either as proteinaceous

vaccine for SRS2 (Pinitkiatisakul *et al.*, 2005), for combinations of SAG1, SRS2, and DG1 and DG2 (Pinitkiatisakul *et al.*, 2005), or as plasmid DNA for SAG1 and SRS2 (Cannas *et al.*, 2003), or presented by vaccinia virus for SRS2 (Nishikawa *et al.*, 2001). Extrapolation of murine models to the problems associated with the disease in cattle needs further study.

In conclusion, *N. caninum* is a major cause of abortion and congenital infection of cattle. Although a killed vaccine is available that reduces the chances of abortion, prevention of transplacental infection is the ultimate goal. Finally, a vaccine for dogs has limitations, since other canids or stray dogs are potential sources of infection that are hard to control.

24.6.2 *Sarcocystis neurona* vaccine for horses

Sarcocystis neurona is the causative agent of a neurological disease, known as EPG (equine protozoal myeloencephalitis), in horses, especially in the Americas. The definitive host for this coccidian parasite is the opossum. Horses contract the disease from sporocysts spread by roaming opossums. Although until recently parasites were detected only in horses that were severely immunocompromised (Long et al., 2002), Rossano et al. recently described the culture of viable merozoites from blood of an immunocompetent horse infected artificially with sporocysts obtained from an opossum (Rossano et al., 2005). The incidence of clinical EPM in the USA is low (< 0.15 percent), but over 40 percent of horses are seropositive in areas where the opossum is prevalent (Dubey et al., 2001). Although it has been suggested that stress factors induced by transportation or heat could predispose animals to manifest clinical disease, this has not been proven to date. Thus, although Cutler and colleagues have shown that dexamethasone-treated horses, when infected, could develop neurological symptoms, the parasite could not be detected as the unambiguous cause of the diseased state (Cutler et al., 2001). Notwithstanding the low chance of clinical disease, a vaccine has been conditionally launched in the USA consisting of killed merogonic stages. The efficacy, however, is not documented, since no challenge model is established (Duarte *et al.*, 2004).

24.6.3 Final comments about other coccidial vaccines

In conclusion, coccidian parasites are highly immunogenic, and have been the target for development of vaccines based on either live or killed parasites due to their role in causing disease in animals and man. However, their great diversity in species and wide spectrum of hosts means that vaccination is not always a feasible choice, and new drugs are also needed. Much effort is currently being expended to form the current solutions into more sustainable products for the future. These efforts will be enhanced by the unraveling of the genomic organization of these parasites, which will undoubtedly pave the path to development of more defined therapeutics and vaccines.

24.7 FUTURE STRATEGIES TO DESIGN NEW VACCINES FOR COCCIDIAL PARASITES IN GENERAL AND *T. GONDII* IN PARTICULAR

It is generally believed that adult acquired infection of humans results in lifelong immunity to *T. gondii*. The evidence would suggest that immunity prevents reactivation of disease from the bradyzoite stage and re-infection by other strains of *T. gondii*. Furthermore, immunity normally prevents congenital transmission from chronically infected individuals even if those individuals are re-exposed to infection. This would suggest that a vaccine is feasible, and should be achieved by mimicking the immune response that occurs during a natural infection.

Immunity to *T. gondii* is complex, and involves many facets of the immune system. The innate immune system is important in containing parasite proliferation during the early stages of infection, and drives the adaptive immune response (Denkers and Gazzinelli, 1998). CD4+ T lymphocytes play an important role in shaping the immune responses and provide IL-2 for the development of CD8+ T lymphocytes, which produce IFN-y and would appear to be the effectors of longterm immunity. Although CD8+ T lymphocytes from experimentally vaccinated mice are capable of killing T. gondii-infected cells in an MHCrestricted, perforin-dependent manner, IFN-y would appear to be the major effector mechanism of long-term immunity in vivo (Denkers et al., 1997; Wang et al., 2004). Thus, if a vaccine is to mimic natural immunity it should comprise the proteins or peptides thereof that are capable of being presented on MHC class I. These should be administered in such a manner that facilitates MHC class I processing and development of CD8+ T lymphocytes. In addition to the immunogen, an appropriate adjuvant is likely to be required. Live attenuated parasites would appear to fulfill many of these requirements. Assuming they maintain their fitness to invade cells, they effectively deliver the complete set of proteins to the class II and class I processing pathways, as occurs during the course of natural infection. In addition, T. gondii has a number of endogenous adjuvants, including cyclophilin 18, which has the ability to bind CCR5 receptor and elicit IL-12 production (Aliberti et al., 2003); profilin, which is a ligand for murine TLR-11 (Yarovinsky et al., 2005); and HSP70, which induces dendritic cell maturation (Kang et al., 2004). Subunit vaccines are likely to be critically dependent on adjuvant for effective delivery to the endogenous class I processing pathway. DNA vaccination or the use of viral vectors may represent alternative means of achieving this end.

Not surprisingly, live attenuated vaccines that mimic a natural infection have been extremely successful in inducing protection in murine models of infection. The use of live attenuated parasites for human and coccidial veterinary vaccines is still the best option if solid immunity is required. However, a number of concerns will need to be overcome regarding a live attenuated *Toxoplasma* vaccine. These include the potential of the parasite to revert to a virulent phenotype, the ability of these parasites to infect cells of the central nervous system, the possibility of persistence, and the potential to cause congenital infection or abortion. However, a live attenuated vaccine (Toxovax) has been used in livestock for some time and has been successful in limiting abortion in sheep. The nature of the defect in this parasite that prevents it from completing a full life cycle is unknown. Consequently, the appeal of a rationally engineered, highly attenuated vaccine with multiple deleted genes conferring multiple auxotrophy or multiple developmental disabilities is obvious.

The imminent completion of the *T. gondii* genome will, for the first time, provide the amino-acid sequence for all potentially immunogenic components of this pathogen. As algorithms are refined it will be possible to make predictions regarding which peptides are likely to have MHC class I and class II epitopes. These peptide predictions will also need to take into account the polymorphisms in MHC molecules, and may be different for humans and the various animal species that it may be desirable to vaccinate. For this information to be fully exploited for a subunit vaccine, adjuvants will be of key importance.

Alternatively, genome information can be used in combination with mass spectrometry to identify relevant sets of proteins (Mann *et al.*, 2001). For example, surface antigens from different *Toxoplasma* life-cycle stages could be isolated, analyzed with MALDI-TOF, and compared with peptide masses from a database for identification. This may yield interesting new vaccine candidates, in particular from bradyzoite stages and gastrointestinal stages.

Another approach to identify novel vaccine antigens would be to use the full theoretical set of genes from *Toxoplasma* to select a subgroup of promising candidates, and have all of them expressed and analyzed for their protective capacity. Such a major task has been undertaken with the genome data from *Meningococcus* (serogroup B), from which 570 surface antigens were identified and tested, resulting in 7 new vaccine candidates (Pizza *et al.*, 2000). Since *T. gondii* has a larger genome, with 6927 genes currently annotated (http://www.toxodb.org [02/06]), this approach cannot be performed by a single group but would require a joint effort by multiple teams.

An ideal vaccine would be able to protect against all strains of T. gondii. This in theory should not be challenging, as T. gondii is remarkably clonal, with most strains examined falling into one of five types (types I-V). However, a number of naturally occurring recombinant strains have been identified in the USA, and a number of exotic strains isolated in South America. Natural infection has generally been thought to protect from a secondary infection. However, there is now some evidence that in at least some circumstances secondary infections can occur with separate strains in mice (Araujo et al., 1997; Dao et al., 2001). In addition, it has been demonstrated in a murine system that cytotoxic CD8+ lymphocytes can be parasite strain-specific (Johnson et al., 2002). This raises the possibility that a vaccine might not be protective against all strains of T. gondii.

Most infections in humans are initiated by bradyzoites or sporozoites, but these stages only persist for a short length of time before giving rise to the tachyzoite form. Tachyzoites multiply extensively for around 14 days before transforming into bradyzoites that reside inside cyst structures. Each of these stages has stage-specifically expressed surface proteins (see, for example, Lyons et al., 2002; Kim and Boothroyd, 2005) and secreted proteins (see, for example, Meissner et al., 2002b; Reichmann et al., 2002). There is a clear advantage in targeting a vaccine against sporozoite or bradyzoite antigens, as this might prevent infection entirely. However, such a strategy may necessitate two components, one targeting sporozoites and one targeting bradyzoites. Most vaccine studies have to date used tachyzoite-derived fractions (and in some cases specific components), which in theory could be protective in infections initiated by either of the infective stages. However, the ability of the tachyzoite to differentiate into the bradyzoite form with a different antigenic profile provides a means of escaping an immune response as it develops against the tachyzoite stage. Targeting the bradyzoite stage in a vaccine may prevent this means of escape and result in sterile immunity. Such a strategy might not be necessary for a vaccine aimed at preventing congenital transmission, as the tachyzoite stage would appear to be responsible for transplacental transmission. Notably, the live attenuated vaccine (S48) used in sheep to prevent congenital transmission consists of tachyzoites. Thus the choice of antigens may be affected by the intended use of the vaccine, but in reality a vaccine may require multiple components from multiple life-cycle stages.

To summarize, there is currently only a commercially available *Toxoplasma* vaccine for sheep and goats, while a vaccine for pigs, cats, and humans is still lacking. In recent years tremendous progress has been made, not only in the cell and molecular biology of *T. gondii* but also in characterizing the immunobiology of the parasite in host species. This information, allied to current biochemical, molecular, and immunological progress, must make us optimistic about the likelihood of developing new, safe, and successful vaccines for both clinical and veterinary medicine.

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Epilogue

As the range of the chapters in this book makes clear, the study of Toxoplasma gondii has evolved rapidly in the past decades. As this book is going to press, initial comprehensive annotation of the T. gondii genome is being completed (www.Apidb.org, www.Toxodb.org). The T. gondii genome project was preceded by sequencing and annotation of the human genome as well as the genomes of other important Apicomplexa responsible for human and veterinary disease, such as Plasmodium species (malaria) and Cryptosporidium. The wealth of these genome studies has led to innovations in technology that are now being applied to T. gondii. Global analyses of gene expression are underway, as are many extensive proteomic studies and subproteome studies. Initial studies have generated new hypotheses and given surprising new insights into the biology of T. gondii and its relationship with its mammalian hosts.

Genome-wide gene-expression studies, including genome-wide chromatin immunoprecipitation (ChIP on chip) studies, will lead to unprecedented understanding of how genes are regulated and how the Apicomplexa manage the many critical developmental metamorphoses they must undertake to complete their life cycles. The Apicomplexa have few of the classic transcription factors described in yeast and the metazoa, yet undergo carefully coordinated developmental programs. Epigenetic phenomena are likely to be critical for modulating these developmental changes. Initial studies reveal that chromatin modifications play essential roles in gene expression in the Apicomplexa. The many advantages of the *T. gondii* model system may facilitate our general understanding of epigenomics of eukaryotes, and how changes in the environment trigger epigenetically regulated gene expression.

A combination of genome-sequencing studies and classical genetics is facilitating the mapping of genetic traits. Comparative genomic studies have been facilitated by the genome data. These studies have revealed surprising results about the evolution of T. gondii strains and their co-evolution with their human hosts. Parasite-specific traits are necessary for virulence, as well as modulation of the host-cell immune response. It is also probable that the host gene repertoire has evolved in the years that humans and T. gondii have coexisted, much in the way that many human traits reflect exposure to diarrheal pathogens or malaria. The genes and metabolic repertoire of T. gondii reflect the intimate interaction of the parasite with its host. Comparative genomic efforts have revealed surprising differences in the Apicomplexa that likely reflect different adaptations and host milieus.

Comparative genomics is also being applied to understanding complex cellular processes and biogenesis of organelles. These studies, combined with early proteomic projects, have greatly enhanced our understanding of the molecular actors involved in host-cell invasion. Proteomics and genomics studies are being combined with chemical biology approaches to identify critical molecules involved in important biological processes such as invasion.

These global projects have also led to identification of novel metabolic pathways and unique adaptations of metabolic pathways that were previously unknown. Toxoplasma gondii and many of the Apicomplexa have acquired many genes from their neighbors and hosts. We anticipate the trend toward larger-scale experiments will continue as efforts to understand complex pathways persist. As an overwhelming amount of data from genome projects, gene-expression experiments (SAGE and EST), and proteomics projects amasses, the challenge will be to integrate the data. As in other fields, in the Toxoplasma field mathematical modeling, bioinformatics, and data management are becoming essential tools in the now emerging era of systems biology.

Studies on innate immunity, the influence of colonization with normal flora on the immune

response to pathogens, and progress in vaccinology are improving our understanding of the immune response to *T. gondii* and other obligate intracellular pathogens. Comparative genomics is now being applied to the discovery of new potential antigens and common epitopes, which should prove useful in furthering our understanding of the immune response that has developed as a consequence of the co-evolution of this parasite and its host. Such studies also are providing strategies for improved vaccine design.

These tools will enable us more thoroughly to understand the parasite and its relationship with its animal hosts. We envisage that the result will be further insights into how *T. gondii* has evolved into such a successful intracellular parasite.

> Louis M. Weiss and Kami Kim Bronx, NY

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