Toxoplasma gondii

Histopathological, Biochemical and Pharmacological Aspects

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Summary

Toxoplasma gondii, a protozoan parasite, can cause severe, life-threatening disease, especially in newborns and immunosuppressed patients. It is an important cause of ocular disease and is considered to be the most common recognisable cause of posterior uveitis in immunocompetent individuals. The clinical and pathological manifestations of congenital toxoplasmosis in infants, children and adults are well characterised. However, until now, this disease has not been extensively studied in fetuses and there remain certain gaps in our understanding of the earliest stages of congenital disease. The first section of this thesis describes the early pathological changes in the eyes of fetuses and infants. The second section provides evidence for two novel biochemical pathways in *T.gondii*. These pathways are more commonly associated with plants and algae and may provide new targets for antimicrobial agents.

This study of fetal and infant eyes represents the biggest most comprehensive study of its kind to date. Eyes from 10 fetuses and 2 infants with congenital toxoplasmosis were studied by light microscopy. In addition, immunohistochemistry was performed for infiltrating inflammatory cells and *T.gondii* antigens. The characteristic findings were of retinitis, retinal necrosis, disruption of the retinal pigment epithelium and choroidal inflammation and congestion. Optic neuritis was present in 5 fetal eyes. The eye obtained from a 32 week old fetus showed a well established retinochoroidal scar. At the edge of the scar the retinal architecture was disrupted with formation of rosettes. This was interpreted as focal retinal dysplasia. Mechanisms leading to abnormal retinal development is discussed. T cells, in particular $CD4^+$ T cells, predominated within the retinal lesions and choroid. This demonstrates that the fetus is capable of mounting an

immune response to *T.gondii*. However, this immune response may be less effective than in the child or adult. *T gondii* organisms were identified in 5 fetuses and two infants by immunohistochemical staining. In one infant eye and one fetal eye parasites were identified in a perivascular location supporting a haematogenous route of dissemination to the eye. These findings demonstrate that ocular toxoplasmosis causes severe irreversible damage to the retina *in utero*. The significance of these findings to current strategies for prenatal diagnosis and management is discussed.

Current treatments are only effective against the rapidly dividing tachyzoite form associated with active disease. There are no treatments capable of eliminating the quiescent cystic bradyzoite stage. This is the source of disease reactivation in the congenitally infected and immunocompromised host. A combination of pyrimethamine and sulphadiazine, both anti-folate agents, is the most effective therapy for active disease currently available. However, there are significant side effects associated with both these drugs. For these reasons, new antimicrobial agents are urgently needed for the treatment of this disease.

In recent years there has been considerable interest in the vestigial plastid organelle of *T. gondii* and Apicomplexans (the subphylum of parasites which include *Plasmodium*, *Theileria*, *Babesia*, *Eimeria*, *Hepatozoon*, *Sarcocystis* and *T.gondii*) in general. In addition certain other plant-like characteristics have been reported in Apicomplexans. These observations may provide exploitable differences between these parasites and their mammalian hosts. In the second part of this thesis, the possibility that *T.gondii* may depend on biochemical processes more normally restricted to plants is investigated.

The shikimate pathway occurs within the plastid of plants and algae. It is a seven step reaction which catalyses the conversion of phosphoenol pyruvate to chorismate. Chorismate is then used for the synthesis of virtually all aromatic compounds, notably, p-aminobenzoic acid which is required for *de novo* folic acid synthesis; ubiquinone an essential component of the mitochondrial electron transport chain; and the aromatic amino acids.

The studies described in this section of the thesis provide chemotherapeutic and genetic evidence for the shikimate pathway in *T. gondii*. Firstly, the *in vitro* growth of *T. gondii* was inhibited by the herbicide glyphosate, also known as N-(phosphonomethyl)glycine (NPMG), a well characterised inhibitor of the shikimate pathway enzyme 5-enolpyruvyl shikimate 3-phosphate (EPSP) synthase. Furthermore, the effect of NPMG on *T.gondii* was reversed by treatment with p-aminobenzoic acid, which suggests that the shikimate pathway supplies folate precursors for their growth. NPMG in combination with pyrimethamine was able to protect mice from death. Finally the gene encoding chorismate synthase, the final shikimate pathway enzyme was cloned and sequenced form *T.gondii*.

Another plant pathway which was investigated was the alternative pathway of respiration. This pathway is present on the inner mitochondrial membrane of plants, *Trypanosoma*, certain fungi and green algae. The alternative pathway of respiration branches from the cytochrome pathway after the ubiquinone complex and channels electrons through the alternative oxidase. Evidence is also presented for the presence of this pathway in *T.gondii*. Thus, *in vitro* growth of *T.gondii* was inhibited by two recognised inhibitors of alternative oxidase, salicylhydroxamic acid and

8-hydroxyquinoline. In addition, antibodies to the alternative oxidase of *Trypanosoma bruceii* and the Voodoo lily, *Sauromatum guttatum,* recognised a band of 66 kD by Western blotting of electrophoresed parasites. Under rigorous reducing conditions a band of 33 kD was also recognised suggesting that, like alternative oxidase of *T.bruceii* and Voodoo lily, *T.gondii* alternative oxidase is a dimeric protein. The description of these two plant like biochemical pathways in *T.gondii* not only increases our knowledge of Apicomplexan biochemical processes, but also provides a rational for the development of novel antimicrobial targets for the treatment of toxoplasmosis.

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Declaration

None of the work in this thesis has appeared in any other submitted thesis to this or any other University. I personally performed all the laboratory work described in this thesis apart from that outlined below.

In Chapter 4 the eyes were collected by Dr. R. McLeod through multinational collaborations. Dr. M. Mets supplied the control eyes. Dr R.B. O'Grady and Mrs C.O'Grady assisted in the processing of tissues for histology. The immunohistochemistry laboratory at Michael Reese Hospital, Chicago, USA and David Ferguson, Oxford, UK, assisted with immunohistochemical staining.

In Chapter 5, Dr. C.W. Roberts assisted with the *in vivo* experiments. I assisted Dr. C.W. Roberts and Ms. J. Johnson with the sequencing of the chorismate synthase from *T.gondii*. Specifically, I assisted with the *in vivo* excision, cloning and sub-cloning. Sequencing was performed by the University of Chicago sequencing facility. The analysis of cDNA and predicted amino acid sequences were performed by Dr. C.W. Roberts.

The following papers have been accepted for publication and contain material included in this thesis:

1. Roberts F., Roberts C.W., Johnson J.J., Milhous W., Kyle D., Krell T., Coggins J., Coombs G., Tzipori S., Ferguson D.J.P., Chakrabarti, D., and McLeod R. (1998). Evidence for the shikimate pathway in Apicomplexan parasites. *Nature*, **393,** 801-805.

2. Roberts, F. & McLeod, R. Pathogenesis of Toxoplasmic Retinochoroiditis, *Parasitology Today*, in press.

Aspects of this work have also been presented at meetings of the following societies:

Fourth International Biennial *Toxoplasma* Conference, Drymen, UK, July, 1996

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Scottish Universities Molecular Parasitologists Meeting, May 1998

CHAPTER 1
DISEASE CAUSED BY *TOXOPLASMA GONDII*

1.1 History

Toxoplasma gondii was first discovered in the spleen of the north African rodent, the gondii (*Ctenodactylus gundi*) (Nicolle & Manceaux, 1908). Initially naming this parasite *Leishmania gondii*, Nicolle and Manceaux later recognised it as a separate genus renaming it *Toxoplasma gondii* (Nicolle & Manceaux, 1909). In the same year the parasite was also seen by Splendore, (1908) in tissues of a rabbit in Brazil. A paper by Janku in 1923 is accepted as the first report of human disease although a number of earlier cases describing disease, which at that time was apparently due to other organisms, were later re-identified as *T.gondii* (Kean, 1972). Congenital toxoplasmosis was first reported in 1937 (Wolf & Cowen, 1937; Wolf *et al.*, 1939) and in 1941 Sabin described the classical tetrad of symptoms, (meningoencephalitis, hydrocephalus, intracranial calcifications and retinochoroiditis), associated with congenital infection. In 1940 the first fatal case of acquired toxoplasmosis in an adult was described (Pinkerton & Weinman, 1940).

Serological tests for *T.gondii* became available in the 1940s. In 1948 the introduction of the Sabin-Feldman dye test (Sabin & Feldman, 1948) allowed large scale epidemiological studies of toxoplasmosis. Use of this test confirmed world-wide distribution of *T.gondii* and showed that there were many asymptomatic carriers both in humans and domestic animals. However, for around 60 years after its discovery, the life-cycle of *T.gondii* remained unknown.

A report by Weinman and Chandler in 1954 claimed that undercooked meat may be a source of infection. This claim was substantiated by Desmonts *et al.*, (1965) who observed almost 100% seroconversion in children fed raw meat in a Parisian

'consumption' (tuberculosis) hospital. In contradiction to this Rawal, (1959) showed that prevalence rates in vegetarians and non-vegetarians were similar.

Attempts to demonstrate infection in excretions and secretions of animals initially failed. However, in 1965, Hutchison showed that cat faeces could be infective to laboratory mice and in 1969 the cat was identified as the definitive host (Hutchison *et al.*, 1970; Hutchison *et al.*, 1969; Hutchison *et al.*, 1971). Subsequent work confirmed the cat as the definitive host and identified most mammals, including man, as intermediate hosts (Frenkel *et al.*, 1970; Miller *et al.*, 1972; Jewell *et al.*, 1972; Janitchke & Werner, 1972).

1.2 Epidemiology

T.gondii infects humans throughout the world. World-wide prevalence of antibodies to *T.gondii* is estimated at 33% although there is considerable variation within and between groups studied (reviewed, Jackson & Hutchison, 1989). Thus, the presence of *T.gondii* antibodies in different populations varies with socio-economic factors, age, and geographical location. For example, infection rates are higher where undercooked meat is frequently consumed or where there is environmental contamination with cat excrement. Geographically, prevalence is highest in tropical areas and is relatively low in hot, arid areas or cold regions (Wallace, 1969). People living at high altitudes have lower rates of infection than those living at sea level (Yamaoka & Konishi, 1993). These variations are attributed to the effects of environmental conditions on oocyst survival. Prevalence of human toxoplasmosis in various groups based on serological evidence is shown in Table 1.1.

Table 1.1 Sero-prevalence of *T.gondii* antibodies in various groups of people (updated from Jackson & Hutchison, 1989)

GROUP	REGION	PREVALENCE (%)	REFERENCES
Women of childbearing age	India	7.7	Mittal *et al.*, 1995
	Mali	34	Maiga *et al.*, 1984
	Turkey	39.9	Durmaz *et al.*, 1995
	Senegal	40.3	Diallo *et al.*, 1996
Pregnant women	Thailand	13-15	Taechowisan *et al.*, 1997
	Glasgow, Scotland	13.4	Williams *et al.*, 1981
	England	14.9	Jackson *et al.*, 1987
	Southern Finland	20.3	Lappalainen *et al.*, 1992
	Denmark	27.4	Lebech *et al.*, 1995
	Southern Spain	30	Gutierrez *et al.*, 1996
	Tanzania	34-37	Doehring *et al.*, 1995
	Slovenia	37	Logar *et al.*, 1992
	Brussels, Belgium	53	Foulon *et al.*, 1984
	Cuba	70.9	Gonzalez-Morales *et al.*, 1995
	Paris, France	84	Desmonts & Couvreur, 1974
Blood Donors	Thailand	1.2-4.6	Morakote *et al.*, 1984
	Scotland	7.6-7.8	Jackson *et al.*, 1987
	Kenya	42	Griffin & Williams, 1983
	Saudi Arabia	52.1	Al-Amari, 1994
Veterinarians	New York, USA	8.3	Sengbusch & Sengbusch, 1976
	California, USA	43.7	Behymer *et al.*, 1973
Abattoir workers	Brazil	60-92	Riemann *et al.*, 1975
Military Recruits	USA	3-20	Feldman, 1965
		3-14	Smith *et al.*, 1996
Cat owners	Washington County, USA	20.9	Peterson *et al.*, 1972
	England	35.8	Woodruff *et al.*, 1982
without dogs	Iceland	18.3	Woodruff *et al.*, 1982

Table 1.1 continued:

GROUP	REGION	PREVALENCE (%)	REFERENCES
Isolated island populations			
with cats	Pacific(tropics)	43 and 56	Wallace, 1969
without cats	Pacific(tropics)	7	Wallace, 1969
Isolated jungle populations	Brazil(tropics)	39-77	Lovelace *et al.*, 1978
	Brazil(tropics)		Ferraroni & Marzochi, 1980
Populations at			
high altitude	Japan	5	Yamaoka & Konishi,
sea level	Japan	10.6	1993
Populations with			
high rainfall	Japan	16.8	Yamaoka & Konishi,
low rainfall	Japan	8.6	1993
Populations			
within Arctic	Alaska, USA	28	Peterson *et al.*, 1974
	Northern Sweden	12	Ljungstrom *et al.*, 1995
outwith Arctic	Gotland Island, Southern Sweden	26	Ljungstrom *et al.*, 1995
Asymptomatic	United Kingdom	27	Holliman, 1990
HIV infected	Mexico	50	Galvan Ramirez *et al.*, 1997
AIDS patients	Zaire	24-33	De Clerq *et al.*, 1986
Medical outpatients	England	35.7	Jackson *et al.*, 1987
Travelling people	Scotland	28	Jackson *et al.*, 1987
Population surveys	Calcutta, India	23.8	Haldar *et al.*, 1993
	Chile	36.9	Contreras *et al.*, 1996
	Parma, Italy	48.5	Valcavi *et al.*, 1995
	Nepal	48	Rai *et al.*, 1996
	Ethiopia	74.4	Guevre-Xabier *et al.*, 1993

Table 1.2 Estimated incidence of human congenital toxoplasmosis in different countries (updated from Jackson & Hutchison, 1989)

REGION	INCIDENCE PER 1000	REFERENCES
Germany	5.3	Kraubig, 1966
Mexico City	2	Roch & Varela, 1966
New York	0.7	Kimball *et al.*, 1971
London	0	Ruoss & Buorne, 1972
Vienna	6-7	Thalhammer, 1973
The Netherlands	6.5	Koppe *et al.*, 1974
Paris	10	Desmonts & Couvreur, 1974
United Kingdom	0.07-0.25	Fleck, 1974
Birmingham, Alabama	1.3	Alford *et al.*, 1974
Austria	8.6	Thalhammer & Heller-Szollosy, 1979
Glasgow	> 0.5	Williams *et al.*, 1981
Europe	3-6[a]	Williams *et al.*, 1981
USA	1-2[a]	Williams *et al.*, 1981
Great Britain	0.9	Henderson *et al.*, 1984
Brussels	> 2	Foulon *et al.*, 1984
USA	1-8	McCabe & Remington, 1988
Slovenia	3	Logar *et al.*, 1992
Southern Finland	0.96	Lappalainen *et al.*, 1992
Guatemala	10.9	Sinibaldi & De, 1992
United Arab Emirates	12	Dar *et al.*, 1997

[a] Computed averages

The incidence of congenital infection is difficult to estimate due to inadequate screening in many countries, but reports world-wide vary between 0.07 and 10 per 1000 births (Table 1.2). However, the incidence of ocular toxoplasmosis is considerably greater than the stated incidence of congenital infection (reviewed, Cook, 1990; Jackson & Hutchison, 1989). Since many of these cases represent previously undiagnosed congenital infection, it is likely that the true incidence of congenital infection is underestimated.

1.3 Clinical Disease

Infection in the immunocompetent host

In the immunocompetent host primary infection with *T.gondii* is frequently asymptomatic (Hughes, 1985). Occasionally it is associated with a febrile illness with or without lymphadenopathy (McCabe *et al.*, 1987). *T.gondii* has been estimated to cause between 3 and 7 % of clinically significant lymphadenopathy in adults (McCabe *et al.*, 1987). Far less commonly toxoplasmosis results in severe illness affecting any organ and in extreme cases can be fatal (Pinkerton & Weinman, 1940; Townsend *et al.*, 1975). Manifestations of severe disease include encephalitis (Grant & Klein, 1987; Lescop *et al.*, 1995; Townsend *et al.*, 1975), myocarditis (Montoya *et al.*, 1997), pneumonitis (Candolfi *et al.*, 1993; Pomeroy & Filice, 1992) and hepatitis (Masur & Jones, 1979; Sijpkens *et al.*, 1994; Weitberg *et al.*, 1979). Ocular disease can also occur in the immunocompetent individual and is described below.

Infection in the immunosuppressed host

T.gondii is a common opportunistic pathogen among immunosuppressed patients. It is a particularly serious problem in patients with HIV-infection (Zangerle *et al.*, 1991), but also affects patients taking immunosuppressive agents following transplant surgery (Israelski & Remington, 1993) or patients receiving chemotherapy for malignancy (Klastersky, 1985).

Toxoplasmosis is believed to be the most common non-viral infection of the brain in patients with AIDS. Without prophylactic chemotherapy, encephalitis eventually develops in up to 50% of AIDS patients with antibodies against *T.gondii* (Zangerle *et al.*, 1991). Whilst cerebral involvement is most frequent in AIDS patients any organ

may be involved and toxoplasmosis may present as a multisystem disease (reviewed, Holliman, 1988; Mariuz *et al.*, 1994).

Toxoplasmosis following transplant surgery is affected by two main factors, the type of organ graft (solid organ or bone marrow) and the degree of immunosuppression associated with the post-operative management (Israelski & Remington, 1993). The organ graft type determines the risk of contamination with *T.gondii* (Sweny, 1993). Most cases of toxoplasmosis associated with solid organ transplant such as heart (Gallino *et al.*, 1996; Holliman *et al.*, 1991), lung (Couvreur *et al.*, 1992; Wreghitt *et al.*, 1989) and to a lesser extent liver (Mayes *et al.*, 1995; Singh *et al.*, 1996) or kidney (Renoult *et al.*, 1997) arise from reactivation of cysts within the graft itself. In contrast, toxoplasmosis following bone marrow transplantation is usually caused by reactivation of the recipient's own previously quiescent *T.gondii* cysts (Chandrasekar *et al.*, 1997; Derouin *et al.*, 1992). This is related to the more intensive immunosuppressive therapy required following bone marrow transplantation. Ocular toxoplasmosis in the immunocompromised host is discussed below.

Congenital infection

Transplacental infection of *T.gondii* to the fetus most frequently occurs when a woman is infected for the first time during pregnancy (reviewed, Roberts *et al.*, 1998a). On rare occasions, congenital infection also can occur if an immunocompetent woman acquires toxoplasmosis 6 to 8 weeks prior to conception (Desmonts *et al.*, 1990; Vogel *et al.*, 1996). Less commonly an immunocompromised mother with chronic toxoplasmosis may transmit this disease to her fetus (D'Ercole *et*

her fetus (D'Ercole *et al.*, 1995; Desmonts *et al.*, 1990). Congenital infection develops in between 30 to 50% of infants born to mothers who have serological evidence of acute toxoplasmosis during pregnancy (reviewed, Stray-Pedersen, 1993). As with other congenital infections this varies depending which trimester infection of the mother occurred (reviewed, Haggerty, 1985). During the first trimester while the infection rate is approximately 10 to 15%, disease manifestations are severe. Conversely in the third trimester almost two thirds of fetuses will be infected but symptoms are usually mild (Table 1.3).

Table 1.3 Inverse relationship between the incidence of fetal infection and severity of fetal damage following acutely acquired maternal infection with *T.gondii* at different stages of gestation (from Remington *et al.*, 1995)

TRIMESTER OF PREGNANCY	FETUSES INFECTED (%)	SEVERITY OF ILLNESS
1	17	Most severe
2	25	Intermediate severity
3	65	Least severe or subclinical

T.gondii infection of the fetus may result in damage to any organ, but most frequently involves the eye and the brain (reviewed, Swisher *et al.*, 1994). The classical tetrad of congenital toxoplasmosis, first described by Sabin, (1941), consists of meningoencephalitis, hydrocephalus, intracranial calcifications and retinochoroiditis. Lesions in the brain are most marked in the cortex, basal ganglia and periventricular areas (reviewed, Remington *et al.*, 1995; Weber, 1983). Extensive necrosis can occur

especially in peri-aqueductal and periventricular regions, where sloughing of necrotic brain tissue into the ventricles may result in obstructive hydrocephalus (reviewed, Frenkel & Friedlander, 1951). Calcifications occur within areas of necrosis (Rosenberg & Kessler, 1993). In surviving children these frequently regress with treatment (Patel *et al.*, 1996).

Ocular infection

Ocular toxoplasmosis is the most common manifestation of congenital infection (Koppe *et al.*, 1986; Remington *et al.*, 1995). Most infants who present with generalised or neurological disease at birth already have extensive ocular disease (Mets *et al.*, 1996). Conversely, ocular disease may be the only sign of congenital infection (Guerina *et al.*, 1994). Serological screening of neonates in Massachusetts, USA showed that 20% of asymptomatic infants, with congenital toxoplasmosis infection, already had ocular involvement (Guerina *et al.*, 1994). Furthermore, longitudinal studies have shown that more than 82% of congenitally infected individuals not treated as infants develop retinochoroidal lesions by adolescence (Koppe *et al.*, 1986; Wilson *et al.*, 1980).

It is commonly believed that most cases of ocular toxoplasmosis are the result of congenital infection. This is based on epidemiological studies in areas of the world such as Micronesia where, despite high seroprevalence, ocular toxoplasmosis is rarely seen (Darrell *et al.*, 1964). In these populations high rates of acquired disease in childhood means that first exposure to *T.gondii* usually occurs before pregnancy and consequently congenital transmission is uncommon. The low rate of ocular disease in

these populations argues against acquired ocular toxoplasmosis. Perkins, (1973) reviewed case reports where acquired toxoplasmosis appeared to be well documented and estimated that ocular involvement occurred in 2 to 3% of patients with symptomatic acquired toxoplasmosis. Perkins assumed that acquired ocular toxoplasmosis without systemic manifestations should be even less common. However, over a period of 13 years, a French epidemiological study identified 43 cases of acquired toxoplasmosis in immunocompetent patients complicated by isolated ocular lesions (Couvreur & Thulliez, 1996). Routine serological screening for toxoplasmosis has been compulsory in France since 1978 and the immune status of the patient's mother was documented in many cases, thus eliminating congenital infection. In addition to serological evidence of recent infection the diagnosis was confirmed by identifying specific local antibody synthesis in the aqueous humor of the eye. This French study suggested that acquired ocular toxoplasmosis was more frequent than had been previously estimated.

In contrast to intracranial disease, which occurs in up to 50% of those with antibodies to *T.gondii*, ocular toxoplasmosis appears to be uncommon in patients with AIDS accounting for between 1 and 3% of retinal infections (Cochereau Massin *et al.*, 1992; Jabs, 1995). In two small studies of patients with both AIDS and intracranial toxoplasmosis, ocular infection occurred in 10 and 20%, respectively (Luft *et al.*, 1983; Snider *et al.*, 1983). In addition, ocular toxoplasmosis may develop without evidence of intracranial disease. Ocular toxoplasmosis following bone marrow transplant (Elkins *et al.*, 1994), chemotherapy (Yeo *et al.*, 1983) or long term corticosteroids (Nicholson & Wolchok, 1976) also has been described.

T.gondii causes a progressive and recurring retinochoroiditis (Holland *et al.*, 1996; Roberts & McLeod, 1998). Patients suffering from active retinochoroiditis usually present with blurred vision and floaters attributable to foci of active retinal inflammation (Figure 1.1A). Lesions heal to leave retinochoroidal scars, which are pale and atrophic with a variable amount of pigmentation at the margins (Figure 1.1B). A retrospective clinical review by the 'Chicago Toxoplasmosis Study Group', defined the ocular manifestations of congenital toxoplasmosis in individuals treated or untreated during the first year of life (Mets *et al.*, 1996). This group of patients included infants, children and adults with congenital toxoplasmosis. The typical ophthalmological presentation, course and sequela of this disease were documented. Their findings are summarised in Table 1.4. Retinochoroidal scars were the most common finding, present in 79% of patients. These scars involved the peripheral retina and macula in 64% and 58% of patients, respectively. If one considers the smaller area of the macula this suggests a definite predilection for the macula in congenital ocular toxoplasmosis. Furthermore, there was significant bilateral visual loss in 29% of patients, and a further 28% had unilateral visual loss.

The clinical presentation of acquired toxoplasmic retinochoroiditis differs from congenital ocular disease. These patients are usually older with unilateral ocular disease and a single, active, retinochoroidal lesion (Montoya & Remington, 1996). Macular involvement is uncommon. This is in contrast to the clustering of lesions, retinochoroidal scars and macular involvement more characteristic of congenital infection.

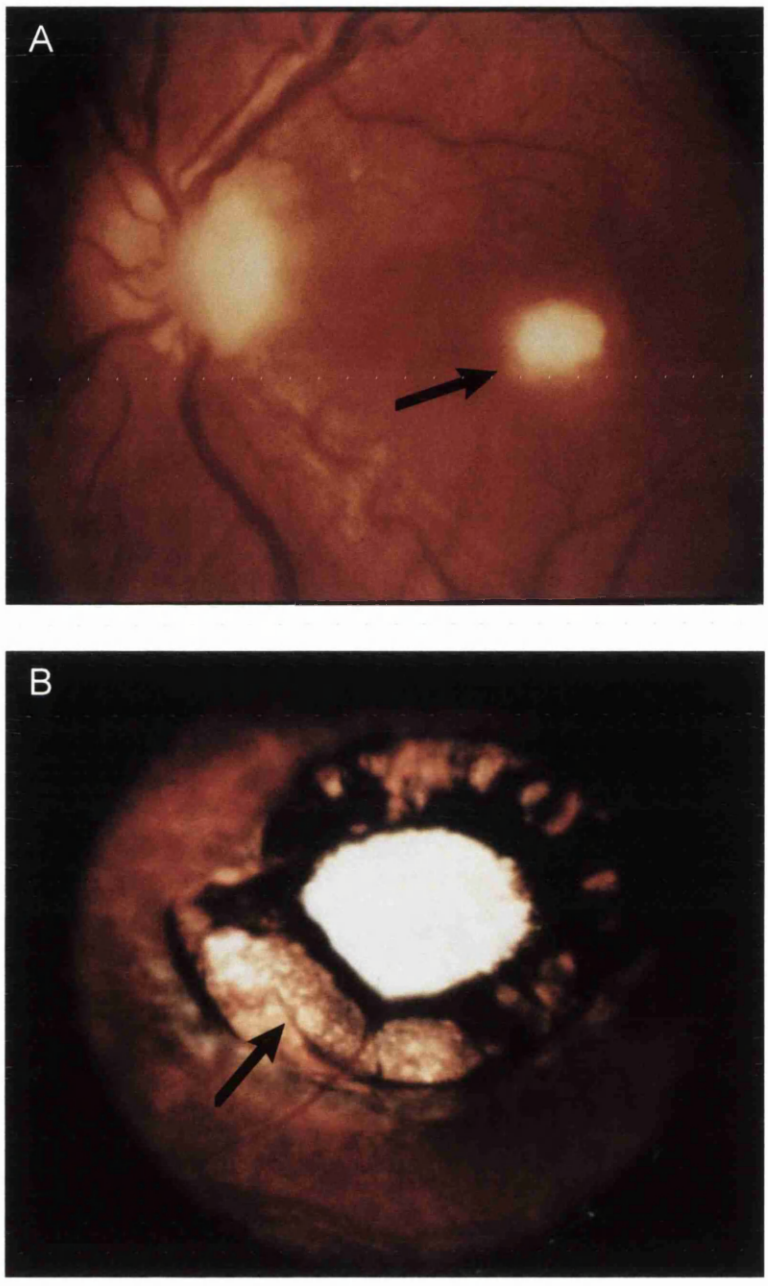

Figure 1.1 **Fundoscopic photographs of toxoplasmic retinochoroiditis with acute lesion (A) and macular scar (B). The acute lesion (A) appears white with an indistinct border (arrow). The retinochoroidal scar (B) is sharply demarcated with a white centre and hyper-pigmented border. There is focal reactivation at the edge of the scar (arrow).**

Table 1.4 The ophthalmological manifestations of congenital toxoplasmosis (adapted from Mets *et al.*, 1996)

Finding	Number of patients(%), total=94	Number of patients with bilateral findings (%)
Strabismus	31(33)	-
Nystagmus	25(27)	-
Microphthalmia	12(13)	4/12(33)
Phthisis	4(4)	2/4(50)
Microcornea	18(19)	9/18(50)
Cataract	9(10)	3/9(33)
Vitritis(active)	5(5)	5/12(42)
Retinitis(active)	10(11)	2/9(22)
Chorioretinal Scars	74(79)	48/74(65)
Macular	52(58)	19/52(37)
Juxtapapillary	46(52)	20/46(43)
Peripheral	57(64)	23/57(40)
Retinal Detachment	9(10)	5/9(56)
Optic Atrophy	19(20)	9/19(47)

The histological features of congenital ocular lesions and their reactivations in adults have been described in reports by (Hogan, 1951; Wilder, 1952; Hogan, 1958; Zimmerman, 1961; Perkins, 1973; Rao & Font, 1977). Histologically, acute lesions show a well defined area of retinal necrosis or inflammatory infiltration. Tachyzoites are occasionally identified in areas of necrosis (Rao & Font, 1977). Tissue cysts may be found at the edge of a lesion or remote from the lesion within apparently normal retina (McMenamin *et al.*, 1986). Distant from the active lesion the retina may show perivascular lymphocytic infiltration, oedema, gliosis, and neuronal degeneration. Usually there is associated choroidal inflammation. Active lesions heal to leave a retinochoroidal scar which consists of gliosis, obliteration of vessels and scattered inflammatory cells. Secondary changes including retinal detachment (Mets *et al.*, 1996), iridocyclitis (Friedmann & Knox, 1969) and cataracts (Mets *et al.*, 1996) have been described. Papillitis, papilloedema and optic atrophy are recognised clinical manifestations of toxoplasmic encephalitis (Mets *et al.*, 1996; Perkins, 1973).

Microphthalmos or microcornea occasionally follows severe congenital infection (Heath & Zueler, 1944; Koch *et al.*, 1943).

In contrast to congenital ocular lesions in children and adults, the early stages of ocular toxoplasmosis during acute infection of the fetus have not been extensively studied. In 1951 Hogan stated that 'There have as yet been no descriptions of the earliest stages of toxoplasmic chorioretinitis'. Since then, there has been only one study of ocular lesions in four fetuses between the ages of 22 to 27.5 weeks (Brezin *et al.*, 1994). In two of these cases the eyes were normal, however the other two cases showed retinal necrosis, neovascularisation and marked chorioretinal inflammation.

In AIDS related ocular toxoplasmosis the lesions differ both clinically and histologically from those of immunocompetent patients (reviewed, Holland, *et al.*, 1988a; Holland, 1989). There is usually no evidence that the disease originated in pre-existing retinochoroidal scars. This suggests that most ocular lesions result from newly acquired disease or organisms which disseminate to the eye from non-ocular sites of reactivation. Lesions are frequently bilateral and multifocal and extensive retinal necrosis is common. Histopathological examination demonstrates scant retinal inflammation in these areas of necrosis with large numbers of parasite similar to that seen in the brain lesions of AIDS associated toxoplasmic encephalitis (Holland *et al.*, 1988a).

1.4 Antimicrobial Treatment of Toxoplasmosis

Anti-folate agents

The most effective available therapeutic regimen is the combination of pyrimethamine and a sulphonamide, usually sulphadiazine (reviewed, Roberts *et al.*, 1998a). Although still unlicensed for the treatment of toxoplasmosis in the United Kingdom, these anti-folate agents have been used since the early 1950's . Both are active against tachyzoites and show synergism with each other (Eyles & Coleman, 1953). Pyrimethamine is a competitive antagonist of dihydrofolate reductase (DHFR) which converts dihydrofolate to tetrahydrofolate (Williams, 1996a). Although more active against parasite DHFR, pyrimethamine is also active against human DHFR and consequently a common side effect of treatment is bone marrow suppression (Williams, 1996a). This can in part be counteracted by simultaneous administration of folinic acid. *T.gondii* is unable to use host cell folinic acid (Giles, 1971). Sulphadiazine blocks an earlier stage in folate synthesis than pyrimethamine: the condensation of p-aminobenzoic acid (PABA) with 6-hydroxymethyl-dihydropterin diphosphate to form dihydropteroic acid (Williams, 1996a). Sulphadiazine is, in fact, metabolised to an analogue of PABA which then competes with PABA for the enzyme site on dihydropteroate synthase. Side effects include hypersensitivity reactions, bone marrow suppression, and both hepatic and renal toxicity (Williams, 1996a).

Recent studies have investigated the use of DHFR inhibitors which are more active against *T.gondii* DHFR than host cell DHFR (Brun Pascaud *et al.*, 1996; Gangjee *et al.*, 1997; Martinez *et al.*, 1996). These new DHFR inhibitors are as effective as pyrimethamine, when combined with sulphadiazine or dapsone, in the treatment of

mice with acute toxoplasmosis (Martinez *et al.*, 1996). In addition, epiroprim, for example, is 650 times more active against *T.gondii* DHFR compared with human DHFR (Martinez *et al.*, 1996). These new DHFR inhibitors are therefore, a potentially less toxic alternative to pyrimethamine.

Clindamycin

Clindamycin has been shown to prevent replication of *T.gondii* grown *in vitro* (Pfefferkorn *et al.*, 1992). Clindamycin is also effective against acute and chronic toxoplasmosis in mice (Hofflin & Remington, 1987; McMaster *et al.*, 1973). The mechanism of its action against *T.gondii* is unclear. In bacteria it inhibits protein synthesis on prokaryotic ribosomes (Williams, 1996b). Ribosomal DNA sequences believed to be derived from the *T.gondii* mitochondrial genome predict resistance to this drug (Beckers *et al.*, 1995). This suggests that clindamycin may act by preventing organellar synthesis in *T.gondii*.

Clinically, it has been used in combination with pyrimethamine for the treatment of AIDS related toxoplasmic encephalitis (Katlama *et al.*, 1996). A randomised, prospective study compared combination pyrimethamine-clindamycin with pyrimethamine-sulphadiazine therapy for the treatment and maintenance of AIDS-related toxoplasmic encephalitis (Katlama *et al.*, 1996a). Pyrimethamine-clindamycin was found to be as effective as pyrimethamine-sulphadiazine during acute therapy. However during maintenance therapy, patients receiving pyrimethamine-clindamycin were twice as likely to relapse than those receiving pyrimethamine-sulphadiazine.

Clindamycin has also been used to treat ocular toxoplasmosis. Oral clindamycin appears to be as effective as pyrimethamine when given in combination with sulphadiazine and corticosteroids for the treatment of active toxoplasmic retinochoroiditis (Rothova *et al.*, 1993). Other studies have suggested that subconjunctival injections of clindamycin may be as effective as combined oral pyrimethamine and sulphadiazine for the treatment of ocular toxoplasmosis (Colin & Harie, 1989; Jeddi *et al.*, 1997). In these studies subconjunctival administration of clindamycin did not produce any side effects which were common with oral pyrimethamine and sulphadiazine. Furthermore, in the short term, recurrences were less common with this therapy. However, these studies involved small numbers of patients in whom the diagnosis of toxoplasmic retinochoroiditis was presumed but not confirmed.

To date, clindamycin is usually recommended as an alternative therapy in conjunction with pyrimethamine, if sulphadiazine therapy cannot be tolerated. However, it must be used with caution in view of the uncommon but serious side effect of pseudomembranous colitis (Swartzberg *et al.*, 1977).

Atovaquone

Atovaquone, a hydroxynaphthoquinone, was considered a major breakthrough when it was suggested that it was effective against bradyzoites within cysts, *in vitro* (Araujo *et al.*, 1991; Huskinson Mark *et al.*, 1991). It also appears to prolong survival and decrease cyst numbers in mice infected with the Me49 strain of *T.gondii* (Araujo *et al.*, 1992a). However, ultrastructural studies on the brains of these mice suggest that

this drug is only active against metabolically active, immature bradyzoites rather than the mature organisms (Ferguson *et al.*, 1994).

Atovaquone inhibits the enzyme dihydroorotate dehydrogenase (Ittarat *et al.*, 1994). This enzyme is linked to the mitochondrial electron transport chain by ubiquinone. Thus, atovaquone interferes with oxidative respiration. Despite its apparent success *in vitro* and in animal models, it has proved less effective in clinical trials. In these trials, after successful induction over 26% of patients receiving maintenance atovaquone therapy for the treatment of AIDS associated toxoplasmic encephalitis relapsed (Katlama et al., 1996b). Lopez *et al.*, (1992) described the successful treatment of ocular toxoplasmosis in a patient with AIDS using atovaquone. However, in view of the high relapse rate seen in AIDS related toxoplasmic encephalitis, it is unlikely to prevent recurrent retinochoroiditis.

Spiramycin

Spiramycin is a macrolide antibiotic which *in vitr* growth on *T.gondii* although it does not kill Pechere, 1988a; Chang & Pechere, 1988b). The i prevent transplacental transmission of *T.gondii*. placenta and treatment is based on the observ maternal and fetal infection (Remington *et al.*, i congenital toxoplasmosis, subinoculation of the placenta into mice was negative in 25% of spiramycin treated mothers compared with 11% of untreated mothers (Couvreur *et al.*, 1988). If spiramycin therapy was combined with pyrimethamine and a sulphonamide 50% of placentas were uninfected. Spiramycin does not however alter

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Spiramycin

Spiramycin is a macrolide antibiotic which *in vitro* has some inhibitory effect on the growth on *T.gondii* although it does not kill the parasite effectively (Chang & Pechere, 1988a; Chang & Pechere, 1988b). The main clinical use of spiramycin is to prevent transplacental transmission of *T.gondii*. Spiramycin is concentrated in the placenta and treatment is based on the observation that there is a lag between maternal and fetal infection (Remington *et al.*, 1995). In a study of 223 cases of congenital toxoplasmosis, subinoculation of the placenta into mice was negative in 25% of spiramycin treated mothers compared with 11% of untreated mothers (Couvreur *et al.*, 1988). If spiramycin therapy was combined with pyrimethamine and a sulphonamide 50% of placentas were uninfected. Spiramycin does not however alter

the course of the disease if the fetus is already infected. In a rhesus monkey model of congenital toxoplasmosis, spiramycin was found in some fetal tissues although at lower concentration than maternal tissues (SchoondermarkVan *et al.*, 1994). However, no spiramycin was found in the fetal brains, one of the most severely affected sites in congenital infection.

Corticosteroids

Corticosteroids have been used in combination with pyrimethamine and sulphadiazine to treat retinitis and vitritis which threaten vision. Corticosteroids do not have any action on the parasite but are used to decrease problems associated with inflammation. There is little controlled data to demonstrate the additional benefit afforded by corticosteroids, but in one small study, worsening of signs and symptoms were not observed when corticosteroid treatment was added to conventional antimicrobial therapy (Acers, 1964). Immunosuppression may potentiate replication of *T.gondii* and, given alone, corticosteroids will exacerbate ocular lesions (O'Connor, 1974; van den Bosch & Rothova, 1997)

Other antimicrobial agents

Many other antimicrobial agents have been shown to be effective against *T.gondii in vitro* or *in vivo.* These include new macrolides such as roxithromycin (Chan & Luft, 1986; Romand *et al.*, 1995), clarithromycin (Alder *et al.*, 1994; Araujo *et al.*, 1992b), azithromycin (Araujo *et al.*, 1988; Dumas *et al.*, 1994) and ketolides (Araujo *et al.*, 1997). In bacteria these drugs inhibit ribosomal protein synthesis and may inhibit organellar protein synthesis in *T.gondii* (Beckers *et al.*, 1995). Clarithromycin has

been used to treat patients with AIDS related toxoplasmic encephalitis (Raffi *et al.*, 1995). Use of the anti-leprosy sulphone drug, dapsone has been described (Derouin *et al.*, 1991; Girard *et al.*, 1993). Similar to sulphadiazine this drug is an inhibitor of dihydropteroate synthase (Williams, 1996a). The anti-malarials, artemisinin ether and cycloguanil, have also been shown to be effective against *T.gondii in vitro* (Holfels *et al.*, 1994). In addition, the anti-HIV drug 2'3'dideoxyinosine has recently been shown to inhibit the growth of *T.gondii in vitro* and to reduce cyst burden *in vivo* (Sarciron *et al.*, 1997). The clinical role of this drug in AIDS patients with toxoplasmic encephalitis has not yet been evaluated.

All the aforementioned drugs have been shown to be more effective when combined with conventional anti-folate drugs. They appear to offer few advantages over existing conventional therapies although they may prove useful where allergy or intolerance limits the use of pyrimethamine or sulphadiazine. Drugs used in the treatment of toxoplasmosis are summarised in Table 1.5.

1.5 Treatment in Specific Clinical Settings

In most symptomatic cases *T.gondii* infection is self-limiting. Furthermore there are no drugs which eliminate tissue cysts and therefore treatment cannot prevent chronic infection. Current guidelines suggest that active treatment is not required for the majority of *T.gondii* infections (reviewed, Boyer & McLeod, 1996; Roberts *et al.*, 1998a). There are specific situations in which treatment is extremely important and may prevent death or blindness. These include infections in pregnant women, newborn infants, immunosuppressed patients and recurrent ocular disease. Table 1.6 summarises the currently recommended therapies for each clinical setting.

Table 1.5 Drugs used experimentally or clinically in the treatment of toxoplasmosis

DRUG	CLASS/ ACTION	ACTIVITY *in vitro*	ACTIVITY *in vivo*	CLINICAL USE	ADVANTAGES	DISADVANTAGES	REFERENCES
ANTIFOLATES							
Pyrimethamine	Inhibits DHFR	Yes	Yes	Yes, treatment of choice	Effective	Bone marrow toxicity	Roberts *et al.*, 1998; Remington *et al.*, 1995
Epiroprim, PS-15	Inhibits DHFR	Yes	Yes	No	Potentially reduced bone marrow toxicity	Not known	Martinez *et al.*, 1996; BrunPascaud *et al.*, 1996
Sulphadiazine	Inhibits DHPS	Yes	Yes	Yes, treatment of choice	Effective	Poorly tolerated Allergy	Roberts *et al.*, 1998; Remington *et al.*, 1995
Dapsone	Inhibits DHPS	Yes	Yes	No	Not known	Not known	Derouin *et al.*, 1991
MACROLIDES							
Clindamycin	Inhibits protein synthesis in bacteria	Yes	Yes	Yes, in sulphadiazine intolerance	Fewer side effects than sulphadiazine	High relapse rate in AIDS patients PM colitis	Hofflin & Remington, 1987; Katlama *et al.*, 1996
Roxithromycin	As for clindamycin	Yes	Yes	No	Not known	Not known	Romand *et al.*, 1995
Clarithromycin	As for clindamycin	Yes	Yes	Yes, trial therapy in AIDS patients	Not known	Not known	Araujo *et al.*, 1992; Raffi *et al.*, 1995

Table 1.5 continued:

DRUG	CLASS/ ACTION	ACTIVITY *in vitro*	ACTIVITY *in vivo*	CLINICAL USE	ADVANTAGES	DISADVANTAGES	REFERENCES
MACROLIDES							
Azithromycin	As for clindamycin	Yes	Yes	No	Not known	Not known	Araujo *et al.*, 1988; Dumas *et al.*, 1994
Ketolides, HMR 3647, HMR 3004	As for clindamycin	Yes	Yes	No	Not known	Not known	Araujo *et al.*, 1997
Spiramycin	As for clindamycin	Poor	Poor	Yes, in maternal infection	Concentrated in placenta	Does not cross placenta or kill *T.gondii*	Chang & Pechere, 1988; Couvreur *et al.*, 1988
OTHER							
Atovaquone	Inhibits DHRDH	Yes	Yes	Yes, in sulphadiazine intolerance	Low toxicity	High relapse rate in AIDS patients on maintenance therapy	Araujo *et al.*, 1991; Durand *et al.*, 1995
Trofloxacin	Inhibits DNA synthesis in bacteria	Yes	Yes	No	Not known	Not known	Khan *et al.*, 1996; Khan *et al.*, 1997
Rifapentine, Rifabutin	Inhibits RNA synthesis in bacteria	Yes	Yes	No	Not known	Not known	Araujo *et al.*, 1994; Araujo *et al.*, 1996
Corticosteroids	Immuno-suppressive	No	No	Yes, adjuvant with antifolates	Uncertain	Immunosuppressive	Acers, 1964; O'Connor, 1974

Abbreviations: DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase; DHRDH, dihydroorotate dehydrogenase; PM, pseudomembranous

Congenital infection

For acute infection during the first or second trimester of pregnancy initial therapy involves administration of spiramycin to prevent transmission of *T.gondii* to the fetus. If the fetus is infected or if maternal infection occurs in the third trimester then the fetus may be treated by administering pyrimethamine, sulphadiazine and folinic acid to the mother. Studies suggest that this treatment, in conjunction with close clinical follow up may improve the outcome of pregnancy.

For example, Daffos *et al.*, (1988) reported a prospective study of 746 cases of acute maternal toxoplasmosis. Congenital infection occurred in 42 fetuses and was diagnosed antenatally in 39 fetuses. Of these 39 pregnancies, 24 were terminated and 15 were continued to term. The mothers were treated with spiramycin, pyrimethamine and either sulphadoxine or suphadiazine. Eleven of the 15 infants with congenital toxoplasmosis were asymptomatic at birth. The other 4 had intracranial calcifications and retinochoroiditis. A further 2 infants developed retinochoroiditis during an 18 month follow-up. Couvreur *et al.*, (1993) compared 52 infected infants whose mothers received pyrimethamine, sulphadiazine and spiramycin with 51 infected fetuses whose mothers received only spiramycin. Treatment with pyrimethamine and sulphadiazine reduced the incidence of placental infection from 77% to 42%. Antibody titres (IgG and IgM) were also significantly lower in the group treated with pyrimethamine and sulphadiazine. However, there was no difference in clinical outcome between the groups. More recently, a prospective study by Berrebi & Kobuch, (1994) examined the outcome of pregnancy for 163 mothers with acute toxoplasmosis. There were 162 liveborn infants and 3 deaths *in utero.* Only 27 of these 162 infants had proven congenital toxoplasmosis. All 27 infants were free of

symptoms and had normal neurological development at 15 to 71 months of age. However, many of these studies do not take into account the most severely affected fetuses which may be aborted, either spontaneously or therapeutically. Furthermore, the longterm outcome for these infants is not yet known.

It is recommended that newborn infants with congenital infection are treated throughout the first year of life, regardless of apparent disease activity (McAuley *et al.*, 1994). Infants treated throughout the first year of life show better neurological and developmental outcomes (Roizen *et al.*, 1995), as well as a reduction in intracranial calcifications (Patel *et al.*, 1996), when compared with untreated infants or those treated for only one month. Similar to *in utero* treatment, the long term outcome particularly as regards ocular disease is as yet unknown.

Infection in the immunosuppressed host

Similar to congenital infection, the first line therapy for AIDS associated toxoplasmosis is pyrimethamine in combination with sulphadiazine (Behbahani *et al.*, 1995). Lifelong maintenance therapy is essential (Wong & Remington, 1994). The relapse rate approaches 100% on withdrawal of therapy, which, as with other recurrent *T.gondii* infections, is attributed to cyst rupture (Cohn *et al.*, 1989). Approximately 20-30% of patients on maintenance therapy relapse, but this may reflect non-compliance (Renold *et al.*, 1992). The use of primary prophylaxis for toxoplasmic encephalitis has also been advocated for patients with CD4 counts lower that 100cells/mm^3 who are seropositive for *T.gondii* (Freedberg *et al.*, 1998). However, no single maintenance regimen that is both efficacious and without adverse

reaction has yet been identified. Pyrimethamine and sulphadiazine have been associated with the lowest relapse rate (Katlama *et al.*, 1996). A higher relapse rate has been reported with pyrimethamine and clindamycin maintenance therapy (Katlama *et al.*, 1996). Patients receiving trimethoprim and sulphamethoxazole for *Pneumocystis carinii* maintenance therapy also have a reduced incidence of toxoplasmic encephalitis (Carr *et al.*, 1992). Similar to pyrimethamine and sulphadiazine, these drugs are associated with adverse reactions.

Ocular infection

Recurrent ocular disease is due to proliferation and tissue invasion by the parasite which usually results from cyst rupture (Mets *et al.*, 1996). In addition, it has been suggested that the immune response to these organisms can also result in additional damage to intra-ocular tissues (Dutton, 1986). In most cases toxoplasmic retinochoroiditis is a self limiting disease albeit with significant pathology. Untreated lesions begin to heal after 1 to 2 months, although the time course is variable (Rothova *et al.*, 1993). The most reliable predictor of disease duration is the size of lesions, with larger lesions taking longer to heal (Rothova *et al.*, 1993). As already mentioned, currently available drugs cannot treat tissue cysts and therefore cannot prevent future recurrences. The goal of therapy is to minimise damage to the retina and optic nerve, thereby preventing permanent visual loss. It is recommended that active lesions are treated with pyrimethamine and sulphadiazine for one month. With early treatment there is usually rapid resolution of active lesions often without associated visual loss (Mets *et al.*, 1996). The controversial role of corticosteroids in ocular toxoplasmosis has been discussed.

Table 1.6 The treatment of toxoplasmosis in different clinical settings (adapted from Boyer & McLeod, 1996)

CLINICAL SETTING	TREATMENT	DURATION OF THERAPY	REFERENCES
Pregnant women with acute toxoplasmosis			Couvreur *et al.*, 1993
First 18 weeks of gestation or until term if fetus is not infected	Spiramycin	Until fetal infection confirmed or excluded at 18-20 weeks	Daffos *et al.*, 1988 Berrebi & Kobuch, 1994
Fetal infection confirmed after 17 weeks of gestation or maternal infection acquired in last few weeks of gestation	Pyrimethamine Sulphadiazine Folinic acid	Until term	
Congenital toxoplasmosis in infants	Pyrimethamine Sulphadiazine Folinic acid	1year	McAuley *et al.*, 1994 Swisher *et al.*, 1994
with CSF protein > 1g/dl or active retinochoroiditis threatening sight	Above with Corticosteroids*	Until resolution of active inflammation	
Ocular toxoplasmosis in older children and adults	Pyrimethamine Sulphadiazine Folinic acid	Until 1-2 weeks after resolution of signs and symptoms	Rothova *et al.*, 1993 Engstrom *et al.*, 1991
	Corticosteroids*	Until resolution of active inflammation	

Table 1.6 continued:

CLINICAL SETTING	TREATMENT	DURATION OF THERAPY	REFERENCES
Acute toxoplasmosis in immunologically normal children or adults			Roberts *et al.*, 1998
Lymphadenopathy	No therapy		
Significant organ damage	Pyrimethamine Sulphadiazine Folinic acid	Until 2 weeks after resolution of signs and symptoms	
Toxoplasmosis in immunocompromised children or adults			Behbahani *et al.*, 1995 Roberts *et al.*, 1998
Non-AIDS	Pyrimethamine Sulphadiazine Folinic acid[#]	Until 4 to 6 weeks after resolution of signs and symptoms	Wong & Remington, 1994
AIDS	Pyrimethamine Sulphadiazine Folinic acid[#]	Lifetime	

[*]The role of corticosteroids in these clinical situations remains controversial
[#]Corticosteroids are contraindicated in the immunocompromised host

1.6 Disease Prevention and Screening

Humans become infected either through ingestion of the parasite in raw or undercooked meat or from contact with cat faeces or contaminated soil (reviewed, Jackson & Hutchison, 1989). Avoidance of infection therefore involves ensuring that all meat is thoroughly cooked and that fruit and vegetables are washed to remove all traces of soil. In addition wearing gloves for gardening and cleaning cat litter trays may prevent infection. Outdoor sand pits should be covered to prevent cats defecating in them. Cat litter trays should be cleaned frequently to prevent oocysts sporulating. Certain occupations may also carry a risk of infection for example, butchers or meat handlers. In addition, farmers may become infected from sheep at lambing time.

Prenatal screening for toxoplasmosis has existed in France and Austria for many years (reviewed, Hall, 1992). The screening is based on determining, by antibody measurement, whether a pregnant woman has a current infection, latent infection, or has not yet been exposed to the parasite. Those with latent infection are immune and require no further screening. Susceptible women require repeated serological testing because of the lack of symptoms or the non-specific nature of symptoms associated with most *T.gondii* infections.

If acute infection occurs during the first or second trimester of pregnancy initial therapy involves administration of spiramycin to prevent transmission of infection to the fetus (Daffos *et al.*, 1988). At 18 weeks gestation amniocentesis may be performed to diagnose fetal infection, either by isolation of the parasite in amniotic fluid or by demonstration of the *T.gondii* B1 gene by the polymerase chain reaction. In France the sensitivity and specificity of amniotic fluid PCR has approached 100%

(Hohlfeld *et al.*, 1994). If the amniocentesis is positive or maternal infection occurs in the third trimester then the fetus should be treated by administration of sulphadiazine and pyrimethamine to the mother (Couvreur *et al.*, 1993). In addition to chemotherapy, repeated fetal ultrasound is performed to detect hydrocephalus (Berrebi *et al.*, 1994). If the fetus is severely affected termination of pregnancy may be recommended (Berrebi *et al.*, 1994).

Screening programmes have proven effective in countries such as France where 80% of women of childbearing age are already seropositive. However, they would be extremely costly in countries such as the United Kingdom where most of the population is still susceptible. Early postnatal treatment also can improve outcome from congenital toxoplasmosis (McAuley *et al.*, 1994). However, significant ocular pathology is already present at birth in 20% of otherwise asymptomatic infants (Guerina *et al.*, 1994). Techniques for measuring *T.gondii* specific IgM in the Guthrie blood spot have been described (Lebech & Petersen, 1992; Parker & Cubitt, 1992; Guerina *et al.*, 1994). However, the reliability of these techniques has not been confirmed.

1.7 Summary

T.gondii, a protozoan parasite can cause severe life threatening disease especially in newborns and immunosuppressed patients. It is an important cause of ocular disease and is considered the most common recognisable cause of posterior uveitis (McCannel *et al.*, 1996). Toxoplasmic retinochoroiditis is a progressive and recurring disease often resulting in loss of vision. Although the pathology of established ocular

toxoplasmosis is well described, the earlier lesions in the fetus have not been extensively studied and this will be addressed in this thesis.

There is no cure for *T.gondii* infection and current treatments are based on controlling disease reactivation. Acute acquired infection in the immunocompetent host does not usually require any treatment. However active treatment is required for congenital infection of the fetus or infant, infection in the immunosuppressed host and for recurrent ocular toxoplasmosis. A combination of pyrimethamine and sulphadiazine, both antifolate agents, is the most effective therapy currently available. There are significant side effects associated with both these drugs. Pyrimethamine may cause bone marrow toxicity. Allergy and intolerance associated with sulphadiazine is common. These drugs are active against the tachyzoite, the rapidly dividing form of *T.gondii*. The quiescent form of *T.gondii*, which resides in tissue cysts, is resistant to all known drugs. New antimicrobial agents and targets are therefore urgently needed.

CHAPTER 2
THE PARASITE, *TOXOPLASMA GONDII*

2.1 Life-Cycle

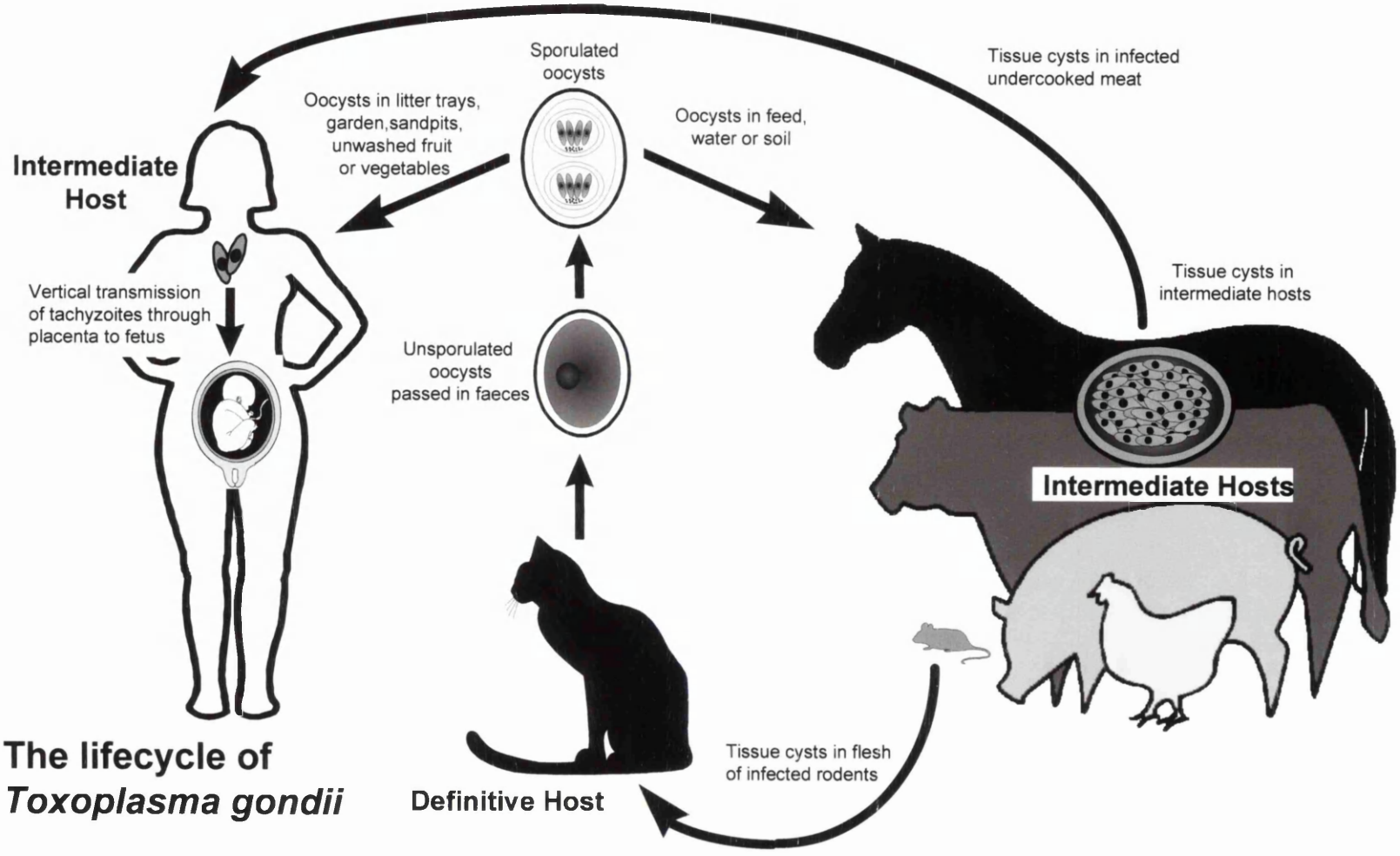
T. gondii is an obligate, intracellular, protozoan parasite. Its full taxonomic classification is phylum *Protozoa*, subphylum *Apicomplexa*, class *Sporozoasida* and order *Euccidiorida*. It undergoes a complicated life-cycle which includes both sexual and non-sexual reproduction with two distinct cycles: (i) The entero-epithelial cycle which only occurs in the small intestines of cats and other members of the *Felidae* (Miller *et al.*, 1972) and (ii) The extra-intestinal cycle which takes place in any warm blooded host (reviewed, Jackson & Hutchison, 1989) (Figure 2.1).

The entero-epithelial cycle

Cats shed oocysts (Figure 2.2A), the product of sexual reproduction, after ingesting any developmental stage of *T.gondii* (Dubey *et al.*, 1970). Most commonly they become infected after ingestion of tissue cysts (Figure 2.2B) from flesh of infected birds or rodents. These tissue cysts, which contain the bradyzoite stage, appear to promote the production of oocysts most effectively (Dubey *et al.*, 1970).

Infection tends to be concentrated in the ileum and occurs in cells at the tips of intestinal villi. Organisms can undergo sexual or asexual reproduction with both processes occurring at the same time (reviewed, Hutchison *et al.*, 1971). In sexual reproduction gametogeny gives rise to microgametes and macrogametes. In asexual reproduction repeated cycles of endopologeny, a modification of schizogony, give rise to merozoites. Merozoites are capable of sexual reproduction on re-invasion of a cell. Microgametes fertilise macrogametes giving rise to oocysts containing two sporocysts. The oocyst is released by rupture of the host cell and is shed from the

Figure 2.1 The life-cycle of *Toxoplasma gondii*
The sexual cycle is initiated in the ileum of the cat upon ingestion of tissue cysts, or occasionally oocysts. This results in release of oocysts in the faeces which are infective to intermediate hosts upon sporulation. After ingestion by the intermediate host these sporocysts transform to tachyzoites and undergo a brief period of rapid multiplication in the reticuloendothelial system, before forming tissue cysts containing many bradyzoites. These cysts are infective to both the intermediate and definitive host upon ingestion. Vertical transmission to the fetus via the placenta can occur in intermediate hosts.



The lifecycle of *Toxoplasma gondii*

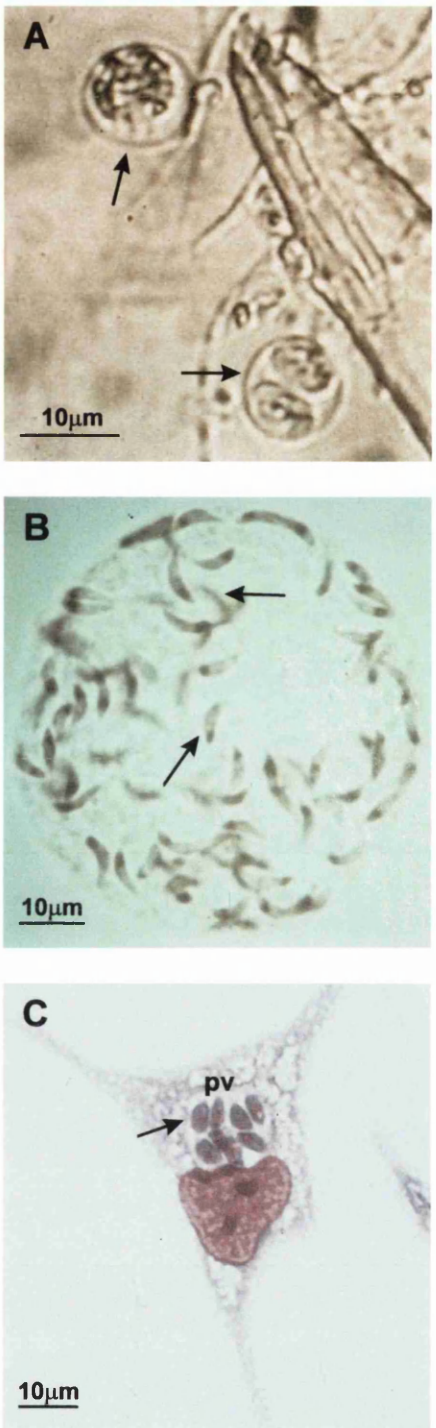


Figure 2.2 **A Oocysts (arrows) in cat faeces. B Bradyzoites (arrows) within a tissue cyst. C Tachyzoites (arrow) within a parasitophorous vacuole (pv) of host cell.**

intestine of the cat in the form of a non-infectious, unsporulated oocyst. During sporulation, each sporocyst gives rise to 8 sporozoites.

Excretion of oocysts may begin within 3 days and for up to 24 days after ingestion, depending on the developmental stage of *T.gondii* ingested by the cat (Dubey *et al.*, 1970). This time is shortest following infection with tissue cysts and longest following infection with oocysts. A cat sheds as many as 10 million oocysts per day, for up to 24 days after initial infection. Oocysts are then shed in lesser numbers for an additional 1 to 3 weeks (Dubey & Frenkel, 1972). Further oocyst shedding may occur if the cat is co-infected with the closely related coccidian parasite, *Isospora felis* (Chessum, 1972). Following excretion sporulation occurs over 2 to 21 days, depending on the ambient temperature. Oocysts can persist and remain infectious in warm moist soil for up to 18 months (Frenkel *et al.*, 1975).

The extra-intestinal cycle

This cycle occurs in any warm blooded host usually following ingestion of oocysts or tissue cysts (reviewed, Jackson & Hutchison, 1989). Tachyzoites (Figure 2.2C), the rapidly dividing stage of the parasite responsible for acute infection, are subsequently released and invade host cells. They replicate within host cells by endodyogeny approximately every 4-6 hours. Following 4 to 5 divisions they destroy the host cell and invade adjacent cells. The differentiation of tachyzoites to bradyzoites occurs around day 10 to 14 following infection and coinciding with the onset of protective immunity. Bradyzoites (Figure 2.2B) are located within tissue cysts. These tissue cysts appear to have a predilection for the central nervous system, skeletal and cardiac

muscle (Jacobs *et al.*, 1960; Remington & Cavanaugh, 1965), although they have been found in almost every tissue including the eye (Rao & Font, 1977) and visceral organs (Jackson & Hutchison, 1989) These tissue cysts may rupture during the lifetime of the host (Pavesio *et al.*, 1992) and released organisms can cause recrudescent infection in the congenitally infected or immunocompromised host.

Other forms of transmission

Congenital transmission of the parasite can occur during primary infection of the mother (reviewed, Jackson & Hutchison, 1989). Tachyzoites invade the placenta to infect the developing fetus. Infrequent forms of transmission include infection following organ transplantation (Israelski & Remington, 1993) or blood transfusion (Siegal *et al.*, 1971) and experimental laboratory accidents (Field *et al.*, 1972).

2.2 Parasite Virulence

All isolates of *Toxoplasma* whether originating from animals or humans have been grouped into a single species based on morphological grounds. Studies on strain diversity by restriction fragment length polymorphism (RFLP) analysis have shown that the population structure of *T.gondii* is essentially clonal (Sibley & Boothroyd, 1992). Despite the presence of a sexual stage in the life-cycle, sexual recombination occurs much less frequently than would be expected and most isolates appear to arise by mitotic expansion of a limited number of genotypes. For example RFLP analysis shows that there are over 1700 different possible combinations of alleles at six independent loci, yet only 15 combinations were identified in a study of over 100 independent strains (Howe & Sibley, 1995). This population clonality comprises three

predominant, clonal lineages which correspond with biological behaviour (Howe & Sibley, 1995). Type I strains account for a significant percentage of human congenital infection. They show the greatest pathogenicity to mice. Type II strains are most commonly associated with human disease in both congenitally infected and immunocompromised patients. They produce high cyst burdens and chronic encephalitis in mice. Type III strains are largely confined to animals.

Table 2.1 Virulence of *T.gondii* based on clonal lineages and zymodeme groups

GROUP*	STRAIN	DISEASE	VIRULENCE	ZYMODENE GROUP#
I	RH	Human, congenital	Kills all strains of mice	1
II	Me49, RRA	Human	Kills some mice, strain dependent	3,4
III	C56	Animals	Less virulent than Groups I and II	2

* Howe & Sibley, 1995; # Darde *et al.*, 1992

Iso-enzyme analysis also supports data suggesting a mainly clonal population for *T.gondii*. Darde *et al.*, (1992) were able to identify 4 main parasite groups in 35 *T.gondii* isolates based on polymorphisms in 6 parasite enzyme systems, known as zymodeme groups. These correspond with the 3 main groups described by (Howe & Sibley, 1995). Type I strains belong to zymodeme 1, type II to zymodeme 2 and 4 and type III to zymodeme 3 (Table 2.1). Two single isolates with different iso-enzyme profiles have also been described, zymodeme 5 (Darde *et al.*, 1992) and zymodeme 6 (Darde *et al.*, 1998).

Although minor differences have been noted in the biochemistry and therefore, potentially the metabolism of different clonal lineages, currently available drugs are effective against all strains of *T.gondii*.

2.3 *T.gondii* Cell Biology

T.gondii is an obligate intracellular parasite and to survive must invade the cells of the host. Invasion is a rapid process taking less than 10 seconds and is energy dependent (reviewed, Joiner & Dubremetz, 1993; Dubremetz, 1998). It can be envisaged as a complex cascade of events with each individual event triggering the next. These events include; target recognition and attachment; invasion; secretion; and formation of the parasitophorous vacuole. Once inside the cell the parasite resides and replicates within a specialised membrane, the parasitophorous vacuole. As the host develops immunity to the acute infection the tachyzoite converts to the bradyzoite form. Understanding these processes is pertinent for rational design of new therapies.

Attachment

In contrast to most other intracellular parasites, *T.gondii* can invade virtually all cells. This suggests that the receptors mediating attachment are ubiquitous or that a variety of different receptors can be utilised. Thus the ultimate goal of designing a therapeutic intervention aimed at blocking attachment will be difficult. Recent work has suggested that parasite-bound, host-cell laminin (Furtado *et al.*, 1992); lectin-like molecules (Robert *et al.*, 1991); and the tachyzoite major surface protein p30 (SAG-1) (Grimwood & Smith, 1992) participate in the attachment process. These studies are based on addition of antibody or peptide to parasites *in vitro*. However, mutant

parasites deficient in SAG-1 show reduced attachment, but can still invade host cells suggesting this is not the only mechanism for parasite attachment (Mineo & Kasper, 1994). Another surface protein SAG-3, expressed by both tachyzoites and bradyzoites, may also have a role in attachment. Mutant parasites in which the SAG-3 gene is disrupted show reduced attachment and invasion of host cells (Tomavo, 1996). SAG-3 shows some structural homology to SAG-1 suggesting a similar function for these proteins (Cesbron Delauw *et al.*, 1994).

Invasion

The morphological steps involved in *T.gondii* invasion are well described (reviewed, Werk, 1985). Following attachment of the tachyzoite, the conoid, positioned at the anterior end of the parasite protrudes forming an indentation in the host cell. A tight junction forms between parasite and host cell which moves posteriorly as the parasite invades. The parasite then shows a series of twisting and gliding movements until it comes to lie within the parasitophorous vacuole.

Invasion can be inhibited by addition of cytochalasin D, which disrupts actin filaments, to parasites in culture (Dobrowolski & Sibley, 1996). Furthermore, cytochalasin D resistant *T.gondii* which have a mutation in their actin gene can invade in the presence of cytochalasin D (Dobrowolski & Sibley, 1996). This confirms that action of cytochalasin D is on the parasite and not the host cell. Myosin also appears to have a role in host cell invasion. Addition of butanedione monoxime (a myosin ATPase inhibitor) to parasites in culture inhibits parasite gliding motility and invasion (Dobrowolski *et al*, 1997). Invasion is therefore an active process powered by the cytoskeleton of the parasite.

Secretion

T.gondii contains three specialised secretory organelles which have a role in invasion and intracellular survival; micronemes, rhoptries and dense granules. Secretion is a regulated, sequential process which begins with exocytosis of the micronemes (Carruthers & Sibley, 1997). Their contents, the MIC proteins, are discharged on initial contact with host cells but ultimately are excluded from the parasitophorous vacuole. Their role may be associated with the attachment process.

Following microneme exocytosis, secretion from the rhoptries begins coincident with the formation of the tight junction with the host cell (Carruthers & Sibley, 1997). The rhoptries are club-shaped apical organelles and are the source of penetration enhancing factor (Lycke & Norrby, 1966), now known as ROP-1. The rhoptries also contain a large amount of lipid, 75% of which is phosphotidylcholine, a major component of plasma membrane and cholesterol which is known to stabilise plasma membranes (Foussard *et al.*, 1991). These components may have a role in formation of the parasitophorous vacuole. Secretion from the rhoptries is completed by the time the parasite is fully enclosed in the parasitophorous vacuole.

Finally, secretion from the dense granules does not begin until several minutes after invasion and continues until around 20 minutes post-invasion (Carruthers & Sibley, 1997). The contents of the dense granules appear to be important for modifying the parasitophorous vacuole for intracellular survival (Cesbron Delauw, 1994; Cesbron Delauw *et al.*, 1996).

In theory, interfering with any of these processes may be a useful strategy for drug development. However, attachment, invasion and to a lesser extent secretion are rapid processes and may be difficult to target. The molecules and regulatory factors involved in attachment and secretion are as yet poorly understood. In addition, invasion utilises the actin cytoskeleton also present in human cells and drugs aimed at this process are unlikely to be parasite specific. However, although these processes are unlikely targets for new drugs, they may prove suitable candidates for development of protective vaccines.

The parasitophorous vacuole

The parasitophorous vacuole allows *T.gondii* to resist microbicidal activity and destruction (Joiner *et al.*, 1990; Jones *et al.*, 1972; Sibley *et al.*, 1985). The parasitophorous vacuole membrane (PVM) is a highly specialised membrane in which transmembrane proteins are reduced or absent and host cell surface markers are rapidly excluded (Jones *et al.*, 1972). Current evidence suggests that it is formed from host cell plasma membrane and pinches off via a fission pore (Suss Toby *et al.*, 1996).

The parasitophorous vacuole does not completely isolate the parasite from the host cell. Interactions between the parasite and the host are important for parasite survival. *T.gondii* is a purine auxotroph and purines needed for growth must therefore be salvaged from the host cell (Schwab *et al.*, 1995). It is likely that *T.gondii* also obtains small molecules such as amino acids, monosaccharides and lipids from the host cell. Interactions between the parasite and the host cell are assisted by small pores within the PVM which allow passage of small molecules (13-19kD) in either direction

between the host cell and the parasite (Schwab *et al.*, 1994). The presence of these small pores within the PVM also provides a means of facilitating drug delivery to this intracellular compartment.

In the infected cell, host mitochondria and endoplasmic reticulum are seen to surround the parasitophorous vacuole (Sinai *et al.*, 1997). The small pores in the PVM may therefore allow the parasite to utilise host cell molecules such as ATP either to meet its energy requirements or for purine synthesis. Recent studies have identified a novel nucleoside triphosphate hydrolase (NTPase) which could utilise ATP as a substrate (Sibley *et al.*, 1994). Constituting 3-4% of parasite protein, it exists in 2 isoforms and appears to have a role in parasite virulence (Asai *et al.*, 1995). These NTPases are located within the dense granule organelles and are secreted into the vacuolar space (Sibley *et al.*, 1994). The type II NTPase is present in all strains of the parasite however the more active type I isoform, is only present in the most virulent strains (Asai *et al.*, 1995). Expression of this highly active NTPase isoform may contribute to intracellular survival and virulence of *T.gondii*.

The vacuolar space and membrane are extensively modified by parasite proteins after *T.gondii* invasion (Sibley & Krahenbuhl, 1988). In addition there is formation of a network of membranous tubules within the lumen of the vacuole which connect with the vacuolar membrane (Sibley *et al.*, 1986). Most of these proteins are derived from the dense granule organelles and are designated GRA proteins. At least 7 GRA proteins are found in the dense granules. Following secretion they are all associated with the parasitophorous vacuole and are either rapidly redistributed on the tubular network or on the vacuolar membrane (reviewed, Cesbron Delauw, 1994).

No definite function has yet been assigned to proteins present within the parasitophorous vacuole space and PVM. GRA 4,5 and 6 show weak homology with extracellular matrix proteins so may have a structural or supportive role (Lecordier *et al.*, 1993; Lecordier *et al.*, 1995). GRA 1 is a calcium binding protein which may have a role in modulating the calcium concentration within the parasitophorous vacuole (Cesbron Delauw, 1994; Charif *et al.*, 1990). GRA 2 is secreted as both a soluble protein from the dense granules and with multilamellar vesicles released from a specialised posterior invagination of the parasite (Sibley *et al.*, 1995). These multilamellar vesicles assemble to form the intravacuolar network which contains an integral membrane form of GRA2 (Sibley *et al.*, 1995). Size polymorphism of the dense granule proteins GRA5 and GRA6 is correlated with parasite virulence (Rauscher *et al.*, 1996). This size variation may be related to different post-translational modifications. However, the role of GRA5 and GRA6 size polymorphism in virulence is unclear. GRA 7 has been recently described (Fischer *et al.*, 1998; Jacobs *et al.*, 1998). In tachyzoites it is associated with the parasitophorous vacuolar membrane. However, in bradyzoite infected cells, GRA 7 is present within the host cell cytoplasm. It has been suggested that this intracellular translocation of GRA 7 may be coupled with parasite stage conversion (Fischer *et al.*, 1998).

Tissue cyst formation

Since all currently available drugs treat only the tachyzoite stage of the parasite the interconversion of tachyzoites and bradyzoites is of considerable interest. The differentiation of tachyzoites to bradyzoites occurs, at around day 10-14 following infection, with the onset of protective immunity. While tachyzoites divide rapidly and synchronously until they lyse the host cell, the slower growing bradyzoites form tissue

cysts which are long lived (Pavesio *et al.*, 1992). These tissue cysts are considered to be responsible for reactivation of disease in the congenitally infected or immunocompromised patient.

Electron microscopic studies have shown that the cyst wall is an adaptation of the parasitophorous vacuole membrane (Ferguson & Hutchison, 1987). This membrane develops invaginations and is thickened on the vacuolar side with a homogeneous, osmiophilic material. In older cysts a number of parallel tubular structures run from the cyst wall to the bradyzoite. Bradyzoite division is infrequent and in older cysts many bradyzoites degenerate and fragment (Pavesio *et al.*, 1992). Ultrastructural studies of the bradyzoite show that the nucleus is more posteriorly situated, the rhoptries are more electron dense and micronemes are increased in number compared with tachyzoites (Ferguson & Hutchison, 1987; reviewed, Dubey *et al.*, 1998). In addition amylopectin granules develop in the cytoplasm.

Not surprisingly, as well as morphological differences there are differences in gene and antigen expression between bradyzoites and tachyzoites (Table 2.2). The main tachyzoite specific surface proteins SAG1 and SAG2 are down-regulated or absent from bradyzoites (Smith, 1995). Several bradyzoite stage-specific molecules have been described. These include surface antigens of 18kD, 21kD, 34kD and 36kD (Tomavo *et al.*, 1991) of which, only the 18kD protein, known as SAG 4, has been studied in detail (Odberg-Ferragut *et al.*, 1996). MAG1 is a 65kD protein which is secreted by the bradyzoite and appears to form part of the cyst matrix or cyst wall (Parmley *et al.*, 1994). Two other apparent cyst wall proteins of 116kD (Weiss *et al.*, 1992) and 29kD (Zhang & Smith, 1995) have been studied at the protein level. BAG1

Table 2.2 Differences in antigen expression between tachyzoites and bradyzoites

PRODUCT	PRESENT IN		TOTAL GENE SEQUENCED	PRODUCT SIZE	LOCATION	FUNCTION (POSTULATED)	REFERENCES
	T	B					
SAG1	Yes	No	Yes	30-35kD	Surface Vacuolar network	May facilitate attachment/invasion	Burg et al., 1988 Grimwood & Smith, 1992
SAG2	Yes	No	Yes	22kD	Surface	Unknown	Prince *et al.*, 1990
SAG3	Yes	Yes	Yes	43kD	Surface	May facilitate attachment/invasion	Cesbron Delauw *et al.*, 1994
p18	No	Yes	Yes	18kD	Surface	Unknown	Odbert-Ferragut *et al.*, 1996
p21	No	Yes	No	21kD	Surface	Unknown	Tomavo *et al.*, 1991
p34	No	Yes	No	34kD	Surface	Unknown	Tomavo *et al.*, 1991
p36	No	Yes	No	36kD	Surface	Unknown	Tomavo *et al.*, 1991
LDH1	Yes	No	Yes	33kD	Cytoplasm	Enzyme	Yang & Parmley, 1997
LDH2	No	Yes	Yes	35kD	Cytoplasm	Enzyme	Yang & Parmley, 1997
BAG1	No	Yes	Yes	28-30kD	Cytoplasm	Heat Shock Protein. stage conversion	Parmley *et al.*, 1995 Bohne *et al.*, 1995
MAG1	No	Yes	Yes	65kD	Cyst Matrix/Wall	Cyst matrix structure	Parmley *et al.*, 1994
p116	No	Yes	No	116kD	Cyst Matrix/Wall	Unknown	Weiss *et al.*, 1992
p29	No	Yes	No	29kD	Cyst Matrix/Wall	Unknown	Zhang & Smith, 1995

Key: T, Tachyzoites; B, Bradyzoites; LDH, Lactate dehydrogenase

is a 28-30 kD cytoplasmic protein, which has been shown to be related to a plant heat shock protein (Parmley *et al.*, 1995). The BAG1 gene has also been identified in a cDNA library prepared from pH-shocked tachyzoites (Bohne *et al.*, 1995) and it has been suggested that stress conditions may trigger expression of this gene and thus bradyzoite conversion. That said, BAG1 is not only expressed during conversion to bradyzoites, but also in mature cysts (Parmley *et al.*, 1995). Finally, there are two isoforms of a lactate dehydrogenase (LDH) enzyme. The genes encoding these enzymes, designated *LDH1* and *LDH2*, respectively, have been cloned and characterised (Yang & Parmley, 1997). While the mRNA of *LDH1* is detected in both the bradyzoite and tachyzoite stages, the mRNA of *LDH2* is only detected in the bradyzoite stage (Yang & Parmley, 1997). However, only one LDH isoform is expressed in each stage, LDH 1 in tachyzoites and LDH 2 in bradyzoites (Yang & Parmley, 1997). The functional activity of LDH has been found to be greater in bradyzoites than tachyzoites (Denton *et al.*, 1996). Furthermore, pyruvate kinase activity is higher in bradyzoites compared with tachyzoites (Denton *et al.*, 1996). These enzymes are required for lactate production. Lactate production does not require the mitochondrial electron transport chain. Based on this data, it has been suggested that bradyzoites lack a functional citric acid cycle and may rely upon glycolysis for energy production.

Much recent work has focused on factors involved in tachyzoite to bradyzoite transformation and bradyzoite reactivation. Factors which promote conversion of tachyzoites to bradyzoites appear to be related to the immune system or to nutritional and metabolic factors in the host environment which stress the parasite. Parasites grown *in vitro* convert from tachyzoites to bradyzoites following heat shock of

cultures (Soete *et al.*, 1994). A high or low pH environment also favours stage conversion (Soete *et al.*, 1994). For example, using a reporter gene construct, Yang & Parmley, (1997) demonstrated upregulation of LDH2 by growing parasites in tissue culture media with alkaline pH while LDH1 was down-regulated. Addition of IFNγ to infected macrophage cultures can also induce tachyzoites to express bradyzoite antigens (Bohne *et al.*, 1993). In addition, nitric oxide (NO) production appears to trigger stage conversion through its action on the electron transport chain (Bohne *et al.*, 1994). In support of this, drugs, such as atovaquone or arsenicals, that interfere with conventional mitochondrial electron transport can trigger bradyzoite formation *in vitro* (Soete *et al.*, 1994; Tomavo & Boothroyd, 1995). This may relate to the suggested increased reliance on glycolysis for energy production in the bradyzoite stage. Humoral immunity may also play a role in the encystment process, as addition of complement and specific antibody against *T.gondii* to infected cultures, stimulates formation of tissue cysts (Shimada *et al.*, 1974).

The rate and frequency of conversion to bradyzoites is also dependent on the strain of the parasite (Soete & Dubremetz, 1996). Expression of bradyzoite antigens *in vitro* is lower for rapidly growing strains such as RH when compared with slower growing strains. The reconversion of bradyzoites to tachyzoites has not been studied *in vitro*, although releasing the culture from any external stress usually leads to rapid resumption of tachyzoite multiplication (reviewed, Soete & Dubremetz, 1996).

In vivo murine studies have done little to confirm the factors that favour bradyzoite formation. However, neutralisation of IFNγ, TNFα or inhibition of NO production all allow reactivation of chronic disease resulting in active disease with tachyzoite

multiplication (Gazzinelli *et al.*, 1992a; Gazzinelli *et al.*, 1994; Hayashi *et al.*, 1996; Suzuki *et al.*, 1989).

The plastid organelle and plant-like properties of *T.gondii*

One of the most successful currently available drugs, pyrimethamine, is aimed at inhibiting a biochemical process which also occurs in host cells, the conversion of dihydrofolate to tetrahydrofolate. Although the host cell can be rescued by administering exogenous folinic acid, there is potential for drug toxicity. Ideally, new drugs would target pathways which differ between host and parasite.

It has recently emerged that *T.gondii*, in common with other Apicomplexan parasites, has three genomes (Wilson & Williamson, 1997). Animal, fungi and most protozoans have only two genomes, one within the nucleus and the other within mitochondria. *T.gondii* also has a nuclear and mitochondrial genome but, in addition, has a second extra-nuclear genome (Wilson & Williamson, 1997). The mitochondrial genome of *T.gondii* has yet to be isolated and sequenced. The second extra-nuclear genome of 35kB has recently been localised to the plastid organelle (McFadden *et al.*, 1996). Recognition of this third genetic compartment offers alternative approaches for drug design.

The plastid organelle was first identified in Apicomplexan parasites in the 1960s. At this time the nature of this organelle was not recognised and historically it has been given various names including Hohozylinder, Golgi Adjunct and Lamellarer Korper (Siddall, 1992). It is now known as the plastid organelle and its presence has been

confirmed in all major Apicomplexans including *Plasmodium, Theileria, Babesia, Eimeria, Hepatozoon, Sarcocystis* and *T.gondii* (McFadden *et al.*, 1997). The evolutionary origin of the plastid has been a subject of much controversy. Some electron microscopic studies have shown the presence of four membranes enclosing the plastid suggesting that it originated as a secondary endosymbiont, derived by ingestion of a eukaryote that itself contained a plastid (McFadden *et al.*, 1996; Wilson *et al.*, 1994). Consistent with this, work on the plasmodium 35kB genome has shown homology with a gene present in the plastid of red algae (Williamson *et al.*, 1994). More recently three different phylogenetic analytical methods strongly suggest a green algal origin for the plastid (Kohler *et al.*, 1997).

As yet no definitive function has been assigned to the plastid. However, the parasite faithfully replicates this organelle and the plastid genome is transcribed (Kohler *et al.*, 1997). It therefore appears to be essential for survival. Furthermore, recent work has demonstrated that the plastid is present in both tachyzoites (reviewed, McFadden *et al.*, 1996) and bradyzoites (Roberts *et al.*, 1998b) (Figure 2.3). Photosynthesis is the most familiar function of plastids in higher plants (where they are known as chloroplasts) and evidence for a chlorophyll binding protein in Apicomplexans has been reported (Hackstein *et al.*, 1995). Photosynthesis seems an unlikely function since the parasites spend most or all of their life-cycle in a dark environment. Furthermore, there are no genes encoding products known to be involved in photosynthesis within the 35kB genome of *Plasmodium* (Wilson *et al.*, 1996) or *T.gondii* (Kohler *et al.*, 1997). However, several non-photosynthetic eukaryotes maintain their plastids which have other key metabolic roles. For example, biosynthetic pathways for porphyrins are confined to the plastid of plants rather than

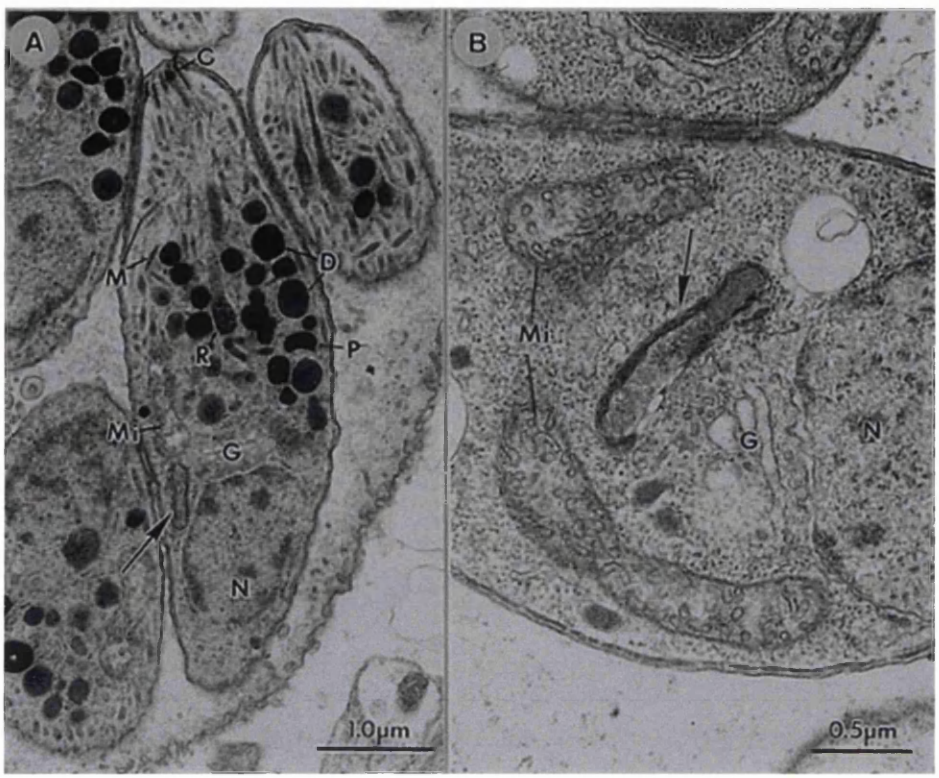


Figure 2.3 **Electron micrographs which demonstrate the apicomplexan plastid in bradyzoites, within tissue cyst of a mouse brain. The plastid is marked with an arrow (low power, A and high power, B). Other characteristic organelles are the conoid (C), dense granules (D), golgi (G), microneme (M) and mitochondrion (Mi), amylopectin granule (P). (Kindly provided by D.Ferguson, Oxford, UK)**

mitochondria as in animals (Howe & Smith, 1991; Kannagara *et al.*, 1988). Biosynthetic pathways for certain amino acids, ubiquinone, nucleotides, lipid and starch also occur in plant plastids (Hrazdina & Jensen, 1992; Wallsgrove, 1991).

Although the function of the Apicomplexan plastid is unknown, it has already been suggested as a target for antimicrobial agents, in particular macrolide antibiotics (Beckers *et al.*, 1995; Fichera *et al.*, 1995; Fichera & Roos, 1997). Earlier work has focused on determining the sequence and function of the Apicomplexan plastid genome with the intent that this would provide novel drug targets. Potential targets based on genes identified in the plastid include plastid ribosomes (Beckers *et al.*, 1995), rifampin-sensitive RNA polymerase (Wilson *et al.*, 1991) the chaperonin-like, a *psb*A homologue (Hackstein *et al.*, 1995) and the ORF 470 gene that has conserved homology with a rhodophyte plastid gene of unknown function (Williamson *et al.*, 1994). This approach of focusing on functions determined by the residual plastid genome may prove a fruitful approach for drug discovery. An alternative approach may be to consider that the presence of a plastid suggests that *T.gondii* utilises metabolic pathways more commonly associated with algae and higher plants. Indeed, in plants, enzymes encoded by nuclear genes are targeted to the plastid where they function (Haucke & Schatz, 1997b). Thus, the conservation of plastid genes in Apicomplexans implies that there may also be conservation of nuclear genes.

In support of this other 'plant'-related molecules have been described in Apicomplexans. For example, phylogenetic analysis of protozoan calmodulins suggests a distant relationship with those of plants (Robson *et al.*, 1993). 'Plant'-like properties of *T.gondii* microtubules also have been described (Stokkermans *et al.*,

1996). Furthermore, the enolase gene of *P.falciparum*, which encodes a metalloenzyme that catalyses one step in the conversion of glucose to lactic acid, contains a pentapeptide insertion, EWGWS (Read *et al.*, 1994). This has near identity to the EWGWC motif in the enolase molecules of higher plants. This pentapeptide motif is not present in any of the enolases sequenced thus far from a wide range of other eukaryotic and prokaryotic non-plant organisms. The recently cloned *T.gondii* bradyzoite gene, BAG-1 shows homology with a small heat shock protein of plants (Parmley *et al.*, 1995). The identification of these 'plant'-like properties in Apicomplexans presents a unique and potentially exploitable difference between host and parasite and could prove useful for development of new antimicrobial agents. Several herbicides, including dinitroaniline and triazines have already been shown to inhibit the growth of *T.gondii in vitro* at concentrations non-toxic to human cells (Hackstein *et al.*, 1995; Stokkermans *et al.*, 1996). Dinitroanilines are potent and specific inhibitors of plant microtubules. Triazines target the plant plastid D1 protein involved in electron transfer.

The shikimate pathway

The shikimate pathway, responsible for the production of aromatic amino acids, ubiquinone and PABA in plants, may also be present in Apicomplexans and provide another potential drug target. This pathway occurs in the plastid of plants (Bentley, 1990; Haslam, 1993). Previous studies which demonstrate the efficacy of anti-folates for the treatment of toxoplasmosis have implied that *T.gondii* has the enzymes necessary to synthesise folates from PABA (Kovacs *et al.*, 1989). However, the

biochemical events that lead to PABA acquisition by these parasites have not been characterised.

Animals and most protista (*e.g. Leishmania*) rely exclusively on exogenous folates and do not utilise PABA. In algae, plants, bacteria and fungi, PABA is synthesised *de novo* via the shikimate pathway which catalyses the seven step conversion of erythrose 4-phosphate and phosphoenol pyruvate to chorismate (Bentley, 1990; Haslam, 1993) (Figure 2.4). Chorismate is than used as a substrate for the synthesis of aromatic compounds including PABA, ubiquinone and the aromatic amino acids. In plants all seven enzymes are encoded in the nucleus made in the cytoplasm and targeted to the chloroplasts where they are functional (Gorlach *et al.*, 1993; Haucke & Schatz, 1997a). Under certain circumstances this pathway can occur in the cytoplasm of higher plants (Bentley, 1990).

The above evidence would suggest that it is entirely possible that *T.gondii* has the enzymes required for PABA synthesis and this will be investigated in this thesis. Furthermore, the shikimate pathway has proved to be an excellent target for herbicides as demonstrated by the enormous commercial success of glyphosate which is a potent and specific inhibitor of EPSP synthase, the sixth enzyme in the pathway (Steinrucken & Amrhein, 1980). Therefore, if *T.gondii* has the enzymes of the shikimate pathway this may prove an ideal target for novel antiparasitic agents.

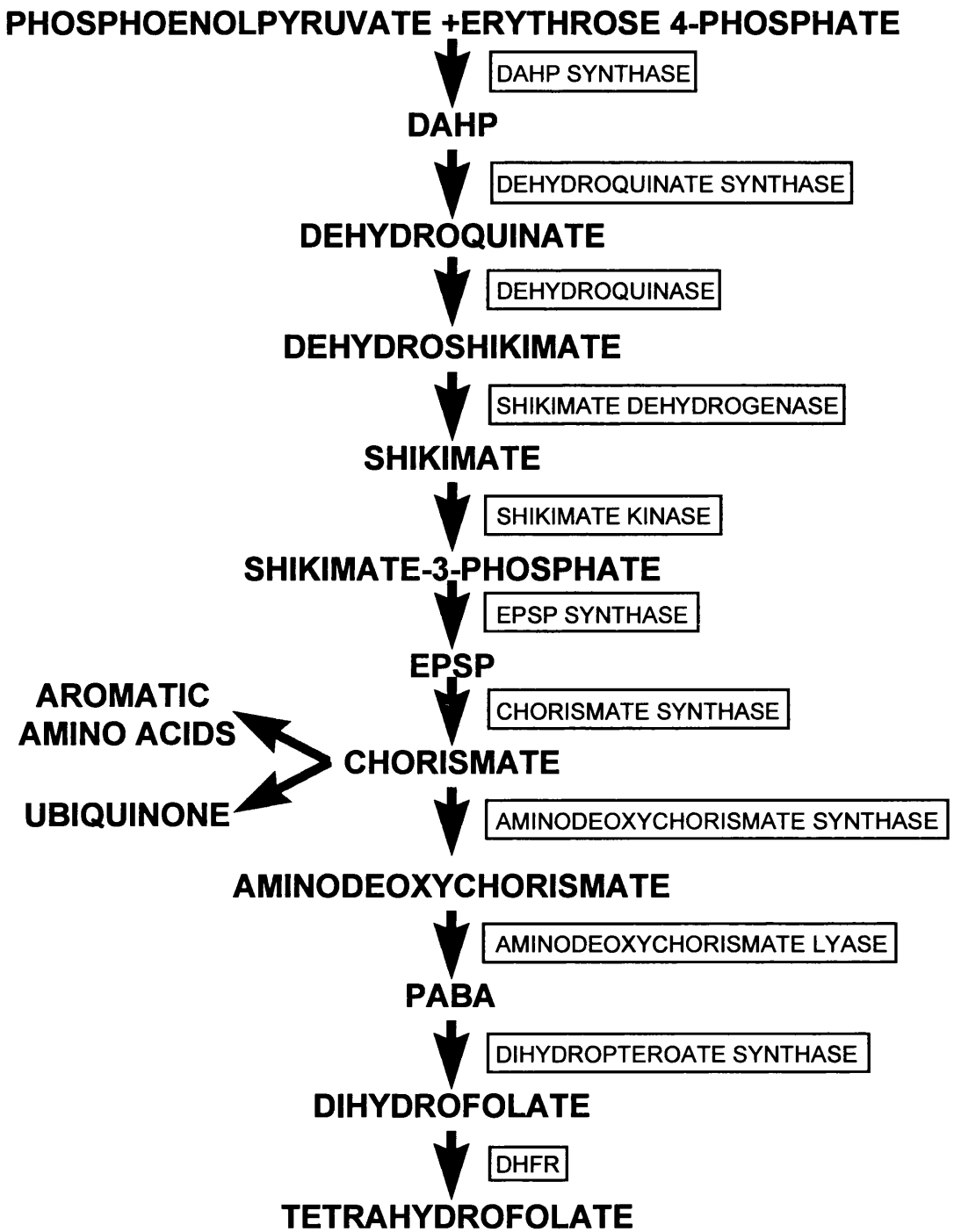


Figure 2.4 **The shikimate pathway which catalyses the seven step conversion of erythrose 4-phosphate and phosphoenol pyruvate to chorismate. Chorismate is then used as a substrate for the synthesis of aromatic compounds including PABA, ubiquinone and the aromatic amino acids. PABA is then used for the synthesis of folate.**

The alternative pathway of respiration

Another plant metabolic pathway worthy of consideration is the alternative pathway of respiration. All higher plants examined to date contain two pathways for mitochondrial electron flow: the cytochrome respiratory pathway, which is common to all organisms, and the alternative respiratory pathway which is absent from all mammals (reviewed, McIntosh, 1994). The alternative pathway of respiration has also been identified in other types of organisms: in protista such as *Trypanosoma* (Chaudhuri *et al.*, 1995), in certain fungi and in the green algae *Chlamydomonas* (Moore & Siedow, 1991).

The enzyme complexes for both pathways are present on the inner mitochondrial membrane (Moore & Siedow, 1991). Conventional mitochondrial respiration via the cytochrome pathway is coupled to ATP synthesis (Figure 2.5). Electrons are transferred through complexes I or II to ubiquinone and then on to the cytochrome oxidases, complexes III and IV. At each of three sites protons are pumped across the inner membrane creating a transmembrane potential for energy generation (reviewed (Moore & Siedow, 1991). The alternative pathway diverges from the cytochrome pathway after the ubiquinone complex. Electrons flowing through the alternative pathway are donated directly to oxygen to form water, apparently in a single, four electron step. Electron flow via this pathway does not contribute to transmembrane potential and two of the three potential coupling sites for proton transport and thus ATP production are lost (reviewed, McIntosh, 1994). Since the energy of electron flow through the alternative pathway is not conserved as chemical energy, it is lost as heat (Meeuse, 1975).

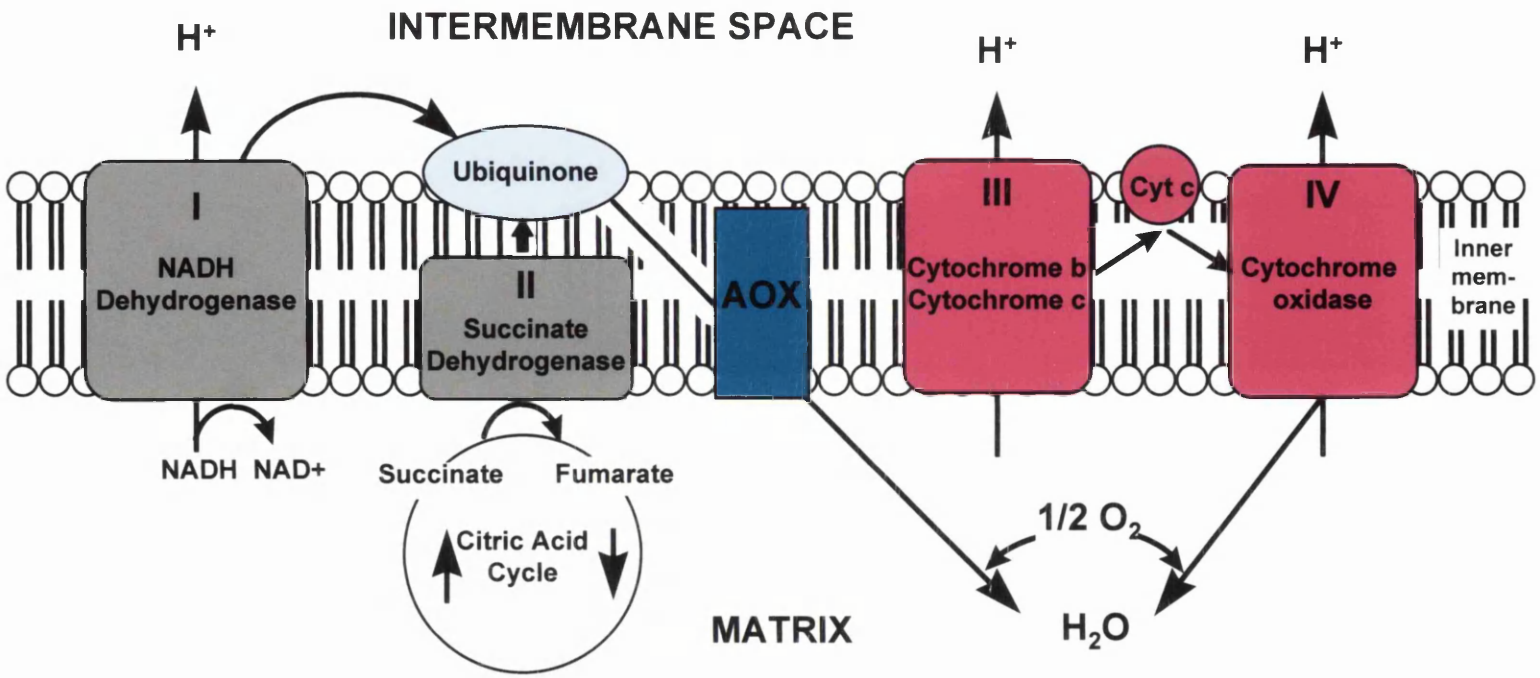


Figure 2.5 The respiratory chain on the inner mitochondrial membrane of plants. In conventional mitochondrial respiration electrons are transferred through complexes I or II to ubiquinone and then on the cytochrome oxidases, complexes III and IV. At each of three sites protons (H^+) are pumped across the inner membrane creating a transmembrane potential for energy generation. Alternative pathway respiration diverges from the cytochrome pathway after the ubiquinone complex. Electrons flowing through the alternative pathway are donated directly to oxygen to form water. Electron flow via this pathway does not contribute to transmembrane potential and two of the three potential coupling sites for proton transport and thus ATP production are lost.

The functional significance of the alternative pathway is not clear. The only confirmed function for alternative pathway respiration is to supply heat in thermogenic blooms of the Araceae, such as *Sauromatum guttatum*, the Voodoo lily (Meeuse, 1975). During flowering the production of heat vaporises odours to attract insect pollinators. In *Trypanosoma* the alternative oxidase is expressed only in bloodstream trypomastigotes which are dependent on glycolysis for ATP production (Chaudhuri *et al.*, 1995).

Relapse of toxoplasmic encephalitis is common in AIDS patients receiving atovaquone (Durand *et al.*, 1995), which inhibits conventional electron transport after ubiquinone. This implies that *T.gondii* must have some mechanism of circumventing the action of this drug. It has been suggested that bradyzoites rely upon glycolysis for energy production (Denton *et al.*, 1996). In addition, *T.gondii* has the enzymes necessary to utilises host cell ATP and therefore may have a reduced requirement to synthesise ATP (Asai *et al.*, 1995; Sibley *et al.*, 1994). Furthermore there is a reduction in the mitochondrial membrane potential of *T.gondii* after invasion of host cells (Tanabe & Murakami, 1984). The existence of this pathway in *T.gondii* will also be investigated.

2.4 Summary

T.gondii undergoes a complex life-cycle which involves an entero-epithelial cycle in the definitive host, the cat, and an extra-intestinal cycle occurring in all other hosts, including man. An understanding of events which occur during infection at a cellular and molecular level is important to increase our knowledge of the disease process and

to identify potential aspects which could be targeted to treat this disease. In particular the process of cyst breakdown represents the cause of disease reactivation and the reason for failure of currently available antiparasitic agents.

The discovery of the plastid organelle and other 'plant'-like properties in *T.gondii* as well as other Apicomplexan parasites presents a truly novel opportunity for identifying targets for antiparasitic agents. This thesis will investigate the shikimate pathway and the alternative pathway of respiration in *T.gondii*.

CHAPTER 3
THE HOST

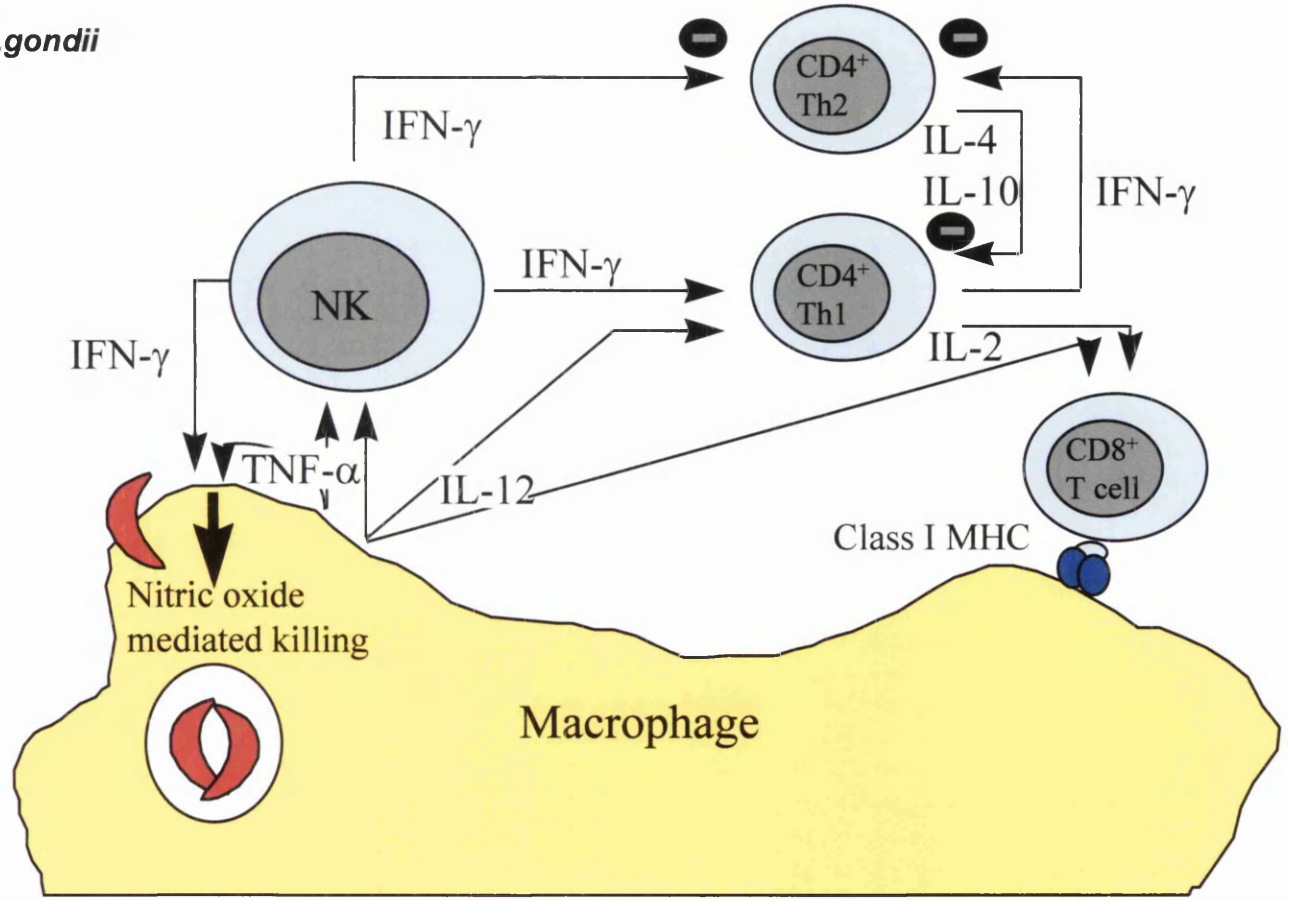
3.1 Immune Response

T.gondii induces a potent immune response that involves macrophages, natural killer (NK) cells, T cells and their monokine and cytokine products. In the immunocompetent host this immune response controls parasite replication and facilitates cyst formation. Different aspects of the immune response to *T.gondii* are illustrated in Figure 3.1 and described below.

Macrophages and natural killer cells

In common with a number of intracellular pathogens, including *Listeria monocytogenes* (Tripp *et al.*, 1993) and *Leishmania major* (Reiner *et al.*, 1994), *T.gondii* can directly stimulate macrophages to produce the monokines, interleukin-12 (IL-12) and tumour necrosis factor- α (TNF-α) (Gazzinelli *et al.*, 1993). Following macrophage activation these monokines, act in synergy to induce NK cells to produce interferon- γ (IFN-γ) (Gazzinelli *et al.*, 1993). IFN–γ can then stimulate the microbicidal activity of macrophages. The effector mechanisms for macrophage killing of *T.gondii* include; production of reactive oxygen intermediates (reviewed, Hughes, 1988) and reactive nitrogen intermediates (Adams *et al.*, 1990); products of arachidonic acid metabolism via the 5-lipoxygenase pathway (Yong *et al.*, 1994); and degradation of intracellular tryptophan, an essential amino acid for *T. gondii*, by induction of indoleamine 2,3-dioxygenase, (Murray *et al.*, 1989). By this mechanism macrophages and NK cells are considered to act as the first line of defence in resistance to *T.gondii* infection before the development of T cell responses (Khan *et al.*, 1994; Sher *et al.*, 1993).

Figure 3.1 The immune response to *T.gondii*



Macrophages are sensitised to tachyzoites and produce TNF-α and IL-12. These cytokines induce NK cells to produce IFN-γ. IFN-γ produced by NK cells can (i) stimulate macrophages to kill *T.gondii* by production of NO and (ii) induce preferential expansion of $CD4^+$ Th1 cells. $CD4^+$ Th1 cells also produce IFN-γ and in addition IL-2. IL-2 in conjunction with IL-12 can induce expansion of $CD8^+$ T cells. These $CD8^+$ T cells show Class I MHC restricted cytotoxicity for *T.gondii* infected cells. $CD4^+$ Th2 cells produce IL-4 and IL-10 which down regulate the effects of Th1 cells and thus promote parasite multiplication. However, they may also be important in down modulating a harmful pro-inflammatory immune response. These T cell subsets are therefore able to cross regulate each others activity. (Re-drawn from Roberts *et al.*, 1996)

T cells

Resistance to *T.gondii* depends on CD4+ and CD8+ T lymphocytes (Gazzinelli *et al.*, 1992a; Parker *et al.*, 1991). Both T helper 1 (Th1) and T helper 2 (Th2), CD4+ T cell subsets, originally defined by (Mosmann *et al.*, 1986) based on their production of cytokines, contribute to immunity against *T.gondii*. Th1 cells produce the pro-inflammatory cytokines, IFN-γ and IL-2 and are often associated with immunity against intracellular pathogens (Reiner & Locksley, 1993). The macrophage activating abilities of IFN-γ have already been described. IL-2 may facilitate expansion of CD8+ T cells (Gazzinelli *et al.*, 1992a). These can be cytotoxic for target cells infected with *T.gondii* (Hakim *et al.*, 1991). Th2 cells produce IL-4, IL-5 and IL-10 and are associated with down regulation of pro-inflammatory Th1 cell cytokines. (Mosmann, 1991; Mosmann & Moore, 1991). Th2 cytokines may down regulate the effects of Th1 cells and thus promote parasite multiplication. However, they are also essential in down modulating a harmful pro-inflammatory immune response during the early stages of toxoplasmosis. For example, mice deficient in IL-4 (Roberts *et al.*, 1996a) or IL-10 (Gazzinelli *et al.*, 1996) show significantly increased mortality during the first 15 days of infection compared with wild-type control mice of the same strains. The increase in mortality in these mice was accompanied by increased production of IFN-γ in stimulated splenocyte cultures. Thus, T cell subsets are able to cross regulate each others activity (Reiner & Locksley, 1993; Romagnani, 1992).

The events which lead to a predominant Th1 or Th2 response are uncertain although initial events in the development of the immune response appear to play a critical role (Reiner & Locksley, 1993; Romagnani, 1992). For example, the production of IFN-γ by NK cells, and IL-12 by macrophages may be instrumental in preferentially inducing

a Th1 CD4+ T cell response. Local immune responses may also differ from the systemic immune response. *In vitro* studies have shown that cells from the mesenteric lymph nodes of mice infected orally with *T.gondii* produce predominantly Th2 cytokine (Chardes *et al.*, 1993). In contrast splenocytes from these mice produce Th1 cytokines (Chardes *et al.*, 1993). The role of different cytokines in resistance to *T.gondii* infection is summarised in Table 3.1

The protective activity of CD8+ T cells is in part mediated through production of cytokines such as IFN-γ (Suzuki & Remington, 1990). In addition a direct cytotoxic effect on extracellular parasites has been reported (Khan *et al.*, 1991; Khan *et al.*, 1988). CD8+ T cells also show Class I MHC-restricted cytotoxicity for *T.gondii* infected cells (Hakim *et al.*, 1991; Subauste *et al.*, 1991; Yano *et al.*, 1989).

Humoral immunity

As an obligate intracellular parasite, innate and T cell mediated immunity is the host's major defence against *T.gondii* infections. Nonetheless, infection with *T.gondii* stimulates production of IgG, IgM, IgA and IgE antibodies (Roberts *et al.*, 1998a). *In vitro*, extracellular tachyzoites coated with antibody and complement can be lysed via the classical complement pathway (Schreiber & Feldman, 1980); or phagocytosed by macrophages (Anderson *et al.*, 1976). *In vivo*, passive transfer of immune serum will protect mice only against less virulent strains of *T.gondii* (Pavia, 1986). However while antibodies may not have a significant role in systemic immunity, they may play a protective role by interfering with the initial interaction of the parasite with the host cell. For example, human secretory IgA, derived from infected mother's breast milk

Table 3.1 The source and actions of cytokines important in *T.gondii* infection

CYTOKINES	MAJOR SOURCES	ACTIONS IN *T.GONDII* INFECTION	EFFECT ON MURINE TOXOPLASMOSIS ACUTE	CHRONIC	REFERENCES
IFN-γ	$CD4^+$ T cells $CD8^+$ T cells NK cells	Inhibits parasite growth by:- (i) Induction of ROI and RNI by macrophages (ii) Eicosinoid production via 5-lipoxygenase pathway in macrophages (iii) Induction of IDO and tryptophan starvation by various cell types	Protective Exogenous IFN-γ prolongs survival in infected mice	Protective Neutralisation of IFN-γ increases cyst rupture and severity of encephalitis in infected mice	Hunter *et al.*, 1994 Subauste, 1991 Yong *et al.*, 1994
TNF-α	Macrophages	Acts in synergy with IL-12 to induce NK cell production of IFN-γ	Protective Neutralisation of TNF-α increases mortality in infected mice	Protective Neutralisation of TNF-α increases cyst rupture and severity of encephalitis in infected mice	Hunter *et al.*, 1994 Gazzinelli *et al.*, 1993
IL-12	Macrophages	Acts in synergy with TNF-α to induce IFN-γ production by NK cells and $CD4^+$ and $CD8^+$ T cells	Protective Exogenous IL-12 prolongs survival in infected mice	Not required Neutralisation of IL-12 has no effect on survival or cyst numbers	Hunter *et al.*, 1994 Gazzinelli *et al.*, 1994 Gazzinelli *et al.*, 1993

Table 3.1 continued:

CYTOKINES	MAJOR SOURCES	ACTIONS IN *T.GONDII* INFECTION	EFFECT ON MURINE TOXOPLASMOSIS ACUTE	CHRONIC	REFERENCES
IL-2	$CD4^+$ T cells	Increased production of IFN-γ by NK cells	Protective	Protective	Hunter *et al.*, 1994
		Enhanced cytotoxicity of $CD8^+$ T cells	Exogenous IL-2 prolongs survival in infected mice	Exogenous IL-2 decreases cyst numbers in brain	Sharma *et al.*, 1985
IL-4	$CD4^+$ Tcells	Down regulation of Th 1 associated, pro-inflammatory cytokines	Protective	Detrimental	Roberts *et al.*, 1996
			Increased mortality in infected IL-4 gene knock out mice compared with wild type mice	Increased cyst numbers and severity of encephalitis in infected IL-4 gene knock out mice compared with wild type mice	
IL-10	$CD4^+$ T cells	Inhibits IL-12 synthesis by macrophages. Down regulation of Th 1 associated, pro-inflammatory cytokines	Protective	Not known	Gazzinelli *et al.*, 1996
			Increased mortality in infected IL-10 gene knock out mice compared with wild type mice		Gazzinelli *et al.*, 1992
TGF-β	Macrophages	Antagonises the actions of IFN-γ	Unclear	Not known	Hunter *et al.*, 1995
			Neutralisation of TGF-β delayed time to death. Exogenous TGF-β led to earlier death in SCID mice		

reduces *in vitro* infectivity of tachyzoites for murine enterocytes (Mack & McLeod, 1992).

Immunity and the eye

The immune response to *T.gondii* infection in the eye is less well studied. The eye is an immunologically privileged site, and as such the local immune response is suppressed to prevent tissue destruction. Immune responses initiated in the eye tend to be dominated by $CD8^+$ T cells and frequently a $CD4^+$ Th2 component (reviewed, Streilein, 1997; Streilein *et al.*, 1997). Induction of this deviant immune response depends on multiple factors. For example there is constitutive intra-ocular expression of Fas Ligand (Fas-L). This may promote deletion of activated T cells in the eye by apoptosis following interaction of Fas molecules on the infiltrating cells with Fas-L expressed on parenchymal cells within the eye (Griffith *et al.*, 1995). This mechanism of killing is specific and does not produce damage to adjacent tissues. There is expression of complement inhibitors, CD59, MCP and DAF on cells that surround the anterior chamber (Bora *et al.*, 1993). Reduced expression of MHC class I and II on parenchymal cells also occurs (reviewed, Streilein, 1995). In addition, even under normal circumstances intra-ocular fluid has been shown to contain cytokines such as transforming growth factor- β (TGF-β) (Cousins *et al.*, 1991; Granstein *et al.*, 1990). Other mediators including α-melanocyte stimulating hormone, vasoactive intestinal peptide, calcitonin gene-related peptide, free cortisol and IL-1 receptor antagonist have also been identified (reviewed, Streilein *et al.*, 1997). All of these compounds have immunosuppressive properties.

These factors which favour immunological unresponsiveness may render the eye vulnerable to certain pathogens especially viruses or parasites. Furthermore, *T.gondii* may promote factors which result in abrogation of immune privilege. For example, IFN-γ, which plays a critical role in resistance to *T.gondii* infection, antagonises the immunosuppressive functions of TGF-β (Hunter *et al.*, 1995a). Indeed, in spite of conditions in the eye that would tend to prevent immunity to *T. gondii*, immunological factors, similar to those which protect against disease in other tissues, appear to protect against murine eye disease. Using a murine model Gazzinelli *et al.*, (1994) found that treatment of chronically infected mice with monoclonal antibodies against IFN-γ, TNF-α, or CD4+ and CD8+ lymphocytes exacerbated ocular lesions and resulted in increased numbers of parasites in the retina. Consistent with a protective role for IFN-γ and TNF-α, inhibition of nitric oxide production in chronically infected mice also results in increased severity of ocular inflammation (Roberts *et al.*, 1998c).

In the eye, disease recurrences are often associated with reduced immunity either locally or systemically. Immunosuppressive therapy may lead to disease reactivation although in patients receiving longterm corticosteroid therapy this is uncommon (Nicholson & Wolchok, 1976). Animal models have provided conflicting data regarding the role of immunosuppression as an initiating factor for toxoplasmic retinochoroiditis. Recurrent retinochoroiditis can be induced in previously infected rabbits following administration of antilymphocyte serum (Nozik & O. Connor, 1970) or in hamsters following total body irradiation or treatment with steroids (Frenkel, 1957). However, Holland *et al.*, (1988b) were unable to induce recurrent eye lesions

in monkeys with healed retinochoroidal lesions by immunosuppression with total body irradiation.

Immunity and the fetus

The fetus and neonate are unduly susceptible to infections with intracellular pathogens such as *T.gondii*. Immaturity of the immune response may contribute to this increased susceptibility. The most severely affected infants acquire *T.gondii* infection during the first trimester. At this time, the numbers of T cells and their repertoire for antigen recognition are limited compared with the fetus in the latter half of gestation (George & Schroeder, 1992).

Although NK cells appear early during gestation and are present in normal numbers by mid to late gestation the phenotype of over half these cells is immature, lacking the CD56 antigen (Phillips *et al.*, 1992). These immature cells have decreased cytotoxic activity compared with adult cells (Phillips *et al.*, 1992; Sancho *et al.*, 1991).

Neonatal monocytes show slower migration to sites of infection (Bullock *et al.*, 1969). In addition both the generation of cytokines and macrophage response to cytokines are deficient in the newborn (Wilson & Haas, 1984). Deficient production of certain cytokines by lymphocytes may be a critical factor in congenital toxoplasmosis. In the normal neonate, while IL-2 is produced in sufficient amounts the production of other cytokines, in particular IFN-γ and IL-4, is markedly reduced compared with adult T cells (Lewis *et al.*, 1991). Since adult virgin T cells also have a reduced capacity to produce these cytokines, the apparent deficiency of neonatal T

cells may reflect their antigenically naïve status rather than an intrinsic immaturity of function. Indeed, studies have shown that neonatal T cells, activated and cultured *in vitro*, can acquire a memory T cell surface phenotype and the capacity to produce cytokines such as IL-4 and IFN-γ (Ehlers & Smith, 1991).

Infants with congenital toxoplasmosis have absent or diminished lymphocyte blastogenic responses to *T.gondii* antigen and production of IL-2 and IFN-γ is reduced (McLeod *et al.*, 1990). Their lymphocytes are, however, capable of responding normally to other stimuli. This anergy to *T.gondii* may reflect differences in the route of acquisition, cytokine environment or co-stimulatory molecules present during gestation. Impairment in the development of antigen-specific T cell responses has also been observed in infants with congenital infection due to CMV (Hayward *et al.*, 1984) and rubella (Buimovici-Klein *et al.*, 1979).

3.2 Genetics

In mice, resistance to toxoplasmic encephalitis and development of large numbers of cysts is controlled by the MHC, Class I, L^d gene (Brown *et al.*, 1995). In contrast mice which lack the L^d gene are susceptible to large cyst burdens and encephalitis (Brown *et al.*, 1995; Brown & McLeod, 1990). Other genes with a more minor influence on parasite burden include *Nramp* and Class II MHC (reviewed, McLeod *et al.*, 1996). Survival of mice following oral *T.gondii* infection is a polygenic trait controlled by at least five genes (McLeod *et al.*, 1989). However, survival following intraperitoneal infection is controlled largely by the H-2 histocompatibility complex (Williams *et al.*, 1978).

Until recently there was only circumstantial evidence that host genetic factors played a role in resistance to human toxoplasmosis. For example there are significant ethnic variations in incidence and severity of disease (McAuley *et al.*, 1994). In addition, clinical studies of twins show that monozygotic twins have a similar incidence and severity of disease which is not seen in dizygotic twins (Couvreur *et al.*, 1976). There is now strong evidence that host genetic factors, at least in part, determine disease severity in humans. In humans, susceptibility to toxoplasmic encephalitis in patients with AIDS is associated with the Class II DQ3 allele. Suzuki *et al.*, (1996) found that the HLA DQ3 allele was present in 85% of AIDS patients with toxoplasmic encephalitis. This allele was present in 40% of AIDS patients who did not develop toxoplasmic encephalitis and 51.8% of the general population. There is also an association of the DQ3 allele in patients with severe congenital infection and hydrocephalus. Mack *et al.*, (1996) found that the HLA DQ3 allele was present in 79% of congenitally infected infants with hydrocephalus. The frequency of this allele in infected infants without hydrocephalus and the general population was 45% and 49% respectively. Furthermore this MHC Class II gene has been shown to increase susceptibility to *T.gondii* in a murine model. Class II knock-out mice with the human transgene DQ3 have an increased parasite burden and more necrotic lesions in their brains, when compared with similar mice with the human transgene DQ1. One study which examined a very small number of cases of ocular toxoplasmosis found no definite MHC association with disease (Nussenblatt *et al.*, 1989). However, a more recent study suggested an association with HLA-Bw62 and severe ocular disease (Meenken *et al.*, 1995).

3.3 Summary

The clinical disease produced by *T.gondii* is a balance between pathogen and host related factors. For example, the more virulent strains of *T.gondii* may cause more severe disease. However, the genetic susceptibility of the host and the ability to mount an immune response also affect the severity of disease. In humans the Class II DQ3 allele appears to be associated with increased severity of congenital infection and in increased susceptibility to AIDS related toxoplasmic encephalitis (Mack *et al.*, 1996; Suzuki *et al.*, 1996). In addition, the immune response controls parasite replication and facilitates cyst formation in the immunocompetent host. However, lack of adequate macrophage, NK cell and T cell immune responses renders the immature fetus and immunosuppressed AIDS patient particularly vulnerable to this disease. Furthermore, certain sites such as the eye have a specialised immune response which may be less effective at controlling this parasite.

Congenital toxoplasmosis causes considerable suffering in afflicted individuals and their families. In addition there is also a major expenditure for health care. In 1975 it was estimated that the lifetime cost for special care of each child with congenital toxoplasmosis was $67 000 (approximately £45 000) (Wilson & Remington, 1980). The cost in 1998 would be considerably greater. Toxoplasmosis was one of the sentinel opportunistic infections observed early in the AIDS epidemic and it remains one of the most frequent and treatable opportunistic infections of the central nervous system in AIDS. These facts alone make this a disease worthy of study.

This thesis is written in two parts. The purpose of the first part is to provide comprehensive histological documentation of the earliest stages of ocular

toxoplasmosis in the fetus and infant. The second part of this thesis investigates biochemical and potentially pharmacological aspects of this disease by describing new pathways in *T.gondii* which may provide potential drug targets.

CHAPTER 4
MORPHOLOGICAL FEATURES OF OCULAR TOXOPLASMOSIS IN THE FETUS

4.1 Specific Objectives

1. To characterise the histopathological features and in addition the nature of the infiltrating inflammatory cells in eyes from fetuses and infants with congenital toxoplasmosis.

2. To compare the histopathological findings with those already described and to correlate them with the recognised clinical signs and symptoms.

4.2 Background

The ocular lesions of congenital toxoplasmosis in infants, children and adults have been well described. The earlier stages of the ocular lesions in the fetus have not been extensively studied. However, much recent work has concentrated on *in utero* or early, postnatal treatment of congenital toxoplasmosis (Berrebi & Kobuch, 1994a; Couvreur *et al.*, 1993; Daffos *et al.*, 1988). Documentation of the ocular lesions in fetuses from pregnancies terminated for congenital toxoplasmosis and early neonatal deaths could provide a baseline for more accurate assessment of the early treatment of this disease. Furthermore insight into the pathogenesis of *T.gondii* infection may be obtained by characterising the early stages of this disease.

The human fetal eyes used in this study have been obtained through multinational collaboration and comprise the largest collection of such tissues. There is therefore great potential for this study to contribute to our understanding of the early stages of this disease.

4.3 Materials and Methods

Eyes

A total of 20 eyes from 10 fetuses, 2 infants and a 2 year old child were available for study. The case histories for the fetuses, infants and child are summarised in Table 4.1. Fetal and infant post mortem findings, where available, are summarised in Table 4.2.

Fetal eyes

Fifteen eyes were collected post mortem from 10 fetuses with congenital toxoplasmosis. These eyes came from France where serological screening for *T.gondii* infection has been carried out since 1978. Maternal infections were confirmed by seroconversion in both the Sabin-Feldman dye test and the IgM-immunosorbent agglutination assay. The Sabin-Feldman dye test detects *T.gondii* specific IgG and may be positive in acute or chronic infection. In contrast, the IgM-immunosorbent agglutination assay is positive during acute infection and is usually negative or at low titre in chronic infection.

Maternal seroconversion occurred at an average gestation of 12.9 weeks (Range 10 to 18.5 weeks). Therefore, the average maximum possible length of infection prior to termination of pregnancy was 11.5 weeks (range 6 to 18 weeks).

The fetuses were aborted between 19 and 32 weeks gestation following a positive diagnosis of toxoplasmosis by polymerase chain reaction (PCR) which demonstrated the presence of the *T.gondii B1* gene in amniotic fluid. Prior to termination of pregnancy, additional evidence of severe *T.gondii* infection, as demonstrated by

intracerebral ventricular dilatation at ultrasound, was obtained in five cases. Following abortion, in seven cases, necropsy and/or subinoculation of placental tissue, amniotic fluid or cerebral tissue into mice was carried out to confirm the diagnosis. For congenital toxoplasmosis, isolation of the organism from the placenta or from fetal tissues is considered diagnostic of infection. These tissues were inoculated into the peritoneal cavities of mice. After 6 weeks, mice were tested for antibody to *T.gondii* or tissue cysts were demonstrated in brain smears. At necropsy one second trimester fetus (Case 8) was also found to be infected with cytomegalovirus (CMV).

In 6 cases the mother received spiramycin therapy in an attempt to prevent transplacental transmission of the parasite. Spiramycin cannot cross the placenta and does not treat the infection in the fetus. In Case 10 the mother had received pyrimethamine and sulphadiazine therapy for 3 weeks prior to abortion.

Infants' and child's eyes

The infants and the child in this study died from severe congenital toxoplasmosis or its complications. Three infant eyes were available from 2 cases. Both infants were born prematurely, one at 34 weeks gestation (Case 12), the other unrecorded (Case 11). The infants survived 7 days and 5 days respectively. For Case 11 the diagnosis was confirmed by necropsy and positive subinoculation of fetal tissues into mice. For Case 12 the diagnosis was confirmed by serological investigations during life and necropsy was not performed.

The 2 year old child had well documented changes of severe ocular and cerebral toxoplasmosis which had been partially treated. These cases were included in the study for comparison with the earlier stages of the disease in the fetus.

Control eyes

Eyes from normal, uninfected, age-matched fetuses (age 21, 24 and 26 weeks gestation), infants (born at 34 weeks gestation and term) and a 2 year old child were used as controls. These cases showed no evidence of congenital toxoplasmosis or other abnormalities.

Histopathology

Cases retrieved from archives were already embedded in paraffin. The remainder of the eyes were obtained in 10% buffered formalin. These were opened horizontally, processed and embedded in paraffin wax. Microtome sections (6μm) were cut, deparaffinised, serially rehydrated and then stained with haemotoxylin and eosin (H&E) or periodic acid schiff (PAS).

Immunohistochemistry

For immunohistochemistry, 6μm sections were deparaffinised and serially rehydrated to water. Endogenous peroxidase activity was blocked by immersing slides in 3% H_2O_2 in distilled water for 10 minutes. Following this slides were washed in running tap water for 5 minutes. For antigen retrieval slides were either pressure cooked or microwaved in 0.01 M sodium citrate buffer, pH 6.0. Non-specific antibody binding was blocked by incubation in 20% normal goat serum (NGS) or 10% normal swine

serum (NSS) in Tris Buffered Saline containing 0.1% Tween-20 (TBS/Tween) for 10 minutes prior to incubation with primary antibodies.

Inflammatory cells

Slides were incubated with primary antibodies diluted in 4% NGS/TBS/Tween for either i) 30 minutes at room temperature, ii) 90minutes at 37°C or iii) overnight at 4°C. After washing in TBS/Tween, slides were incubated with the secondary antibody, biotinylated IgG-goat anti-mouse (Ventana, Tucson, AZ, USA) diluted in TBS/Tween , at room temperature, for 30 minutes. The slides were again washed and then incubated with an Avidin-Biotin complex (Ventana) for 30 minutes at room temperature. Immunohistochemical staining was visualised with 3-3'diaminobenzidine (DAB) and the slides were then counterstained with haemotoxylin. The primary antibodies used were pan-B cell (CD20), pan T cell (CD3), CD4+T cells (OPD4), CD8+ T cells (CD8), and macrophages (CD68). The primary antibodies were used at the following dilutions; CD20 (1:200); CD3 (1:10); OPD4 (1:25); CD8 (1:50); CD68 (1:100) (all from DAKO, Santa Barbara, CA, USA).

T.gondii antigens

Similarly slides were incubated with murine monoclonal anti-*T.gondii*, L43 (donated by E.Petersen, Statens Seruminstitut, Copenhagen, Denmark) or rabbit polyclonal anti-*T.gondii* (also donated by E.Petersen). Both monoclonal and polyclonal antibodies recognise bradyzoite and tachyzoite life cycle stages. The primary antibodies were used at a 1:100 dilution. The slides treated with the monoclonal antibody were washed and incubated with monoclonal rabbit anti-mouse antibody (DAKO). After washing they were incubated with an alkaline phosphatase, anti-

alkaline phosphatase (APAAP) complex for 30 minutes. The last two steps were then repeated. Slides treated with the polyclonal antibody were incubated with mouse anti-rabbit antibody (DAKO) and then treated as for the monoclonal antibodies. Immunohistochemical staining was visualised using APAAP substrate solution (0.2M Tris-HCl, pH 9.0 containing 4% new fuschin in 2N HCl, 4% sodium nitrite, and napthol-AS-BI-phosphate dissolved in dimethylformamide). This gives a red reaction product which allows distinction of extracellular organisms from melanin granules of the disrupted RPE. The slides were counterstained with haemotoxylin.

Figure 4.1 Case histories for fetuses, infants and child with congenital toxoplasmosis

Case No.	Age (g or pn)	Maternal Seroconversion	Diagnosis	Treatment
1	19 weeks g	12-14 weeks	AF (17 weeks)-PCR,I US normal. N, Brain-I	Maternal Spiramycin from 14 weeks
2	21 weeks g	7.5-13 weeks	AF(18.5 weeks)-PCR,I Placenta- I. N	Maternal Spiramycin from 15 weeks
3	21 weeks g	10-13 weeks	AF(19 weeks)-PCR,I VD at US, Placenta-I. N	Maternal Spiramycin from 16 weeks
4	21 weeks g	6.5-14 weeks	AF(20 weeks)-PCR VD at US	None
5	22 weeks g	12-13 weeks	AF(20 weeks)-PCR VD at US. N, Brain-I	Maternal Spiramycin from 13 weeks
6	22 weeks g	13-17 weeks	AF(21 weeks)-PCR VD at US	Maternal Spiramycin from 18 weeks
7	23 weeks g	9.5-11.5 weeks	AF(23 weeks)-PCR VD at US. N	None
8*	23 weeks g	17-20 weeks	AF(22 weeks)-PCR N, Brain-I	None
9	26 weeks g	<10 weeks	AF(20 weeks)-PCR, I Intracranial densities at US	Maternal Pyrimethamine and Sulphadiazine from 23 weeks
10	32 weeks g	14-17 weeks	AF(29 weeks)-PCR VD at US. N	Maternal Spiramycin from 22 weeks
11	5 days pn (premature)	Not known	N	None
12	7 days pn (premature)	Not known	Serology	Pyrimethamine and Sulphadiazine
13	2 years pn	Not known	Initial diagnosis as neonate N	Pyrimethamine and Sulphadiazine

Abbreviations: g, gestation; pn, postnatal; AF, amniotic fluid; PCR, polymerase chain reaction to detect *T.gondii* B1 gene; I subinoculation of placenta, brain or amniotic fluid into mice; VD, cerebral ventricular dilatation; US, ultrasound; N necropsy; * Fetus also positive for CMV by PCR

Table 4.2 Post mortem findings in fetuses, infants and child with congenital toxoplasmosis

Case No	Placenta	Brain	Heart	Lungs	Thymus	Liver & Spleen	Adrenal	Skeletal Muscle	Presence of *T.gondii*
1	NE	VD, C&PV necrosis, calcifications	Myocarditis	Pneumonitis	Depletion of thymocytes	HSM with inflammation	Necrosis	Myositis	Lungs, skeletal muscle
2	Chorio-amnionitis	C&PV necrosis	Myocarditis	Pneumonitis	Depletion of thymocytes	No HSM inflammation	Normal	Myositis	Brain, heart, skeletal muscle
3	Chorio-amnionitis	VD, C&PV necrosis, abscess	Myocarditis	Pneumonitis	Depletion of thymocytes	HSM with inflammation and necrosis	Haemorr-hage	Myositis	Brain, spleen, skeletal muscle
4	No necropsy								
5	Chorio-amnionitis	VD, necrosis, calcifications	Normal	Normal	Normal	No HSM inflammation necrosis	Normal	Normal	Brain
6	No necropsy								
7	NE	Necrosis, menigitis, calcifications	Myocarditis	Pneumonitis	Depletion of thymocytes	Hepatitis with necrosis	Necrosis, calcifications	Myositis	Heart, liver, skeletal muscle
8*	Chorio-amnionitis	VD, C&PV necrosis, IV haemorrhage	Myocarditis	Pneumonitis, CMV inclusions	Depletion of thymocytes	Normal	Normal	Normal	Placenta, brain
9	No necropsy								
10	Chorio-amnionitis	VD, necrosis, calcifications	Normal	Amniotic fluid aspiration	Normal	Normal	Normal	Focal myositis	Placenta, brain
11	NE	VD, necrosis	Myocarditis	Pneumonitis	Depletion of thymocytes	Inflammation	NE	NE	Heart
12	No necropsy								
13	NE	VD, necrosis, calcifications	Normal	Normal	Normal	Normal	Normal	Normal	None

Abbreviations: NE, not examined; VD, ventricular dilatation; C&PV, cortical and perivenular; HSM, hepatosplenomegaly; IV, intraventricular. * Fetus also infected with cytomegalovirus (CMV)

4.4 Results

The histopathological and immunohistochemical findings are summarised in Tables 4.3 to 4.5 and are illustrated in Figures 4.1 to 4.7.

Fetal eyes

Gross

For eyes obtained in formalin, the gross findings ranged from completely normal to white fluffy areas suggestive of acute retinitis accompanied by a cloudy vitreous. The eye from the 32 week old fetus showed an established retinochoroidal scar in the superior temporal region.

Histopathological features

Two eyes, one from a 21 week old fetus (Case 2), and the other from a 26 week old fetus (Case 9) were normal. In all cases there was a mild inflammatory cell infiltrate composed principally of lymphocytes and plasma cells in the iris and ciliary body. Where present the cornea and lens were normal.

The retina showed appropriate maturation for the gestational age. Focal retinal lesions were identified in eight of the 15 eyes. Some lesions consisted of frank retinal necrosis with disruption of the underlying RPE (Figure 4.1A). Free pigment granules were scattered throughout the necrotic areas. Other lesions were devoid of retina and consisted of disruption and proliferation of the retinal pigment epithelium with scattered pigment granules in the overlying vitreous. The choroid underlying lesions was congested with chronic inflammation (Figure 4.1B). In addition, there was a diffuse choroiditis. Lesions varied in size from less than 1mm in a 21 week old fetus to

over 7mm in a 22 week fetus. Of four cases where both eyes were available, lesions were bilateral in only one case. The most frequent locations of lesions was in the posterior pole which was involved in 5 eyes. In three of these cases the lesions were confined to the peripapillary area. The remaining eyes had lesions in the peripheral retina. In 2 cases both peripapillary and peripheral retina were involved.

The lesion in the peripheral retina of the 32 week old fetus was unusual compared with other retinal lesions. Unlike other cases, which showed evidence of continuing inflammation, this lesion was almost devoid of inflammatory cells. At the edge of the lesion the retina showed abnormal maturation with loss of distinction of nuclear layers and formation of Flexner Wintersteiner rosettes (Figure 4.2). Distant from this lesion the retina showed appropriate maturation for gestational age. These appearances were interpreted as focal retinal dysplasia.

In addition to focal retinal lesions there was retinal gliosis in 5 eyes (Figure 4.3D). In 3 eyes with no focal lesions, the retina was normal in 2 eyes, but showed widespread gliosis in one eye. No CMV inclusions were detected in Case 8. Inflammation extended into the vitreous in 7 eyes with condensation bands, scattered inflammatory cells and necrotic debris. Remnants of the tunica vasculosa lentis were identified in 9 eyes. In addition the hyaloid artery and primary vitreous also were present in 2 eyes (Figure 4.4).

The optic nerve was present in sections from 8 eyes and was normal in three cases, (Case 1, 9 and 10). In 5 eyes there was a leptomeningitis associated with optic neuritis

(Figure 4.5). In 3 of the eyes this also had resulted in distortion of the normal nerve architecture. In 2 of the eyes with optic neuritis there was a peripapillary lesion.

Infants' eyes

Gross

Gross examination of both eyes from the 7-day old infant (Case12) showed bilateral retinal detachment with large white fluffy areas in the retina and a cloudy yellow vitreous.

Histopathological features

In all three eyes the cornea, angle, iris and ciliary body were normal.

The gross findings in Case 12 were reflected in the histological sections which showed a large area of retinal detachment with atrophy of the photoreceptors and a subretinal serous exudate. There was bilateral extensive retinal necrosis. In the left eye the retinal lesion was over 8mm diameter extending from the pars plana to the posterior pole. The other eye showed a 3mm lesion at the posterior pole and a 4mm lesion in the peripheral retina, superiorly. Within the areas of retinal necrosis there were scattered pigment granules and foci of calcification.

Underlying the lesions, the RPE was disrupted showing tubuloacinar proliferation and there was a diffuse choroiditis. Elsewhere the RPE was intact however, the retina showed oedema, gliosis and mononuclear cell infiltration. There was an organisation within the vitreous with continuing inflammation. The optic nerve was not present in the sections examined.

In case 11 there was also a large retinal detachment with subretinal exudate and atrophy of photoreceptors (Figure 4.3A). However, there were only small foci of retinal necrosis and the RPE was intact. The retina showed changes similar to those described above but in addition there were collections of intracellular organisms (Figure 4.3B). The optic nerve showed a leptomeningitis with chronic inflammation confined mainly to the prelaminar region and the lamina cribrosa. Condensation bands were present in the vitreous with necrotic debris and mononuclear cells.

Child's eyes

Gross

Both eyes showed features of end-stage disease. The left eye contained two retinochoroidal scars situated in the posterior pole and superior region of the peripheral retina. The larger scar in the posterior pole measured 5x3mm, the smaller scar measured 3mm in maximum diameter. Overlying these lesions the vitreous was cloudy.

The right eye was firm, partially collapsed and smaller than the left eye. On opening the anterior chamber was shallow with an opaque lens displaced into the posterior chamber. In the posterior chamber the vitreous was cloudy and the cavity was distorted by firm white material.

Histopathological features

The anterior chamber of the left eye was normal. The anterior chamber of the right eye was collapsed with displacement of the lens into the posterior chamber. The lens showed cataractous degeneration.

In the right eye there were two well established retinochoroidal scars. The centre of the scar consisted of an organising exudate containing foreign body giant cells and scattered chronic inflammation. This had destroyed the normal structures of the retina and choroid. The retinal pigment epithelium was disrupted and at the edge of the scar showed tubulo-acinar proliferation (Figure 4.1C). The retina at the edge of the scars was oedematous and disrupted. Elsewhere it was gliotic. There was focal, mild chronic inflammation in the choroid. The vitreous showed evidence of organisation overlying the scars but was otherwise normal.

The left eye showed changes of end-stage disease. The retina was gliotic and the RPE was disrupted with tubulo-acinar proliferation. There was a large subretinal exudate with cholesterol clefts, foreign body giant cells and scattered pigment. The optic nerve showed fibrosis and atrophy.

Control eyes

The uninfected, age-matched, control eyes from the 21, 24 and 26 week old fetuses, 34 week old and term infants and 2 year old child were normal. They showed no focal lesions or inflammation.

Immunohistochemistry

Inflammatory cells

Findings were similar for both fetal and infant eyes. The inflammatory cells present in the lesions consisted of lymphocytes, plasma cells and macrophages. Polymorphonuclear leucocytes were not present in significant numbers.

Immunohistochemical staining showed both B and T lymphocytes. Overall T lymphocytes predominated with only scattered B lymphocytes. There was a diffuse infiltrate of inflammatory cells in the choroid composed of both T and B lymphocytes. This infiltrate was most severe in the choroid underlying lesions where it was composed almost entirely of T cells (Figure 4.6). These collections of T lymphocytes consisted of both CD4+ and CD8+ subtypes although, CD4+ T cells predominated. T cells were also present in the retina adjacent to lesions. In addition in the cases with optic neuritis the nerve was infiltrated by B and T cells of both CD4+ and CD8+ subtypes although, again CD4+ T cells predominated. The eye from the 32 week old fetus and both eyes from the 2 year old child contained only rare lymphocytes.

In 2 fetal eyes from Case 5 numerous macrophages were identified in the choroid underlying retinal lesions (Figure 4.6F). In all other eyes, only occasional macrophages were identified within the choroid. Only very occasional inflammatory cells were identified within control eyes.

T.gondii antigens

No tissue cysts were seen by light microscopy in any of the cases although, collections of intracellular organisms were identified in Case 11 (Figure 4.7A). Immunohistochemistry for *T.gondii* antigen detected extracellular organisms and amorphous antigens in 10 eyes (7 fetal, 3 infant and 0 child) and 9 eyes (6 fetal, 3 infant and 0 child), respectively. Parasites were almost exclusively confined to the retina and necrotic debris (Figure 4.7). In the fetal eyes the parasite were most numerous within the retina immediately adjacent to areas of necrosis. Parasites were not identified in Case 9 or 10. In Case 11, in addition to the groups of intracellular

parasites seen on light microscopy, numerous extracellular parasites were apparent with immunohistochemical staining, within the abnormal retina. In one area parasites were identified surrounding an inner retinal vessel (Figure 4.7B and C). In Case 12 very few parasites were identified despite the presence of extensive necrosis.

No parasites were identified within the choroid despite the presence of numerous inflammatory cells in all but one case. In Case 6, a few extracellular parasites were identified in the perivascular space around the central retinal vessels. However, no parasites were identified within the substance of the optic nerve in any case.

Immunohistochemical staining for *T.gondii* was negative in all the control eyes.

Table 4.3 Summary of histopathological findings in the eyes of fetuses, infants and child with congenital toxoplasmosis

Age	No. of Cases	No. of Eyes	No. of Eyes with lesions	Location of Lesions			Retinal Necrosis	Retinal Detachment	RPE Disruption	Choroiditis	Optic Neuritis
				PP	PeriP	PR					
Second trimester	9	12	7	5	3[1]	4	6	0	7	9	5[2]
Third trimester	1	1	1	0	0	1[3]	0	0	1	1[4]	0[5]
Infants	2	3	3	3	1	2	3	3	2	3	0[5]
Child	1	2	2[6]	2	0	1	0	1	2	2[4]	0
Total	**13**	**18**	**12**	**10**	**4**	**8**	**9**	**3**	**12**	**15**	**5**

[1] In three cases three the lesion at the posterior pole was confined to the peripapillary area.
[2] Five cases showed optic neuritis but in addition 3 of these showed disruption of the normal nerve architecture
[3] Post inflammatory retinal dysplasia was also present at the edge of this lesion
[4] These cases were associated with only occasional inflammatory cells in the choroid
[5] Although no optic neuritis was present, these cases were associated with a leptomeningitis
[6] The left eye from the 2 year old child was severely disrupted without focal lesions.

Table 4.4 The histopathological findings for fetuses, infants and child with congenital toxoplasmosis

Case No.	Age	Eye	Location and Size of Discrete Lesions	Type of Discrete Lesion S,N or R	Other Findings		
					RC	G	ON, OA, LM
1	19w (g)	L	None	N/A	RC	no	LM
2	21w (g)	L	PR, 1mm	R	no	no	N/A
		R	None	N/A	no	no	N/A
3	21w (g)	N/K	PeriP, 1mm, PR, 2.5mm	R	no	no	N/A
4	21w (g)	L	PeriP, 2.5mm	R	RC	no	ON, LM, A
		R	None	N/A	RC	no	ON, LM, A
5	22w (g)	L	PP, 6mm	R	RC	G	N/A
		R	PP, 6mm	R	RC	G	N/A
6	22w (g)	L	PeriP, 4mm, PR, 7mm	N, R	RC	no	ON, LM
7	23w (g)	R	PR, 5mm	N, R	RC	G	ON, LM, A
8*	23w (g)	R	None	N/A	RC	G	ON, LM
9	26w (g)	L	None	N/A	no	no	LM
10	32w (g)	L	Pr, 6mm	S, R	no	no	N/A
11	5d (pn)	L	PeriP, 3mm, PP, 8mm	N, R	RC, D	G	N/A
		R	PP, 3mm, PR, 4mm	N, R	RC, D	G	N/A
12	7d (pn)	N/K	None	N/A	RC, D	G	LM
13	2yrs (pn)	L	PP, 3mm, PR, 5mm	S, R	no	G	no
		R	None#	NA	D	G	OA

Abbreviations: w, weeks; g, gestation; d, days; pn, postnatal; yrs, years; L, left; R, right; NK, not known; PR, peripheral retina; PeriP, peripapillary; PP, posterior pole; S, established retinochoroidal scar; N, retinal necrosis; R, disruption of retinal pigment epithelium; N/A, not applicable; RC, retinochoroiditis ; D, retinal detachment; G, gliosis; ON, optic neuritis; OA, optic atrophy; LM, leptomeningitis; A distortion of normal architecture; * fetus also infected with CMV; # end-stage eye in which whole retina was involved.

Table 4.5 Immunohistochemical staining for inflammatory cells and *T.gondii* in congenital ocular toxoplasmosis

Case No.	Age	Eye	Inflammatory cells					*T.gondii*	
			B (CD20)	T (CD3)	T (CD4)	T (CD8)	M (CD68)	EC organsims	A Antigens
1	19w(g)	L	1	1	1	0	0	0	0
2	21w(g)	L	0	3	2	2	1	0	0
		R	0	0	0	0	0	0	0
3	21w(g)	N/K	2	3	3	0	0	3	2
4	21w(g)	L	2	3	3	2	0	2	3
		R	1	3	3	2	0	3	3
5	22w(g)	L	2	3	2	0	3	3	1
		R	2	3	3	1	3	1	0
6	22w(g)	L	1	3	3	1	1	3	3
7	23w(g)	R	1	3	3	2	0	2	3
8*	23w(g)	R	0	1	1	1	0	0	0
9	26w(g)	L	0	0	0	0	0	0	0
10	32w(g)	L	0	1	1	0	0	0	0
11	5d(pn)	L	0	3	2	2	0	1	1
		R	0	3	2	2	0	1	1
12	7d(pn)	N/K	0	3	2	2	0	1	1
13	2yrs(pn)	L	0	1	1	0	2	0	0
		R	0	1	1	0	2	0	0

The number of positive cells or organisms identified were scored as follows: O, none; 1, few; 2, moderate numbers; 3, large numbers.
Abbreviations: w, weeks; g, gestation; d, days; pn, postnatal; yrs, years; L, left; R, right; NK, not known; EC, extracellular; A, amorphous; * fetus also infected with CMV.

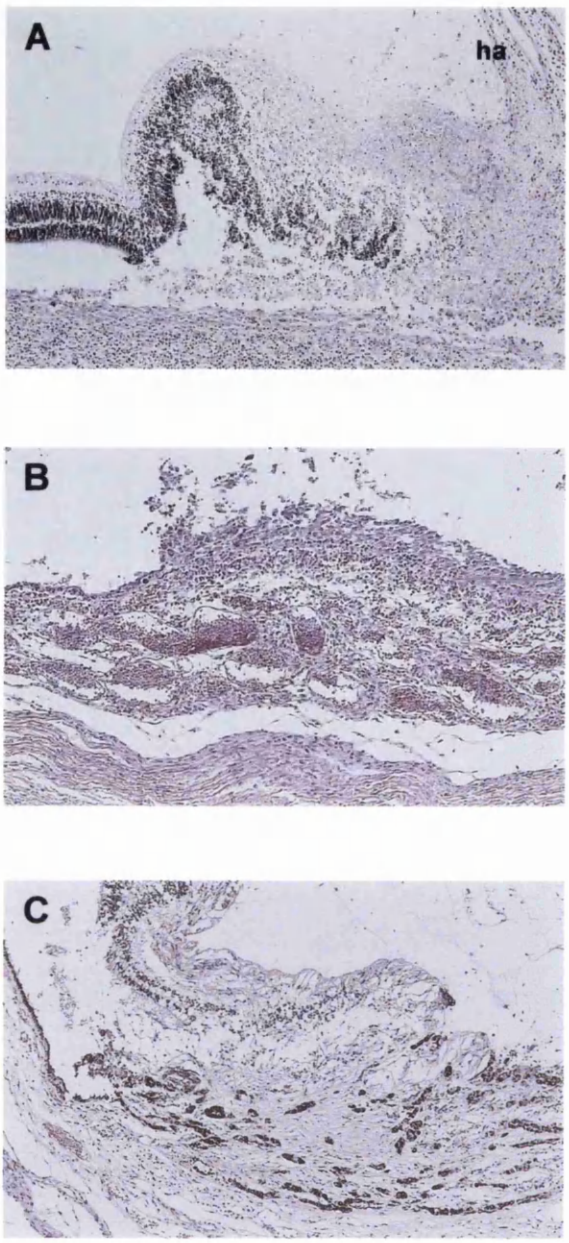


Figure 4.1 Retinochoroidal Lesions

A Well-demarcated area of retinal necrosis at the posterior pole of a 22 week gestation fetus (Case 6). The hyaloid artery (ha) is still present. (H&E, x 250). B Focal disruption and proliferation of the retinal pigment epithelium in a 22 week gestation fetus (Case 5). The underlying choroid shows marked congestion and chronic inflammation. (H&E, x 250). C Edge of a large retinochoroidal scar from the eye of the 2 year old child. The scar is well demarcarated with tubulo-acinar proliferation of the RPE at the edge of the scar. The centre of the scar is devoid of retina. (H&E, x 250).

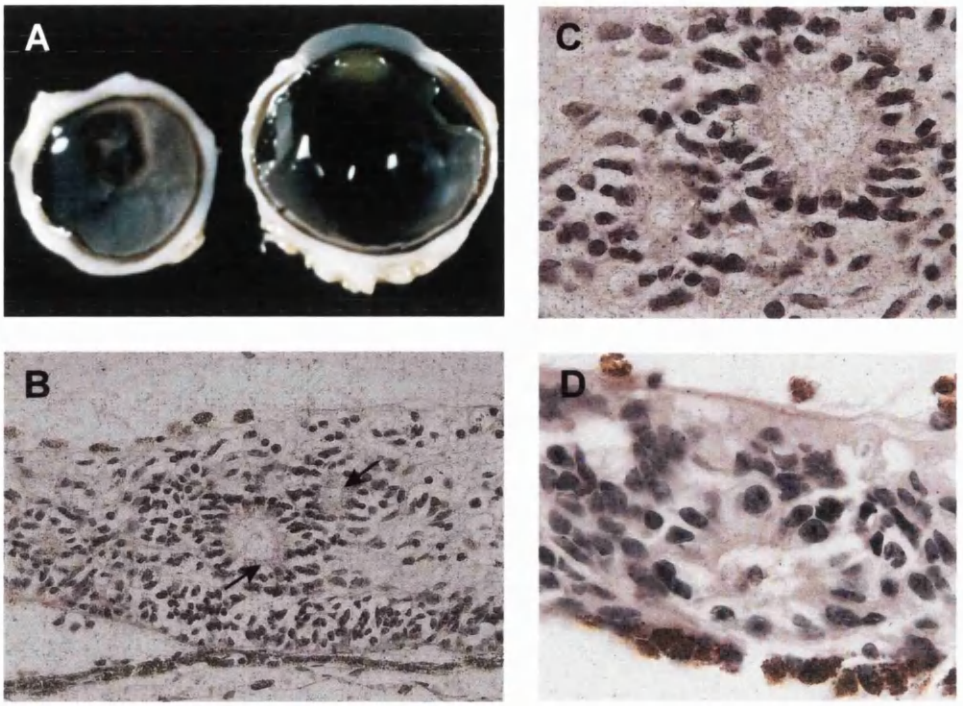


Figure 4.2 Retinal Dysplasia adjacent to retinochoroidal scar **A** Eye from a 32 week gestation fetus (Case 10), showing a large hyperpigmented scar, with a white rim, in the superio-temporal region of the eye. **B** The retina from the edge of the scar shows disorganisation with formation of rosettes (arrows) (H&E, x250). **C** and **D** Rosettes within abnormal retina (H&E, x 400).

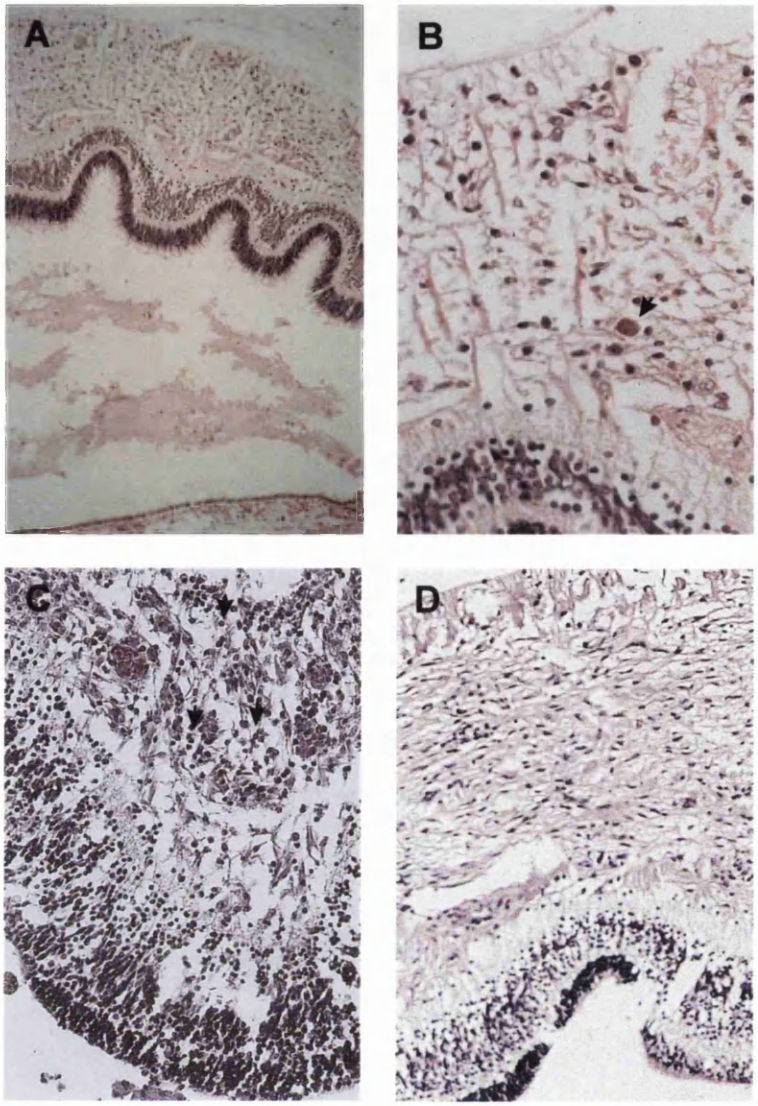


Figure 4.3 Retinal detachment, retinitis and gliosis
A and **B** Retina from a 5 day old infant, (Case12), showing detachment with an exudate between the retina and choroid (A), (H&E,x 100). The inner retinal layers are oedematous and inflamed (B). Collections of intracellular organisms are identified (arrows), (H&E, x 400). **C** Retina from a 21 week gestation fetus, (Case 3), showing numerous inflammatory cells within the retina (arrows), with prominent inner retinal vessels, (H&E, x 400). **D** Retina from a 22 week gestation fetus (Case 6) showing gliosis, (H&E, x 400).

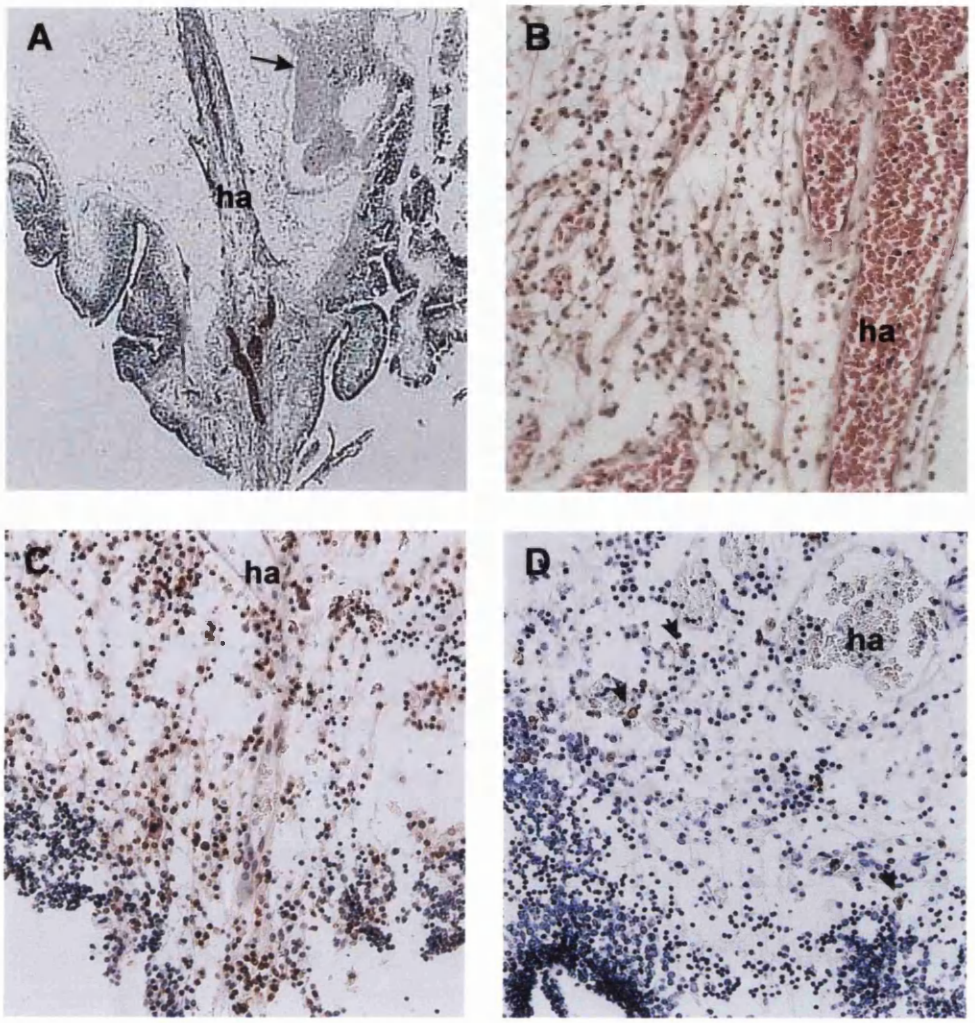


Figure 4.4 Inflammation in the primary vitreous
A Eye from a 23 week gestation fetus (Case 7) showing a moderate inflammatory cell infiltrate within the primary vitreous and surrounding the hyaloid artery (ha). An area of necrosis is present (arrow) (H&E, x 20). **B** The inflammatory infiltrate is composed principally of small lymphocytes and plasma cells (H&E, x 250). **C** Immunohistochemical staining shows that the majority of lymphocytes are T cells, (CD3, x 250). **D** A smaller proportion of B cells are also present (arrows), (CD20, x 250).

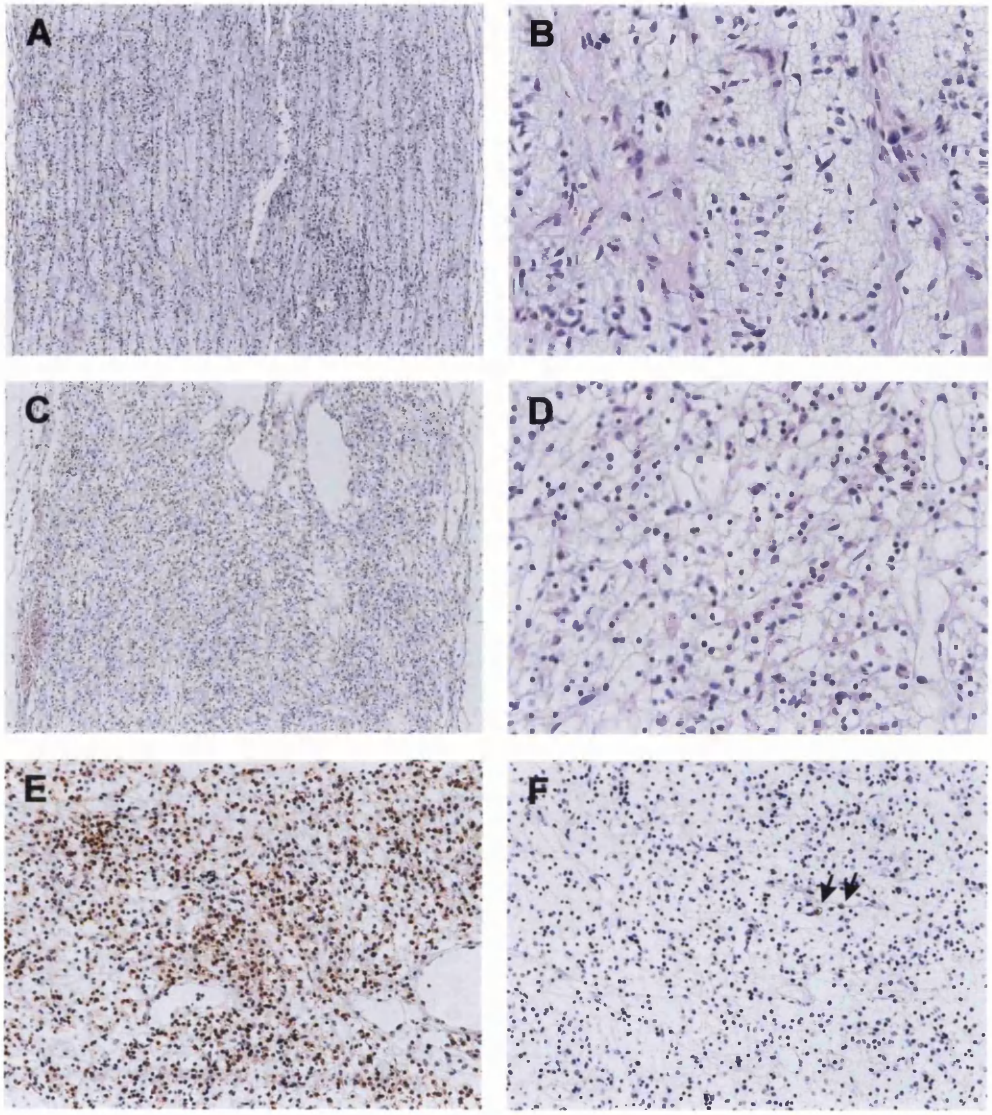


Figure 4.5 Optic Neuritis

A and B Optic nerve from a 24 week gestation, uninfected fetus showing normal nerve architecture. (H&E, x 100 and x 400).

C and D Optic nerve from a 23 week gestation fetus with congenital toxoplasmosis. The nerve architecture is disrupted with vacuolation and infiltration by inflammatory cells. (H&E, x100 and x400).

E Immunohistochemical staining for T cells shows numerous positive lymphocytes within the optic nerve. (CD3, x 400). F Immunohistochemical staining for B cells show very few positive lymphocytes (arrows). (CD20, x 400).

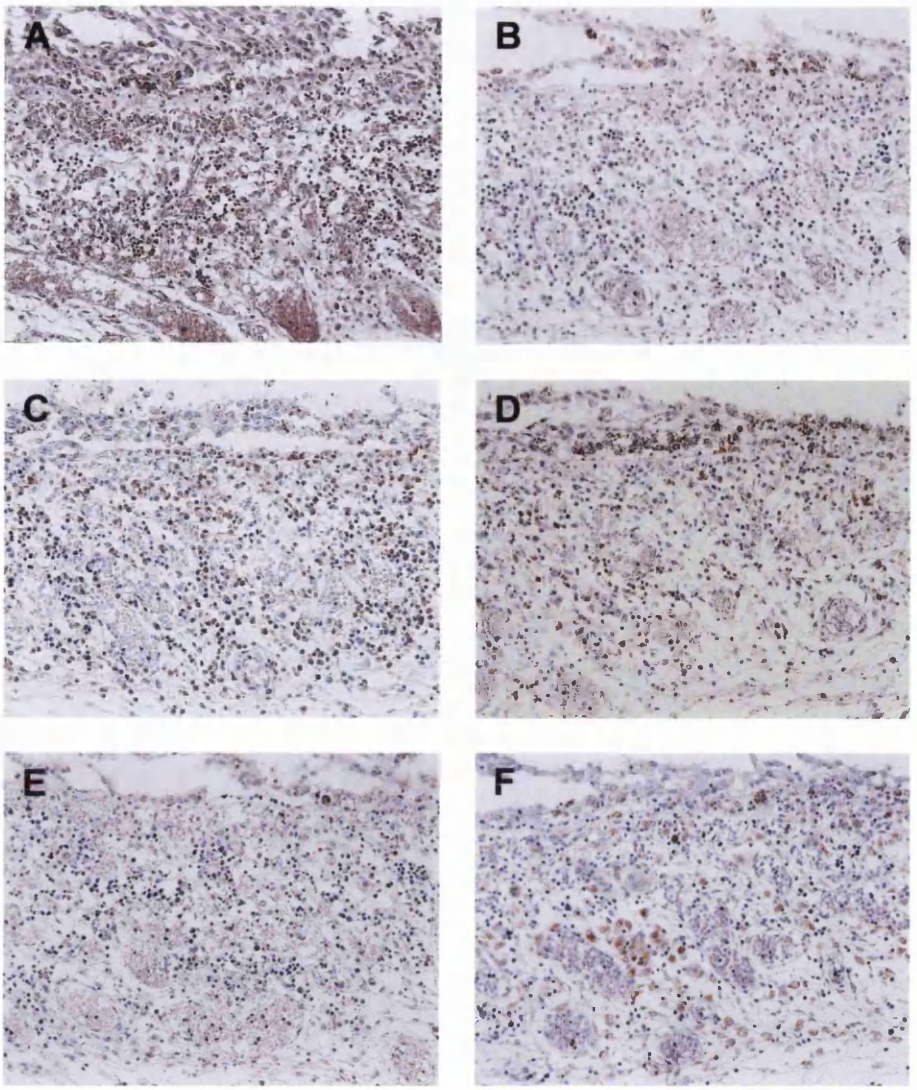


Figure 4.6 Inflammatory cells present in ocular toxoplasmosis **A** Discrete ocular lesion from a 21 week gestation fetus (Case 2) showing disruptionof the RPE, choroidal congestion and inflammation (H&E, x 400). **B** Immunohistochemical negative control for same case (Negative, x 400). **C** and **D** Numerous CD3 positive and CD4 positive T cells are present within the choroid (CD3 and CD4, x 400). **E** No CD8 positive T cells are present (CD8, x 400). **F** CD68 positive macrophages are numerous within the choroid underlying the RPE disruption (CD68, x 400).

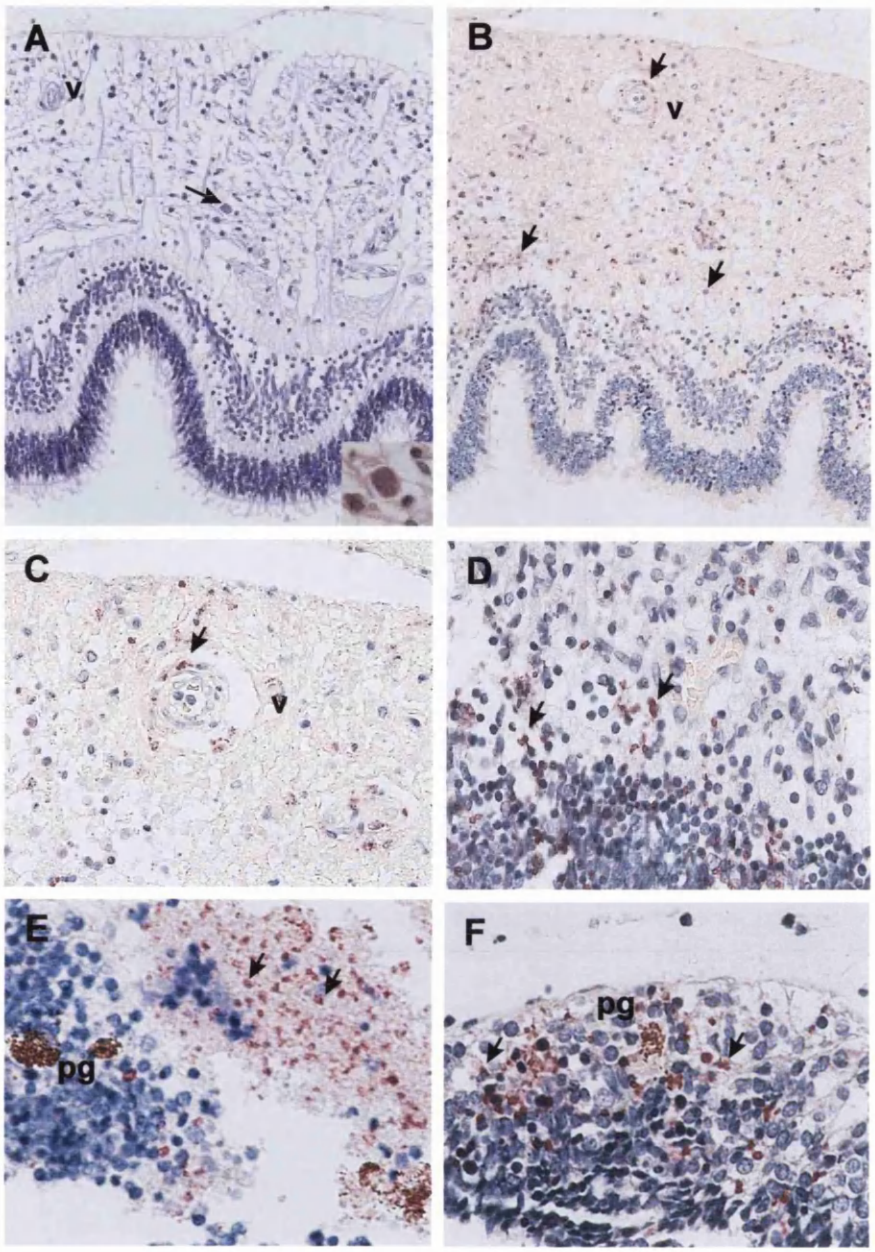


Figure 4.7 Presence of *T.gondii*

A Retina from a 7 day old infant (Case 12) showing a collection of intracellular *T.gondii* within the retina (arrow), (H&E; x 100). The insert shows a high power view of these organisms, (H&E; x 400). **B** and **C** Retina from Case 12 showing immunohistochemical staining for *T.gondii* antigen. Many extracellular *T.gondii* are identified (arrows). In addition organisms are present in a perivascular location (v), (L43; x 100 and x 250). **D** Gliotic retina from a 22 week gestation fetus (Case 5) showing extracellular organisms (arrows), (Polyclonal antibody; x250). **E** Numerous extracellular *T.gondii* (arrows) within necrotic debris from a 23 week gestation fetus (Case 7), (L43; x 400). The red staining product allows distinction from melanin pigment granules (pg) of disrupted RPE. **F** Extracellular *T.gondii* within inflamed disrupted retina from a 21 week gestation fetus, (Case 4) (L43; x 400). Again the organisms are distinct from pigment granules (pg) of the the RPE.

4.5 Discussion

Retinitis, retinal necrosis, disruption of the RPE with marked choroidal inflammation and congestion are the characteristic findings in the eyes of fetuses with congenital toxoplasmosis. These lesions represent irreversible *in utero* damage to the retina. They frequently affect extensive areas including the peripapillary area, posterior pole and peripheral retina. The two infants in this study showed severe ocular disease with retinal necrosis, retinal detachment and large subretinal exudates. Within the surviving retina there was neovascularisation and gliosis. Again much of this damage must have occurred *in utero* since postnatal survival was only a few days.

It is almost impossible to document the kinetics of *T.gondii* infection *in utero.* The time from maternal seroconversion to fetal diagnosis and subsequent termination of pregnancy estimated the maximum length of fetal infection to be on average 11.5 weeks. In reality, however, it is probably much shorter than this because there is a delay between maternal and fetal infection (Remington *et al.*, 1995). The length of this delay varies, but can be longer than 16 weeks (Remington *et al.*, 1995). In addition, delay between fetal infection and ocular involvement may occur. Furthermore, unlike an animal model, it is rarely feasible to precisely document the dose, clonal derivation of the parasite (*i.e.* Type I, II or III, as defined by Sibley & Boothroyd, (1992)) or parasite life cycle stage with which the mother has been infected. Despite these different parameters the findings in the fetal eyes are remarkably similar and appear to represent a continuum of the same process. Specifically, retinal necrosis occurs in the acute lesions of ocular toxoplasmosis. As these lesions heal they leave an early scar devoid of normal retina but with proliferation of the RPE. The end stage of this process is formation of a

retinochoroidal scar. This has a hyperpigmented border due to tubulo-acinar proliferation of the RPE at the edge of the lesion. End-stage scars such as these were seen in the eyes from the 2 year old child.

Acute retinitis with necrosis may also be seen in children and adults following reactivation of ocular toxoplasmosis (Zimmerman, 1961). This is rarely as extensive as that seen in the fetus except in the immunocompromised host (Holland *et al.*, 1988a)

Clinically established retinochoroidal scars have been identified at birth in some infants, implying that ocular damage, healing and repair can all occur *in utero* (Guerina *et al.*, 1994; Mets *et al.*, 1996). Indeed a retinochoroidal scar, almost devoid of inflammatory cells, was the main abnormality detected in the eye of the 32 week old fetus. Adjacent to this scar the retinal disorganisation and rosettes, interpreted as a form of retinal dysplasia, was an unusual finding. Retinal dysplasia occurs most frequently with multiple congenital anomalies associated with chromosomal aberrations (Fulton *et al.*, 1978; Godel *et al.*, 1981). At necropsy this fetus (Case 10) was not dysmorphic and showed no evidence of other malformations. In addition the abnormal retina was confined to the area adjacent to the scar. Retinal dysplasia also has been described following X-irradiation of the fetus both in clinical situations and in animal models (Goldestein & Wexler, 1931; Shively *et al.*, 1970). Various cytotoxic agents including 5-iododeoxyuridine (Percy, 1975), cytosine arabinoside (Percy & Danylchuk, 1977) and alkylating agents such as methylnitrosourea (Smith & Yielding, 1986), can produce retinal dysplasia in animal models. However, this is the first report of retinal dysplasia following *T.gondii* infection .

There have been several reports of infectious agents other than *T.gondii*, causing retinal dysplasia in animals. For example, retinal dysplasia, develops in 2 day old hamsters, but not 25 day old hamsters following intracerebral injection of measles virus (Khalifa *et al.*, 1991). However, 25 day old hamsters did show retinal haemorrhages and focal retinitis. Similarly, retinal dysplasia was the most common finding following intra-ocular injection of feline leukaemia virus into fetal kittens (Albert *et al.*, 1977). Finally, canine herpes virus infection in neonatal pups produces severe ocular inflammation with subsequent retinal dysplasia (Albert *et al.*, 1976).

The mechanism whereby these infectious agents cause abnormal retinal development remains to be determined. It has been postulated that progressive disorganisation of the retina and necrosis is followed by differential repair of the retinal layers (Albert *et al.*, 1976; Albert *et al.*, 1977; Parhad *et al.*, 1980). Furthermore the RPE appears to be important in normal retinal development (Fulton *et al.*, 1978; Godel *et al.*, 1981). Therefore disruption of the RPE also may contribute to retinal dysplasia and rosette formation, perhaps due to loss of polarity of retinal cells or lack of specific growth or maturation factors produced by the RPE (Steinberg, 1985; Tanihara *et al.*, 1997). For example, retinoids are transported from the RPE to photoreceptors (Steinberg, 1985) and retinal dysplasia is recognised in fetal vitamin A deficiency syndrome (Ghyselinck *et al.*, 1997). Both retinal necrosis and RPE disruption occur in ocular toxoplasmosis. Retinal dysplasia may therefore represent a rare complication of early *T.gondii* infection where the insult occurs prior to organisation of the retinal layers.

The location of lesions is important because patients are at risk for blindness if tissues critical for vision, such as the macula or optic nerve, are involved. In clinical studies

there appears to be a definite predilection for the macular region in patients with congenital toxoplasmosis. In one study, peripheral scars were present in 64% of patients whereas macular scars were present in 58% (Mets *et al.*, 1996). Considering the much smaller area of the macula, these results support a definite predilection for the macular region in these patients. During embryogenesis, development of the macular region is markedly retarded compared with the rest of the retina and differentiation of this region continues for at least the first four years of life (Yuodelis & Hendrickson, 1986). In view of this lack of specialisation and its small size we were unable to identify the macular region in the fetal or infant eyes. However where lesion were extensive and involved the posterior pole it seems likely that the macula was involved.

In congenital infection, *T.gondii* probably reaches the eye by a haematogenous route. The identification of parasites around an inner retinal vessel in Case 11 supports a haemotogenous route. Furthermore a predilection for the posterior pole may reflect the fact that it is vascularised earlier in development than other portions of the retina. In addition although the macula is avascular, it obtains its blood supply from end arterioles which form a capillary plexus surrounding the macula. Lodging of parasites in these capillaries may facilitate establishment of infection in this delicate region of the eye.

The optic nerve was present in sections from 8 fetal eyes. Optic neuritis was present in 5 of these with distortion of the normal nerve architecture in 3 cases. Furthermore, similar to the focal retinal dysplasia, distortion of the nerve architecture may represent another instance whereby inflammation of the nerve early in gestation results in

abnormal development. Due to a lack of appropriate neural connections optic atrophy may be the clinical outcome in surviving patients. In one study, optic atrophy was present in 20% of patients with congenital toxoplasmosis (Mets *et al.*, 1996). However, fetuses in this study were known to have intracranial disease with ventricular dilatation on ultrasound, and it may be that the presence of optic neuritis simply reflects the severity of their encephalitis. Papillitis, papilloedema, and optic atrophy are recognised clinical manifestations of toxoplasmic encephalitis (Mets *et al.*, 1996; Perkins, 1973). True optic neuritis is a rare complication of disease reactivation although *T.gondii* may involve the optic disc or retina immediately adjacent to the optic disc resulting in a papillitis sometimes referred to as Jensen juxtapapillary retinitis (Holland *et al.*, 1996). This may represent a clinical correlate of the two cases of optic neuritis associated with a peripapillary lesion in the fetal eyes.

Optic neuritis with necrosis and numerous parasites has been described in a patient with HIV-associated ocular toxoplasmosis(Holland *et al.*, 1988a) and a patient with fulminant congenital infection (Manshot & Daamen, 1965). This has led to the suggestion that congenital infections may be transmitted to the eye from the brain via the optic nerve. Alternatively, if infection occurs very early in embryogenesis, the parasite may accompany the optic anlage from the developing forebrain. However, in the fetal cases with optic neuritis, although there was inflammation and distortion of the normal nerve architecture there was no necrosis or parasites. Except in a few isolated cases, such as those already described, it seems unlikely that the optic nerve is the usual means of *T.gondii* spread to the eye. Indeed the presence of parasites in the optic nerve may simply reflect haematogenous spread via the central retinal artery. The presence of a few parasites surrounding the central retinal artery in Case 6 and

inner retinal vessels in Case 12 support a haematogenous route of dissemination to the eye for *T.gondii*.

Effective immunity to *T.gondii* requires an immune response that involves macrophages, NK cells and their monokine and cytokine products (Subauste & Remington, 1993). In particular, IFN-γ appears to be a critical cytokine for effective immunity (Subauste, 1991). The fetus and neonate are unduly susceptible to infections with intracellular pathogens such as *T.gondii*. This is due to multiple factors including a limited T cell repertoire for recognition of antigens (George & Schroeder, 1992) and reduced production of certain cytokines, in particular, IFN-γ and IL-4 (Lewis *et al.*, 1991). Nonetheless, the presence of inflammatory cells in the eyes of infected fetuses suggests that the fetus is capable of mounting, albeit a less effective, immune response to *T.gondii* infection. Murine models have shown that the cytokines IFN-γ, TNF-α and CD4+ and CD8+ lymphocytes are important in acquired ocular toxoplasmosis (Gazzinelli *et al.*, 1994). Since T lymphocytes, of CD4+ and to a lesser extent CD8+ subtypes, are clearly present in the fetal eye at the time of infection, a decreased production of IFN-γ or other cytokines by these lymphocytes may contribute to tissue destruction through uncontrolled parasite proliferation. Alternatively, it has been suggested that T lymphocytes and their cytokine products participate in the retinal destruction associated with ocular toxoplasmosis (Dutton, 1986). While this may occur during disease reactivation in the immunocompetent adult, it is less likely to occur during primary infection of the fetus where the magnitude and quality of the immune response is reduced compared with the adult.

Tissue cysts were not identified in the eyes of any of the fetuses or infants although they were identified at necropsy in the brains of 5 out of 7 fetuses. In the

immunocompetent adult, tissue cysts may be found at the edge of retinochoroidal scars or within apparently normal retina at some distance from previous lesions (McMenamin *et al.*, 1986). Encystment usually occurs with the onset of effective immunity. Studies have shown a role for immune mediators, such as IFN-γ, in tachyzoite to bradyzoite conversion and thus cyst formation (Bohne *et al.*, 1993). Extracellular organisms were present in lesions particularly adjacent to areas of necrosis and numerous intracellular parasites were present in one infant case. This may reflect the early acute nature of this disease in the eye as well as a lack of effective immunity. Despite the extensive areas of retinal necrosis in Case 11 only a few parasites were identified. This may be because the infant had received high dose pyrimethamine and sulphadiazine during life. Parasites were not identified in the choroid in any case, confirming that this is primarily an infection of the retina.

This study has shown that significant irreversible ocular disease can occur *in utero* and has substantial implications for fetal and infant treatment. In countries, such as France, which have prenatal screening programs, studies suggest that antimicrobial treatment *in utero* may reduce ocular disease due to *T.gondii* at least in the short term (Hohlfeld *et al.*, 1989; Berrebi *et al.*, 1994). Similarly, early postnatal treatment of toxoplasmosis is associated with prompt resolution of active retinochoroiditis (Mets *et al.*, 1996). The long term outcome for *in utero* treatment and early postnatal treatment is unknown. Documentation of the early stages of this disease in unsuccessful pregnancies is therefore important to accurately assess the effectiveness of *in utero* therapy and to allow comparisons with the long term clinical outcome in treated patients.

CHAPTER 5
NOVEL BIOCHEMICAL PATHWAYS IN *T.GONDII*

5.1 Specific Objectives

1. To determine whether *T.gondii* utilises the shikimate pathway for the synthesis of folate.

2. To determine whether *T.gondii* has an alternative pathway of respiration.

5.2 Background

Toxicity, allergy and intolerance limit the use of antimicrobial agents currently available to treat toxoplasmosis. There is no cure for *T.gondii* infection and current treatments are based on controlling disease reactivation. The quiescent form of the parasite, the bradyzoite, which resides in tissue cysts is resistant to all known drugs. This is the usual source of recrudescent disease in congenitally infected and immunocompromised individuals. Therefore new therapeutic agents and targets are urgently needed.

The identification of 'plant'-like properties in Apicomplexans presents a unique and potentially exploitable difference between host and parasite which could prove useful for development of new antimicrobial agents. In particular the shikimate pathway required for *de novo* folate synthesis in plants and the alternative pathway of respiration were considered potential candidates for further investigation. Identification of these novel biochemical pathways in *T.gondii* would further our understanding of parasite biology and potentially provide new targets for antimicrobial agents.

5.3 Materials and Methods

In vitro experiments

Cells

Human foreskin fibroblasts (HFF) were grown in tissue culture flasks in Iscoves' Modified Dulbecoes Medium (IMDM), supplemented with 10% heat inactivated (60min;56°C) fetal calf serum, L-glutamine (1mM), penicillin (100U/ml), streptomycin (100μg/ml), (all from Gibco, Grand Island, NY, USA), at 37°C in 100% humidity and 5% CO_2. Confluent cells were removed by trypsinisation. Cells were first washed in Hanks' balanced salt solution (HBSS) without magnesium (Gibco). HBSS without magnesium containing 0.25% trypsin (Sigma, St Louis, MS, USA) was then added and the flask rocked gently for 1 minute. The flask was then agitated to dislodge cells from the surface. An equal volume of supplemented IMDM was added to the cells to stop enzyme activity. Following centrifugation at 1000g for 10 minutes, the cell pellet was resuspended in supplemented IMDM. Viable cells were counted in a haemocytometer by the ability to exclude trypan blue (0.4% solution; Sigma). The cell concentration was adjusted to $1x10^4$/ml for toxicity or $1x10^5$/ml for parasite growth inhibition assays.

Parasites

Tachyzoites of the RH strain were obtained from the peritoneal exudate of ND4 mice, age 8-12 weeks (Jackson laboratories, Bar Harbor, ME, USA) infected intraperitoneally 3 days previously. Following centrifugation at 1000g for 10 minutes the solution containing the tachyzoites was resuspended in supplemented IMDM. Aliquots of the tachyzoites were then added to 100% confluent HFF in a 24 well

plate. Tachyzoites were passed up to 5 times in culture prior to use in the parasite inhibition assays.

For parasite inhibition assays, the tachyzoites were grown until moderate numbers of plaques were identified with minimal disruption of the fibroblast monolayer. This ensured the maximum number of live parasites. To harvest the parasites, the monolayer was disrupted with a sterile pipette tip and the media from the infected well collected in a syringe. This media was passed through a 14 gauge needle 3 times to disrupt cells and release the tachyzoites. Viable tachyzoites were counted in a haemocytometer by their ability to exclude trypan blue. The tachyzoites were then diluted to $2x10^4$/ml in supplemented IMDM for parasite growth inhibition assays.

Inhibitor compounds

N-phosphonomethyl glycine (NPMG) was used as an inhibitor of EPSP synthase, a key enzyme in the shikimate pathway. This compound was dissolved in IMDM to make a 100mM solution (4.23mg/ml) and adjusted to pH 7.0 using 10N NaOH.

Salicylhydroxamic acid (SHAM) and 8-hydroxyquinolone (8HQ) were used as inhibitors of the alternative oxidase. SHAM is not water soluble therefore it was initially dissolved in 100% ethanol. The ethanol solution was then diluted in supplemented IMDM to give a final concentration of 50μg/ml. 8HQ was dissolved in supplemented IMDM to give a concentration of 2mg/ml.

A combination of pyrimethamine and sulphadiazine, the currently recommended therapy for toxoplasmosis was used as a control for inhibition of parasite growth.

Pyrimethamine was initially dissolved in 100% ethanol then diluted 1 in 1000 in IMDM to give a 0.4μg/ml solution. Sulphadiazine was initially dissolved in HBSS then diluted 1 in 100 in IMDM to give a 50μg/ml solution. An equal volume of pyrimethamine and sulphadiazine was used in the parasite inhibition assays to give a final concentration of 0.1μg/ml and 12.5μg/ml, respectively. These doses of pyrimethamine and sulphadiazine have been previously shown to be non-toxic to a fibroblast monolayer (Mack & McLeod, 1984).

All the inhibitor compounds, pyrimethamine and sulphadiazine were obtained from Sigma. After preparation each solution was passed through a 0.2 micron filter (Gelman, Ann Arbor, MI, USA) for sterilisation. Stock solutions of NPMG and SHAM were kept at -20°C. 8HQ, pyrimethamine and sulphadiazine were prepared fresh for each assay.

Toxicity assays

HFF were obtained from tissue culture flasks. The concentration was adjusted to $1x10^4$/ml and 200μl per well added to a 96-well tissue culture plate. HFF were then incubated until 10% confluent (36 to 48 hours). At this point up to 100μl of media was removed from each well and replaced by various concentrations of inhibitor compounds which were added to triplicate wells. Similarly ethanol diluted in supplemented IMDM to the same concentration as was present in the SHAM solution was also added to triplicate wells. The plates were then incubated for four days. During the last 18 hours of culture the cells were pulsed with tritiated thymidine (2.5μCi/well)(Amersham, Arlington Heights, IL, USA). The cells were then harvested

using an automated cell harvester (PHD, Cambridge, MA, USA) and thymidine uptake, and thus cell growth was measured by liquid scintillation on a β-counter (Packard CA2000, Meridan, CT, USA).

The mean counts per minute (CPM) for each concentration of inhibitor were compared with control CPM, using a students t-test. P values less than 0.05 differed significantly from control wells and were thus considered toxic to the fibroblast monolayer. Maximum concentrations which did not affect cell growth (*i.e.* $p>0.05$) were used as the starting point for making serial dilutions of inhibitor compounds for the parasite growth inhibition assays.

Parasite growth inhibition assays

HFF were grown in tissue culture flasks. The concentration was adjusted to $1x10^5$/ml and 200µl per well added to a 96-well tissue culture plate. HFF were then incubated until fully confluent (48-72 hours). At this point up to 150µl of media was removed from each well and the wells were then infected with $2x10^3$ tachyzoites of the RH strain. Control cultures were left uninfected. Following infection the plates were incubated for 1 hour to ensure that parasites were intracellular. Serial dilutions of inhibitor compounds (50µl) were then added to triplicate wells and cultures were incubated for 4 days. Control cultures were incubated with pyrimethamine and sulphadiazine or were left untreated. During the last 18 hours of culture, cells were pulsed with tritiated uracil (2.5µCi/well) (Amersham). The cells were then harvested using an automated cell harvester and uracil uptake, and thus parasite growth measured by liquid scintillation on a β-counter.

The mean counts per minute (CPM) for each concentration of inhibitor were compared with control CPM, using a students t-test. P values less than or equal to 0.05 were considered significant inhibition of parasite growth.

In addition, $1x10^5$ HFF were grown in four 1ml chamber Lab-Tek slides (Miles, Naperville, IL, USA). When fully confluent (48-72 hours) up to 750µl of media was removed from each well. Cultures were infected with $1x10^4$ tachyzoites of the RH strain and incubated for one hour as for the 96-well plates. Inhibitor compounds (250 µl) were added and the slides were incubated for four days. After this time each well was washed 3 times in HBSS. The slides were then fixed in 0.4% aminoacridine in 50% ethanol for 1 hour, stained with 10% giemsa for 10 minutes and mounted with a cover slip for microscopic evaluation.

Product rescue assay to evaluate the specificity of NPMG

To confirm that NPMG was inhibiting the shikimate pathway and ultimately PABA synthesis, parasite inhibition assays were performed in the presence of exogenous PABA, a product of the shikimate pathway. PABA (Sigma) was initially dissolved in 100% ethanol and then diluted in IMDM to give a 10mM solution. The solution was adjusted to pH 7.0 with 10N NaOH and filter sterilised. A toxicity assay was performed for PABA and for ethanol at corresponding dilutions.

In the parasite growth inhibition assay $2x10^3$ tachyzoites were added to confluent HFF in a 96 well plate. After an incubation period of one hour, PABA was added at concentrations ranging from 2.5µM to 100µM to triplicate wells followed immediately by NPMG (4.5mM), sulphadiazine (12.5µg/ml) or pyrimethamine

(0.1μg/ml). Cultures were incubated for four days. During the last 18 hours of culture, cells were pulsed with tritiated uracil (2.5μCi/well). The cells were then harvested and tritiated uracil uptake measured by liquid scintillation on a β-counter.

Synergy assays

The ability of NPMG to act in combination with conventional anti-folate drugs and SHAM was tested. Using the parasite growth inhibition assay, a concentration which achieved approximately 50% inhibition of parasite growth was identified for NPMG (3.125mM), pyrimethamine (0.025μg/ml), sulphadiazine (6.25μg/ml) and SHAM (0.19μg/ml). These concentrations were then used in the parasite growth inhibition assay alone and in combination to determine whether combinations of these drugs resulted in an additive, synergistic or inhibitory interaction. Inhibitor compound combinations were considered synergistic if the actual to predicted ratio of parasite growth inhibition was less than 0.5. The predicted inhibition of parasite growth was calculated as [(CPM Drug A x CPM Drug B)/CPM of untreated cultures].

In vivo survival experiments

The highly virulent RH strain of *T.gondii* was used in survival experiments. Tachyzoites of the RH strain were obtained from the peritoneal exudate of ND4 mice (Jackson laboratories) infected intraperitoneally 3 days previously. ND4 mice, age 8-10 weeks, were then infected intraperitoneally with 500 tachyzoites. This inoculum is lethal in untreated mice, usually by day 8 to 10. Immediately following infection, mice were administered either NPMG (100mg/kg/day), pyrimethamine (12.5mg/kg/day) or sulphadiazine (85mg/kg/day) as a drinking water additive. SHAM (40mg/kg/day) was

administered by intraperitoneal injection. A combination of NPMG (100mg/kg/day) and pyrimethamine (12.5mg/kg/day) was also given as a drinking water additive. Mortality was then recorded daily. After 30 days the drugs were withdrawn from surviving mice. The number of surviving mice in untreated control and treated groups was compared using a non-parametric Mann Whitney U-test.

Sequencing of chorismate synthase from *T.gondii*

The WashU-Merck Toxoplasma EST project database (Washington University, St.Louis, MO, USA) was searched for sequences with homology with any shikimate pathway enzyme. A clone was found with homology to chorismate synthase of tomato. This EST clone, TgESTzyllc05.r1, (putative chorismate synthase), was obtained from the *T.gondii* EST project, . This clone was supplied as a phage stock which contained a cDNA insert from the tachyzoite stage (RH strain). The insert had been cloned into the XhoI (3') to EcoRI (5') site of the Lambda Zap II phage (Stratagene, La Jolla, CA, USA).

In vivo excision

E.coli for use in the *in vivo* excision protocol were prepared as follows. *E.coli* (XL1-Blue, Stratagene) were grown in NZY (Select agar, Gibco, casamino acids and yeast extract, both from Becton-Dickinson, Franklin Lakes, NJ, USA) containing 0.2% maltose and 10mM $MgSO_4$ in a shaking incubator at 37°C until an OD_{600} of 0.5 was obtained. Cells were then centrifuged for 10 minutes at 1000g and the resulting pellet resuspended in half the original volume of 10mM $MgSO_4$. These cell suspensions were then stored at 4 °C until ready for use.

Phagemid particles containing the putative chorismate synthase clone were obtained by following the *in vivo* excision protocol as recommended by Stratagene. Briefly 200µl of the *E.coli*, XL1-blue, cell suspension was combined with 200µl of the Lambda Zap II phage stock, containing the EST clone and 1µl of R408 helper phage (Stratagene). This mixture was incubated at 37°C for 15 minutes. Following this, 5mls of 2 times YT media (yeast extract, Becton-Dickinson, and tryptone extract, Difco) was added and cells suspensions incubated for a further 3hours at 37°C with shaking. Next, the cell suspension was placed in a water bath at 70°C for 20 minutes to lyse the cells, and then centrifuged for 5 minutes at 4000g. The resulting supernatant which contained the pBluescript phagemid as filamentous phage particles was decanted and stored at 4°C until ready for use.

Cloning

Aliquots (200µl) of the supernatant containing pBluescript phagemid were used to infect 200µl of XL1-blue host cells. Following a 15 minute incubation, 100µl of each suspension was plated onto Luria Broth Agar (Gibco) containing ampicillin (50µg/ml) (LB/Amp) and incubated overnight at 37°C. Colonies were picked from these plates and used to inoculate 5ml cultures of LB/Amp. These cultures were incubated overnight at 37°C with shaking. Plasmid DNA was purified from these cultures using a Wizard mini-prep kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

The cDNA insert was then obtained from the plasmid DNA by simultaneously digesting the plasmid with restriction enzymes, Xho1 and EcoR1 (Boehringer

Mannheim, Indianapolis, IN, USA). The digests were performed for 1 hour at 37°C. Digest products were separated by 1% agarose gel electrophoresis and visualised using ethidium bromide. Analysis revealed an insert of approximately 2.4kB in 8 out of 10 cultures examined.

A sample of one of these original cultures was used to subinoculate 25mls cultures of LB/Amp and incubated at 37°C, overnight with shaking. Sequencing quality plasmid DNA was purified from this culture using a Qiagen maxi-prep kit (Qiagen, Santa Clarita, CA, USA). The absorbance of the resulting DNA was measured at 260 and 280nm and purity assessed by OD_{260}/OD_{280}. The concentration of the DNA was then calculated as OD_{260}x50x100 (dilution factor). The concentration of the DNA was then adjusted to 200μg/ml for sequencing.

Sequencing

Sequencing was performed using the automated sequencer at the University of Chicago, Molecular Biology Facility. Templates consisted of the full length clone or subclones in Bluescript SK or KS plasmids (Stratagene). Primers were M13, or reverse (Stratagene), or were custom made according to sequencing information already obtained. The sequence of custom made primers is shown in Table 5.1.

Table 5.2 Nucleotide sequences of custom designed primers

PRIMER	SEQUENCE
1	5' TGT CCA AGA TGT TCA GCC T 3'
2	5' AGG CTG ATC ATC TTG GAC A 3'
3	5' TCG GGT CTG GTT GAT TTT 3'
4	5' GAG AGA GCG TCG TGT TCA T 3'
5	5' ATG AAC ACG ACG CTC TCT C 3'
6	5' CAT GTC GAG AAG TTG TTC 3'
7	5' GAA CAA CTT CTC GAC ATG 3'
8	5' ACT TGT GCA TAC GGG GTA C 3'
9	5' GTA CCC CGT ATG CAC AAG T 3'
10	5' TGA ATG CAA CTG AAC TGC 3'
11	5' GCA GTT CAG TTG CAT TCA 3'
12	5' AGC CGT TCC GTG TAT AAT C 3'
13	5' CTA CGG CAC CAG CTT CAC 3'
14	5' CGT CCT TCC TCA ACA CAG TG 3'
15	5' GTG AAG CTG GTG CCG TAG 3'
16	5' CGC CTC TGA TTT GGA AGT G 3'
17	5' TCT GCC GCA TTC CAC TAG 3'
18	5' GAA GCC AAG CAG TTC AGT T 3'

Sub-cloning

The plasmid DNA was then digested with combinations of PstI, EcoRI, HincII, and XhoI (Boehringer Mannheim). Specifically, simultaneous digestions were performed on 10μg of the plasmid DNA with HincII and PstI, and PstI and XhoI for 1 hour at 37°C. Digest products were separated by 1% agarose gel electrophoresis and visualised using ethidium bromide. Analysis of the HincII and PstI digest showed 3 bands of 500kB, 800kB, 300kB and a >4000kB band corresponding to the pBluescript. Analysis of the PstI and XhoI digest showed 2 bands of 800kB and 1600kB as well as a >3000kB band corresponding to the pBluescript. This allowed construction of a restriction map (Figure 5.1).

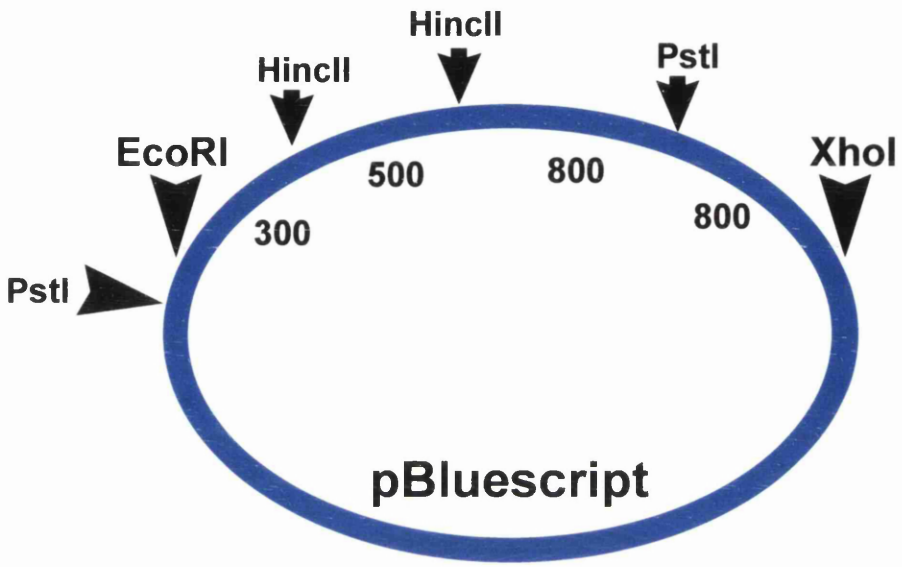


Figure 5.1 Restriction map for putative *T.gondii* chorismate synthase cDNA insert in pBluescript SK plasmid.
The large arrowheads indicates restriction sites on the plasmid. The *T.gondii* cDNA insert has been cloned into the XhoI (3') to EcoRI (5') site. The small arrows indicate restriction sites identified within the cDNA insert by simultaneous enzyme digests. The sizes of the resulting fragments are shown in kB.

The 800bp fragment from the HincII and PstI digest (corresponding with 800-1600bp) and the 800bp fragment from the PstI and XhoI digest (corresponding with the last 800bp) were purified from this gel using a Qiax gel purification kit according to the manufacturer's instructions (Qiagen). These portions of cDNA were then ligated into Bluescript SK (Stratagene) using T4 ligase (Promega) at 14 °C overnight. These ligation reactions were then used to transform subcloning efficiency DH5α competent cells (Gibco). Specifically 10μl of ligation reactions were added to 50μl of competent cells and gently mixed. The cells were incubated on ice for 30 minutes after which they were heat shocked for 20 seconds at 37°C . Following heat shock, cells were placed on ice for 2 minutes after which 0.95mls of room temperature LB was added. The cells were then incubated for 1 hour at 37°C with shaking.

A 50μl aliquot of the transformation mixture was spread on an LB agar plate containing 100μg/ml of ampicillin and 50μg/ml of X-gal (Sigma). The plate was incubated overnight at 37°C. The following day white colonies were picked and used to inoculate 5ml LB/Amp cultures. These cultures were incubated overnight at 37°C with shaking. Plasmid DNA was purified from these cultures using a Wizard mini-prep kit (Promega) according to the manufacturer's instructions.

To confirm the identity of the subclones, restriction enzyme digests were performed on the plasmid DNA followed by agarose gel electrophoresis as before. Samples of corresponding cultures was then used to subinoculate 25mls cultures of LB/Amp for the preparation of sequencing quality plasmid DNA. Sequencing of the subclones was performed as previously described.

Analysis of cDNA and predicted amino acid sequences

Sequencing chromatograms were edited and nucleotides assembled using Sequencer Version 3.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Both strands of the entire clone were sequenced at least once but usually twice. The resulting nucleotide sequence was analysed for open reading frames (ORF) using Sequencer. A list of chorismate synthases from other organisms was obtained from Genbank (Accession numbers: *Synechocystis*, P23353; *Solanum lycopersicum*, Z21796; *Saccharomyces cerevisiae*, P28777; *Neurospora crassa*, Q12640; *Haemophilus influenza*, P43875; *Plasmodium falciparum*, AF008549). The predicted amino acid sequence was aligned with chorismate synthases of other organisms using CLUSTAL X (Thompson *et al.*, 1997).

Demonstration of alternative oxidase by SDS-PAGE and western blotting

Samples

Tachyzoites (RH) were obtained from the peritoneal exudate of infected ND4 mice. After centrifugation the pellet was subjected to 3 cycles of freeze thawing. Protein concentration was measured using the Bradford assay (Bradford, 1976). 30μg of tachyzoites were loaded for each centimetre of gel.

A *T.gondii* mitochondrial enriched extract was donated by Stan Tomavo, Pasteur Institute, France. 10μg of protein was loaded for each centimetre of gel.

Sauromatum guttatum (Voodoo lily) mitochondrial protein (donated by Lee McIntosh, Michigan State University, USA) and *T.bruceii* mitochondrial protein

(donated by George Hill, Meharry Medical College, Tennessee, USA) were used as positive controls. 30μg of Voodoo lily mitochondrial protein and 10 μg of *T.bruceii* mitochondrial protein were loaded for each centimetre of gel.

Prior to use samples were stored at 4°C. For SDS-PAGE they were boiled for 5 minutes in sample buffer (75mM Tris, 5% glycerol, 1% SDS and 0.001% bromophenol blue, pH 6.8). For Urea-SDS-PAGE samples were boiled for 10 minutes in sample buffer containing 6.0M urea. 2-mercaptoethanol (1%) was added to all samples immediately before loading.

Primary Antibodies

Murine polyclonal antibody to *T.bruceii* alternative oxidase (serum), murine monoclonal antibody to *T.bruceii* alternative oxidase (7D3) and murine monoclonal antibody to *T.bruceii* alternative oxidase (IA2) (both hybridoma tissue culture supernatants) were all donated by George Hill, Meharry Medical College, Tennessee, USA. The polyclonal antibody was used at a dilution of 1 in 1000. Both monoclonal antibodies were used at a 1 in 100 dilution.

Murine polyclonal antibody to Voodoo lily alternative oxidase (serum) and murine monoclonal antibody to Voodoo lily alternative oxidase (a hybridoma tissue culture supernatant) were donated by Lee McIntosh, Michigan State University, USA. The polyclonal and monoclonal antibodies were used at a dilution of 1 in 1000 and 1 in 500 respectively.

All primary antibodies were diluted in Phosphate Buffered Saline, 0.1%TWEEN-20 (PBS/Tween).

Secondary Antibody

Horse Radish Peroxidase (HRP) conjugated goat anti-mouse antibody (Sigma) was diluted 1 in 800, in PBS/TWEEN.

SDS-PAGE

Gels containing 10% (separation gel) or 3% (stacking gel) acrylamide were prepared from a stock solution of 30% acrylamide and 0.8% N,N'-bis-methylene acrylamide. The separation gel also contained 0.375M Tris-HCl (pH 8.8) and 0.1% sodium dodecylsulphate (SDS). Similarly the stacking gel contained 0.125M Tris-HCl (pH 6.8) and 0.1 % SDS. The gels were polymerised by the addition of 0.025% tetramethylethylenediamine (TEMED) (Sigma) and ammonium persulphate (Sigma). When polymerised the gel was immersed in electrode buffer (pH 8.3) containing 0.025M Tris, 0.192M glycine and 0.1% SDS. Samples were loaded onto the gel and electrophoresed for 1 hour at 200V. Following electrophoresis the samples were transferred to a PVDF membrane (Micron Separations Inc., Westborough, PA,USA) at 4°C using a semi-dry electro-blotter.

Urea- SDS-PAGE

Urea-SDS-PAGE was performed as for SDS-PAGE with the addition of 2.5M urea to both separation and stacking gels.

Western blotting

After protein transfer, the PVDF membrane was blocked in PBS, 10% dried milk, 0.1%TWEEN 20, for 1 hour at room temperature. Following three washes in PBS/TWEEN the membrane was incubated with primary antibody for 1 hour at room temperature on a shaking plate. Negative controls were incubated in PBS/TWEEN without primary antibody. The membrane was then washed three times in PBS/TWEEN prior to a similar 1 hour incubation with the secondary antibody. The membrane was washed a further 3 times in PBS/TWEEN followed by a final wash in PBS.

Detection of proteins was performed by enhanced chemoluminesence (ECL). The membranes were incubated with ECL reagents 1 and 2 (Amersham) for 2 minutes. The membrane was then immediately applied to Hyperfilm ECL (Amersham) and left in the dark for up to 1 hour before developing.

5.4 Results

The Shikimate Pathway

In vitro experiments

Toxicity assays

NPMG was found to be non-toxic to HFF at doses of 10mM and below, $p>0.304$ (Figure 5.2A). Serial dilutions of NPMG (4.5 to 1.56mM) were then used in the parasite growth inhibition assays.

Parasite growth inhibition assays

NPMG inhibited the growth of *T.gondii* tachyzoites in a dose dependent manner (4.5mM, $p<0.0001$), (Figure 5.2B). Similarly inhibition of parasite growth was visualised in giemsa stained preparations. Only small numbers of parasites were seen in NPMG treated cultures and the fibroblast monolayer was otherwise intact and appeared normal. In contrast, in control cultures there was extensive destruction of the monolayer and remaining groups of cells infected with tachyzoites (Figure 5.2C).

Product rescue assays

PABA was found to be non-toxic to HFF at doses of 5mM and below, ($p>0.956$). Ethanol was also non-toxic to HFF at this dilution (1 in 73; $p>0.977$). Addition of exogenous PABA ablated the inhibitory effects of NPMG on the growth of *T.gondii* in a dose-dependant manner (Figure 5.3A). For example, at a concentration of 10μM, PABA reduced NPMG inhibition of parasite growth by 60%. However 2.5μM PABA was unable to 'rescue' parasites from the inhibitory effects of NPMG. As a direct antagonist, PABA was also able to ablate the effects of sulphadiazine in a dose

dependent manner. However, as anticipated, it was unable to ablate the effects of pyrimethamine which acts on dihydrofolate reductase an enzyme further downstream in the folate synthesis pathway (Figure 5.3B).

Synergy assays

NPMG was found to act synergistically with both sulphadiazine and pyrimethamine to restrict the growth of *T.gondii in vitro* (Table 5.1). Thus at a concentration of 3.25mM NPMG and 6.25μg/ml sulphadiazine both inhibited parasite growth by 60%. In combination they were able to inhibit 97% of parasite growth which was greater than the 86% predicted if the effect was purely additive. Similarly pyrimethamine was completely ineffective at restricting parasite growth at a concentration of 0.025μg/ml. However in combination with NPMG it increased the inhibition of parasite growth from 60% to 86%.

In vivo survival experiments

The survival of mice treated with NPMG or pyrimethamine alone was not significantly enhanced compared with that of untreated control mice. However, simultaneous administration of NPMG and pyrimethamine protected approximately 50% of mice from death. This difference in survival was significant when compared with untreated mice or mice administered NPMG or pyrimethamine alone ($p<0.05$), (Figure 5.4). Furthermore, surviving mice showed no apparent illness and remained sleek and healthy in appearance during the 30 days of treatment after infection. Following withdrawal of the drugs the mice remained well and showed no signs of disease reactivation. Control mice treated with high dose sulphadiazine all survived.

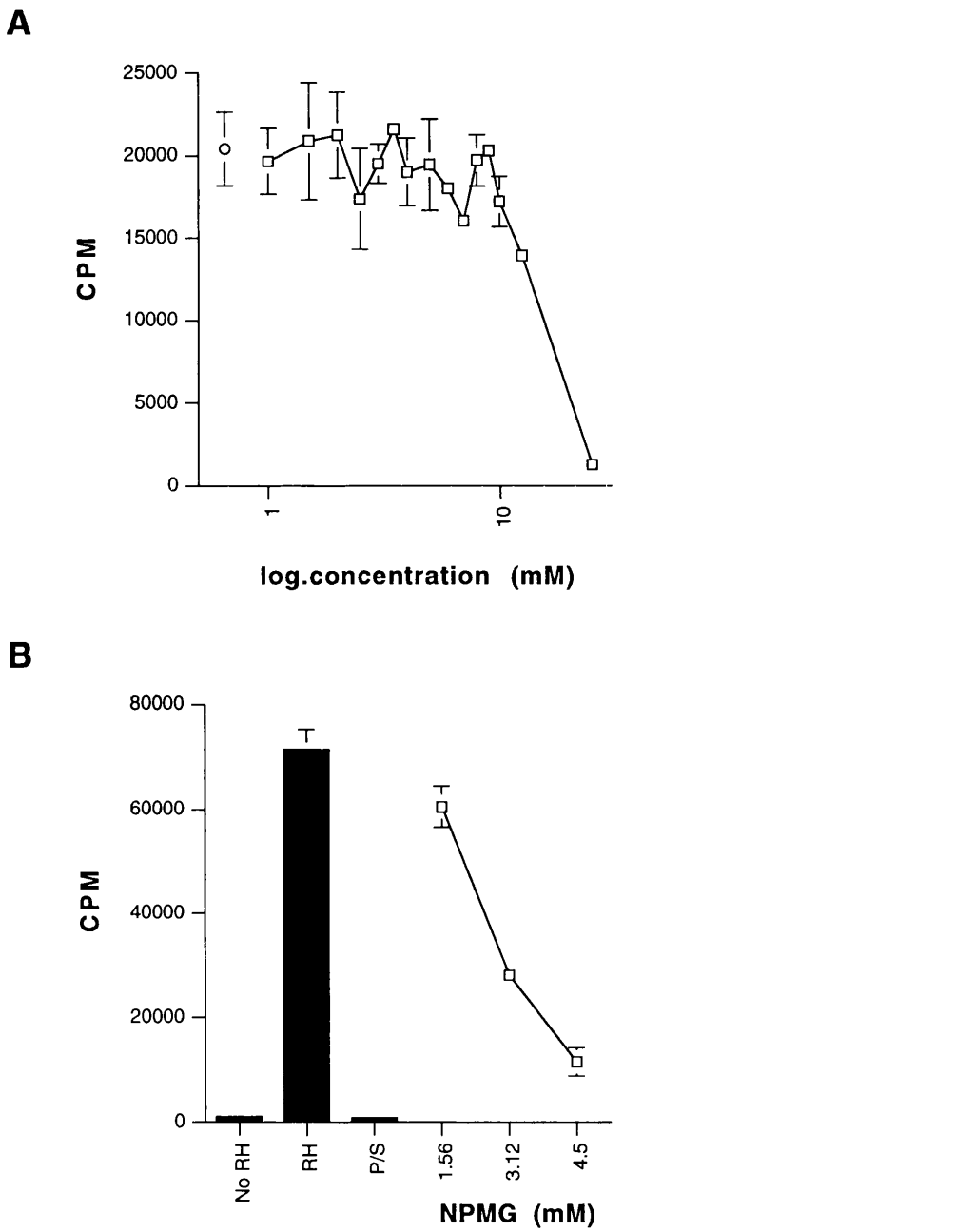


Figure 5.2 **A** The toxicity of NPMG to HFF as measured by uptake of tritiated thymidine. Concentrations of 10mM and below were non-toxic to the fibroblast monolayer ($p>0.304$). (○, control cultures; ❑, NPMG treated cultures). **B** The effect of NPMG on the growth of *T.gondii* in HFF as measured by uptake of tritiated uracil. At a concentration of 4.5mM NPMG inhibited over 85% of parasite growth ($p<0.0001$). Abbreviations: No RH, uninfected control cultures; RH, control cultures infected with RH; P/S infected control cultures treated with pyrimethamine and sulphadiazine.

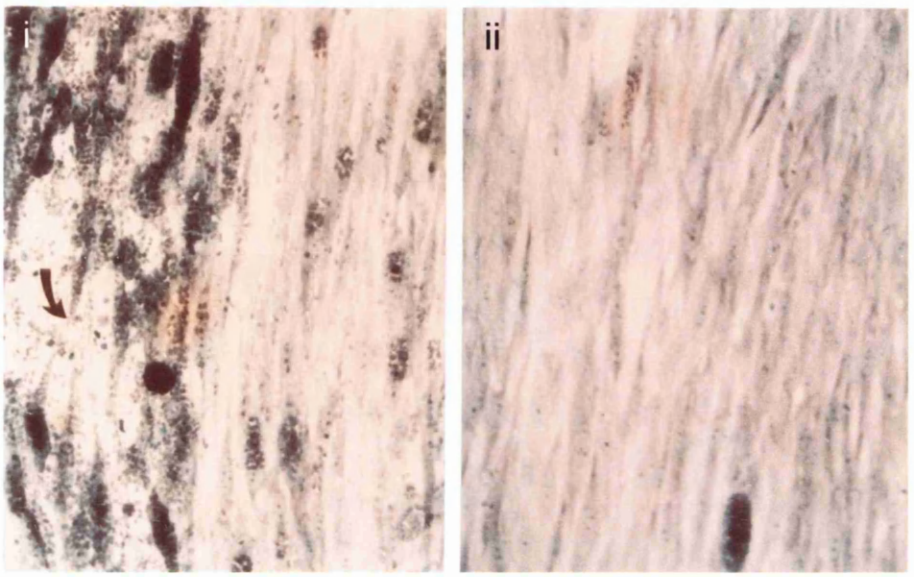


Figure 5.2 ***continued*** **C The effect of NPMG on the growth of *T.gondii* in HFF. (i) Control cultures showing large groups of parasites, destruction of the fibroblast monolayer and numerous extracellular parasites (arrow). (ii) Cultures treated with NPMG (4.5mM). Only small groups of parasites are identified and the fibroblast monolayer is intact. (Giemsa, x 250).**

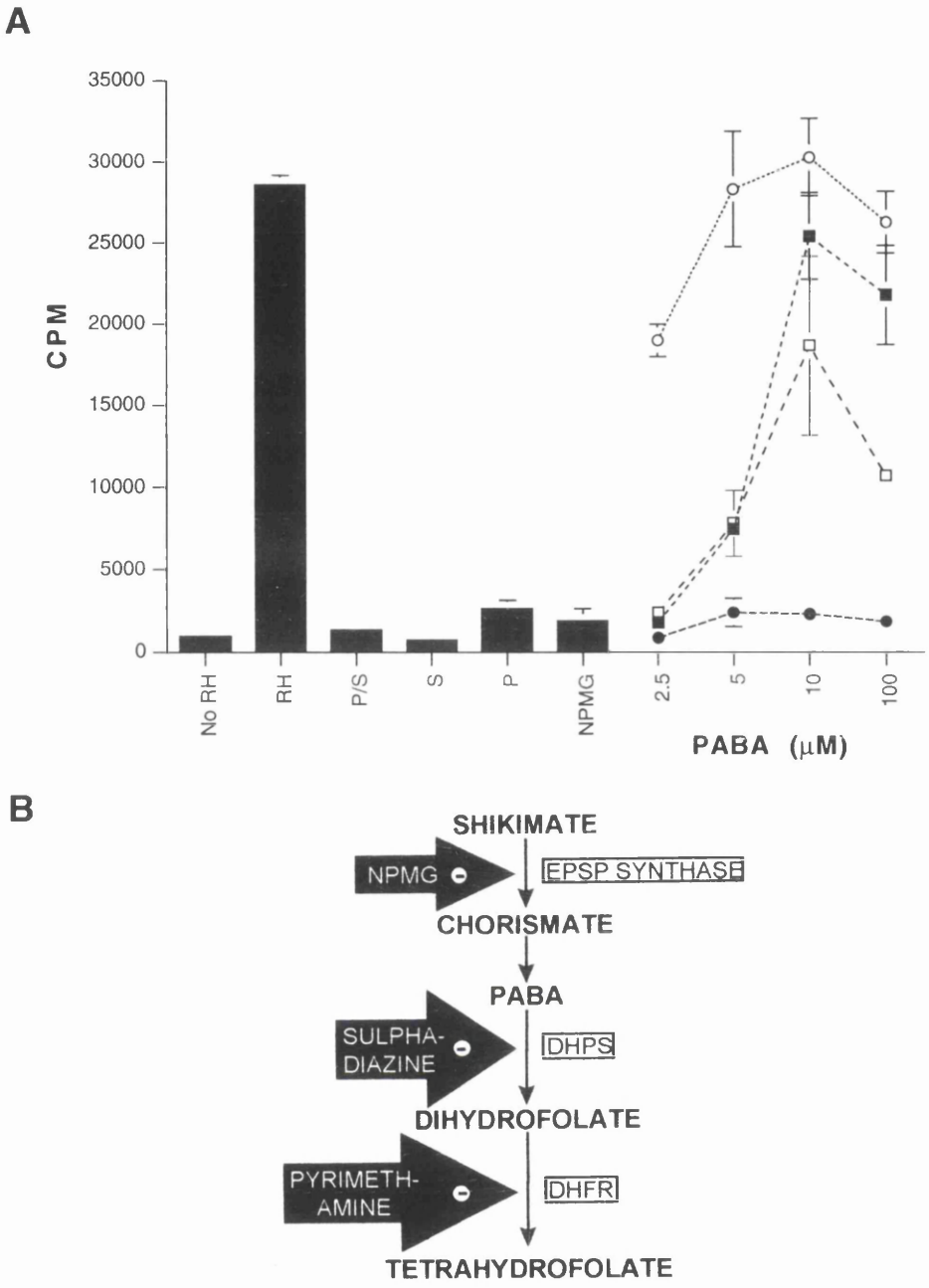


Figure 5.3 **A** Product rescue of the inhibitory effect of NPMG on the growth of *T.gondii.* Addition of PABA reduced the effects of NPMG and sulphadiazine in a dose dependent manner. PABA had no effect on pyrimethamine treated cultures. Key: PABA(○); PABA+NPMG(❑); PABA+sulphadiazine(■); and PABA+ pyrimethamine(●). Abbreviations: No RH, uninfected control cultures; RH, control cultures infected with RH; P/S, infected cultures treated with pyrimethamine and sulphadiazine; S, P and NPMG, infected cultures treated with sulphadiazine, pyrimeth-amine and NPMG, respectively. **B** Diagram of the shikimate pathway and folate synthesis pathway showing site of action of NPMG, sulphadiazine and pyrimethamine. NPMG inhibits EPSP synthase above PABA in the shikimate pathway. Sulphadiazine competes with PABA for dihydropteroate synthase (DHPS). Pyrimethamine acts below PABA on dihydrofolate reductase (DHFR).

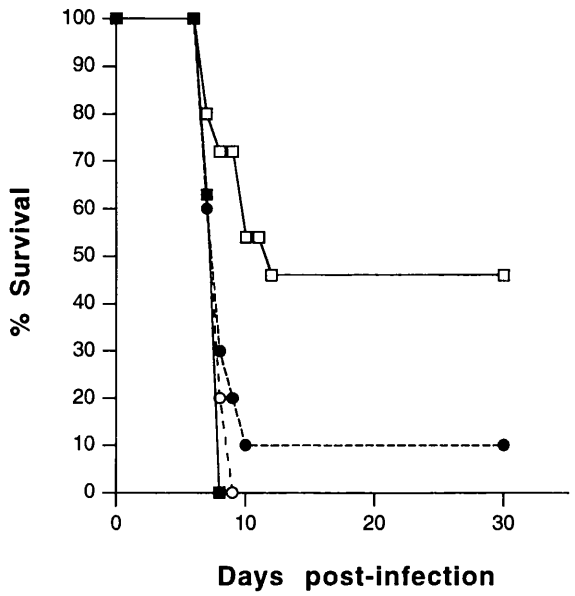


Figure 5.4 ***In vivo*** **activity of NPMG and pyrimethamine against** ***T.gondii*** **infection in mice. Treatment with either drug alone was not sufficient to prevent death (■, NPMG; ●, pyrimethamine) compared with control mice (○). In contrast 50% of mice administered NPMG and pyrimethamine simultaneously (❑) survived infection (at 7 days post-infection, $p<0.05$).**

Table 5.2 Effects of Inhibitors alone and incombination on the growth of *T.gondii in vitro*

Inhibitor A	Inhibitor B	CPM Untreated	CPM for A	CPM for B	CPM for A+B		Ratio
					Actual	Predicted	Actual:Predicted*
NPMG	Sulphadiazine	71449+/-3763	28138+/-2216	25026+/-4365	2368+/-418	9856	0.24
NPMG	Pyrimethamine	64343+/-1222	25097+/-1398	69217+/-3253	9354+/-2126	25097	0.37
NPMG	SHAM	64343+/-1222	25097+/-1398	42993+/-1098	7554+/-970	16769	0.45

Predicted CPM for Inhibitor A+ Inhibitor B (if effect is only additive) is calculated as (CPM Inhibitor A x CPM Inhibitor B)/CPM of untreated cultures. Concentrations were: NPMG (3.25mM); Sulphadiazine (6.25μg/ml); Pyrimethamine (0.025μg/ml); SHAM(0.19μg/ml)

* A ration of Actual:Predicted of <0.5 is considered synergistic. A ratio of Actual:Predicted of >0.5 is considered to be additive.

Sequencing of chorismate synthase from *T.gondii*

Both strands of the putative *T.gondii* chorismate synthase were sequenced and found to be 2312 bases in length (Figure 5.5a). Analysis revealed a large open reading frame of 1608 base pairs which would encode a 536 amino acid protein. The deduced amino acid sequence has considerable identity (44.5 to 51.4%) with chorismate synthases of diverse species (Figure 5.5b). Furthermore the putative *T.gondii* chorismate synthase shares all the amino acids known to be highly conserved in chorismate synthases of other organisms. The main difference between the putative *T.gondii* protein and other known chorismate synthases is in length. Chorismate synthases from other organisms range in length from 357 to 432 amino acids. The larger size of the *T.gondii* protein is mainly due to a number of insertions which on the basis of their amino acid composition appear to form exposed loops.

```
                                                  CT CAT CTT CTC GGT TTC  17

ACT TTT CTT TGA GTG CCT GTG TGA GAG ACG GTC GTC GCA ACA AGA ATC  65

TCC TCC GCT CAC GCC TTT CCT CAC AGT CCT GTT TTT CCT CCA GCT GTC  113

ACA CAT CCC GCT CGT TCC GCT GCA TCT CCT CAC ATT TCT TGC AGT CAG  161

ATG TCT TCC TAT GGA GCC GCT CTG CGC ATA CAC ACT TTC GGT GAA TCT  209
 M   S   S   Y   G   A   A   L   R   I   H   T   F   G   E   S   16
CAC GGC TCA GCC GTT GGG TGT ATA ATC GAC GGG CTG CCT CCT CGC CTC  257
 H   G   S   A   V   G   C   I   I   D   G   L   P   P   R   L   32
CCT CTT TCT GTC GAA GAT GTT CAG CCT CAA TTA AAT CGC AGA AGA CCC  305
 P   L   S   V   E   D   V   Q   P   Q   L   N   R   R   R   P   48
GGC CAA GGG CCT CTC TCG ACG CAG CGG AGA GAG AAA GAT CGA GTC AAC  353
 G   Q   G   P   L   S   T   Q   R   R   E   K   D   R   V   N   64
ATA CTC TCC GGT GTT GAA GAC GGA TAT ACA CTC GGT ACT CCC CTG GCG  401
 I   L   S   G   V   E   D   G   Y   T   L   G   T   P   L   A   80
ATG CTC GTC TGG AAT GAA GAC CGG CGG CCC CAG GAC TAC CAC GCC CTC  449
 M   L   V   W   N   E   D   R   R   P   Q   D   Y   H   A   L   96
GCG ACA GTC CCG CGT CCA GGT CAC GGG GAT TTC ACC TAC CAT GCA AAG  497
 A   T   V   P   R   P   G   H   G   D   F   T   Y   H   A   K   112
TAC CAC ATT CAC GCG AAA AGC GGG GGC GGT CGG AGC AGC GCG CGG GAG  545
 Y   H   I   H   A   K   S   G   G   G   R   S   S   A   R   E   128
ACT TTG GCG CGC GTC GCC GCT GGA GCA GTC GTT GAG AAG TGG CTA GGC  593
 T   L   A   R   V   A   A   G   A   V   V   E   K   W   L   G   144
ATG CAC TAC GGC ACC AGC TTC ACA GCT TGG GTC TGT CAG GTT GGT GAT  641
 M   H   Y   G   T   S   F   T   A   W   V   C   Q   V   G   D   160
GTC TCT GTG CCC CGA TCG CTC CGA AGA AAG TGG GAG CGG CAG CCG CCA  689
 V   S   V   P   R   S   L   R   R   K   W   E   R   Q   P   P   176
ACT CGC CAA GAC GTC GAT CGC CTT GGC GTG GTC CGC GTG AGC CCA GAT  737
 T   R   Q   D   V   D   R   L   G   V   V   R   V   S   P   D   192
GGA ACC ACA TTT CTC GAC GCG AAC AAC CGC CTT TAC GAC GAG CGA GGA  785
 G   T   T   F   L   D   A   N   N   R   L   Y   D   E   R   G   208
GAG GAA CTC GTC GAG GAG GAA GAC AAA GCC AGG CGT CGG CTT CTT TTC  833
 E   E   L   V   E   E   E   D   K   A   R   R   R   L   L   F   224
GGA GTC GAC AAC CCG ACG CCA GGA GAA ACA GTG ATT GAG ACC AGG TGC  881
 G   V   D   N   P   T   P   G   E   T   V   I   E   T   R   C   240
CCG TGC CCC TCC ACA GCT GTT CGC ATG GCT GTG AAA ATC AAC CAG ACC  929
 P   C   P   S   T   A   V   R   M   A   V   K   I   N   Q   T   256
CGA TCT CTG GGC GAT TCG ATT GGC GGA TGC ATC TCC GGT GCA ATC GTG  977
 R   S   L   G   D   S   I   G   G   C   I   S   G   A   I   V   272
CGG CCA CCG CTG GGC CTC GGC GAG CCG TGT TTC GAC AAA GTG GAG GCG  1025
 R   P   P   L   G   L   G   E   P   C   F   D   K   V   E   A   288
GAG CTG GCG AAG GCG ATG ATG TCG CTC CCT GCT ACG AAA GGG TTT GAG  1073
 E   L   A   K   A   M   M   S   L   P   A   T   K   G   F   E   304
ATT GGC CAG GGC TTT GCG AGT GTC ACG TTG CGA GGC AGC GAG CAC AAC  1121
 I   G   Q   G   F   A   S   V   T   L   R   G   S   E   H   N   320
GAC CGC TTC ATT CCC TTC GAG AGA GCG TCG TGT TCA TTC TCG GAA TCA  1169
 D   R   F   I   P   F   E   R   A   S   C   S   F   S   E   S   336
GCC GCG AGC ACG ATC AAG CAT GAA AGA GAT GGG TGT TCA GCT GCT ACA  1217
 A   A   S   T   I   K   H   E   R   D   G   C   S   A   A   T   352
CTC TCA CGG GAG CGA GCG AGT GAC GGT AGA ACA ACT TCT CGA CAT GAA  1265
 L   S   R   E   R   A   S   D   G   R   T   T   S   R   H   E   368
GAG GAG GTG GAA AGG GGG CGG GAG CGC ATA CAG CGC GAT ACC CTC CAT  1313
 E   E   V   E   R   G   R   E   R   I   Q   R   D   T   L   H   384
GTT ACT GGT GTA GAT CAG CAA AAC GGC AAC TCC GAA GAT TCA GTT CGA  1361
 V   T   G   V   D   Q   Q   N   G   N   S   E   D   S   V   R   396
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TAC ACT TCC AAA TCA GAG GCG TCC ATC ACA AGG CTG TCG GGA AAT GCT  1409
 Y   T   S   K   S   E   A   S   I   T   R   L   S   G   N   A   416
GCC TCT GGA GGT GCT CCA GTC TGC CGC ATT CCA CTA GGC GAG GGA GTA  1457
 A   S   G   G   A   P   V   C   R   I   P   L   G   E   G   V   432
CGG ATC AGG TGT GGA AGC AAC AAC GCT GGT GGA ACG CTC GCA GGC ATT  1505
 R   I   R   C   G   S   N   N   A   G   G   T   L   A   G   I   448
ACA TCA GGA GAG AAC ATT TTT TTT CGG GTG GCC TTC AAG CCT GTT TCT  1553
 T   S   G   E   N   I   F   F   R   V   A   F   K   P   V   S   464
TCC ATC GGC TTG GAA CAA GAA ACT GCA GAC TTT GCT GGT GAA ATG AAC  1601
 S   I   G   L   E   Q   E   T   A   D   F   A   G   E   M   N   480
CAG CTA GCT GTG AAA GGC CGC CAC GAT CCC TGC GTC CTT CCG CGA GCC  1649
 Q   L   A   V   K   G   R   H   D   P   C   V   L   P   R   A   496
CCT CCT CTG GTT GAG AGC ATG GCT GCC CTT GTG ATT GGC GAT CTG TGC  1697
 P   P   L   V   E   S   M   A   A   L   V   I   G   D   L   C   512
CTC CGC CAG CGC GCC CGG GAA GGG CCG CAC CCC CTT CTC GTC CTT CCT  1745
 L   R   Q   R   A   R   E   G   P   H   P   L   L   V   L   P   528
CAA CAC AGT GGT TGC CCA TCT TGC TGA GCT CTA CCT TGT TCC AAA AAC  1793
 Q   H   S   G   C   P   S   C   *                               536
TTG TGC ATA CGG GGT ACA CCA GGT TCC TCA CAA GGA GAA TCG TGA GGC  1841

GGT GAC TGG CCA GCG CCA CAG ATT GCT GTT CAT GCA CAA GAA AGA AAA  1889

CAG CGC ATT TCC GCC ACA ACC CAG CTG CAT GAA GTT GCT GGA TAT CGT  1937

TCC GGC GGT GCT CGG CCT TCT TCT CTA CGC TCG CGA TGA TAC GTC GCG  1985

AGC TTC ATC AAG CTC CTT TTG CAT TGT TAG TGG CTC CCA ACA GAA CCC  2033

TTT GTG GAA GGG AAT CTG GTC TCA CGC TTG CAG GAG AGA GTT CGC CTT  2081

TGT TCA CGA AAT AAC GAA GCC AAG CAG CTC AGT TGC ATT CAG CCT GCA  2129

CAC AGT TGC ATT CAG CCT GCA CAC TAA ACA CGG GCG AAA TCG TCG CGT  2177

GAT ATG TAG TTC TTC GGT TGT CAC GGT GAT TGT CGT CGT GTT TGA ACA  2225

ACT AAA CGT TTC TAA TGC TGG ATC TTA AAA AAA AAA AAA AAA AAA AAA  2273

AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA         2312
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Figure 5.5A The cDNA and deduced amino acid sequences of *T.gondii* chorismate synthase. The cDNA sequence was found to be 2312 base pairs in length. It contains a long open reading frame of 1608 base pairs (bold) that encodes a 536 amino acid protein. The deduced amino acid sequence of this open reading frame is shown beneath the cDNA sequence.

```
Synechocystis       ----------------------------------------------------MGNTFG
S.lycopersicum      MASFVPTKQFVGASSSSDIGSSRLVSLQLPSKFSSSNFHLPSRPSQLKRLEIQAAGSTFG
S.cerevisiae        ----------------------------------------------------MSTFG
N.crassa            ----------------------------------------------------MSTFG
H.influenza         ---------------------------------------------------MAGNTIG
T.gondii            ----------------------------------------------------MSSYG
P.falciparum        ----------------------------------------------------MSTYG
                                                                           *

Synechocystis       SLFRITTFGESHGGGVGVIIDGCPPRLEISPEEIQVDLDRRRPGQSKITTPRKEADQCEI
S.lycopersicum      NYFRVTTFGESHGGGVGCIIDGCPPRLPLSESDMQVELDRRRPGQSRITTPRKETDTCKI
S.cerevisiae        KLFRVTTYGESHCKSVGCIVDGVPPGMSLTEADIQPQLTRRRPGQSKLSTPRDEKDRVEI
N.crassa            HYFRVTTYGESHCKSVGCIVDGVPPGMELTEDDIQPQMTRRRPGQSAITTPRDEKDRVII
H.influenza         QLFRVTTFGESHGIALGCIVDGVPPNLELSEKDIQPDLDRRKPGTSRYTTPRREDDEVQI
T.gondii            AALRIHTFGESHGSAVGCIIDGLPPRLPLSVEDVQPQLNRRRPGQGPLSTQRREKDRVNI
P.falciparum        TLLKVTSYGESHGKAIGCVIDGFLSNIEINFDLIQKQLDRRRPNQSKLTSNRNEKDKLVI
                            ****    *   **           *    ** *        * * *   *

Synechocystis       LSGVFEGKTLGTPIAILVRNKDARSQDYN--EMAVKYRPSHADATYEAKYGIRNWQGGGR
S.lycopersicum      SSGTADGLTTGSPIKVEVPNTDQRGNDYS--EMSLAYRPSHADATYDFKYGVRSVQGGGR
S.cerevisiae        QSGTEFGKTLGTPIAMMIKNEDQRPHDYS--DMDKFPRPSHADFTYSEKYGIKASSGGGR
N.crassa            QSGTEFGVTLGTPIGMLVMNEDQPPKDYGNKTMDIYPRPSHADWTYLEKYGVKASSGGGR
H.influenza         LSGVFEGKTTGTSIGMIIKNGDQRSQDYG--DIKDRFRPGHADFTYQQKYGIRDYRGGGR
T.gondii            LSGVEDGYTLGTPLAMLVWNEDRRPQDYH--ALATVPRPGHGDFTYHAKYHIHAKSGGGR
P.falciparum        LSGFDENKTLGTPITFLIYNEDIKKEDYN--SFINIPRPGHGDYTYFMKYHVKNKSGSSR
                     **     * *         * *    **          ** * * **  **      * *

Synechocystis       SSARETIGRVAAGAIAKKILAQFNGVEIVAYVKSIQDI----------------------
S.lycopersicum      SSARETIGRVAAGAVAKKILKLYSGAEVLAYVSQVHQVVLP------------------
S.cerevisiae        ASARETIGRVASGAIAEKFLAQNSNVEIVAFVTQIGEIKMN------------------
N.crassa            SSARETIGRVAAGAIAEKYLKPRYGVEIVAFVSSVGSEHLFPPTAEHP-----------
H.influenza         SSARETAMRVAAGAIAKKYLREHFGIEVRGFLSQIGNIKIAP-----------------
T.gondii            SSARETLARVAAGAVVEKWLGMHYGTSFTAWVCQVGDVSVPRSLRRKWE-RQPPTRQDVD
P.falciparum        FSGRETATRVAAGACIEQWLYKSYNCSIVSYVHSVGNIKIPEQVSKELENKNPPSRDLVD
                     * ***  *** **     *

Synechocystis       ------------EATVDSNTVTLE-----------------------------------Q
S.lycopersicum      ------------EDLIDHQNVTLE-----------------------------------Q
S.cerevisiae        ------RDSFDPEFQHLLNTITRE-----------------------------------K
N.crassa            ------SPSTNPEFLKLVNSITRE-----------------------------------T
H.influenza         -------------QK-VGQIDWE-----------------------------------K
T.gondii            RLGVVRVSPDGTTFLDANNRLYDE-----------------------------------R
P.falciparum        SYGTVRYNEKEKIFMDCFNRIYDMNASMLKTDEYNKNTLTIPSIDNTYINVKTNECNINQ

Synechocystis       VESN------------------------IVRCPDEECAEKMIERIDQVLRQKDSIGGVV
S.lycopersicum      IESN------------------------IVRCPDPEYAEKMIAAIDAVRVRGDSVGGVV
S.cerevisiae        VDSMG-----------------------PIRCPDASVAGLMVKEIEKYRGNKDSIGGVV
N.crassa            VDSFL-----------------------PVRCPDAEANKRMEDLITKFRDNHDSIGGTV
H.influenza         VNSN------------------------PFFCPDESAVEKFDELIRELKKEGDSIGAKL
T.gondii            GEELVEEEDKARRRLLFGVDNPTPGETVIETRCPCPSTAVRMAVKINQTRSLGDSIGGCI
P.falciparum        VDNNHNNYINDKDN-TFNNSEKSDEWIYLQTRCPHPYTAVQICSYILKLKNKGDSVGGIA
                                                    **          *       ** *

Synechocystis       ECAIRNAPKGLGEPVFDKLEADLAKAMMSLPATKGFEFGSGFAGTLLTGSQHNDEYYLDE
S.lycopersicum      TCIVRNLPRGLGTPVFDKLEAELAKACMSLPATKGFEFGSGFAGTFMTGSEHNDEFYMDE
S.cerevisiae        TCVVRNLPTGLGEPCFDKLEAMLAHAMLSIPASKGFEIGSGFQGVSVPGSKHNDPFYFEK
N.crassa            TCVIRNVPSGLGEPAFDKLEAMLAHAMLSIPATKGFEVGSGFGGCEVPGSIHNDPFVSAE
H.influenza         TVIAENVPVGLGEPVFDRLDADLAHALMGINAVKGVEIGDGFAVVEQRGSEHRDEMTPNG
T.gondii            SGAIVRPPLGLGEPCFDKVEAELAKAMMSLPATKGFEIGQGFASVTLRGSEHNDRFIPFE
P.falciparum        TCIIQNPPIGIGEPIFDKLEAELAKMILSIPPVKGIEFGSGFNGTYMFGSMHNDIFIPVE
                           * * * * **   * **         ** * * **      ** * *
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Synechocystis       AGE-----------------------------------------------------
S.lycopersicum      HGR-----------------------------------------------------
S.cerevisiae        ETN-----------------------------------------------------
N.crassa            NTEIPPSVAASGAARNGI--------------------------------------
H.influenza         FES-----------------------------------------------------
T.gondii            RASCSFSESAASTIKHERDGCSAATLSRERASDGRTTSRHEEEVERGRERIQRDTLHVTG
P.falciparum        NMSTKKESDLLYDDKGECKNMSYHSTIQNNEDQILNSTKGFMPPKNDKNFNNIDDYNVTF

Synechocystis       ----------------------------------------------WRTRTNRSGGVQGG
S.lycopersicum      ----------------------------------------------IRTRTNRSGGIQGG
S.cerevisiae        ----------------------------------------------RLRTKTNNSGGVQGG
N.crassa            ------------------------------------------PRPKLTTKTNFSGGIQGG
H.influenza         ----------------------------------------------------NHAGGILGG
T.gondii            VDQQNGNSEDSVRYTSKSEASITRLSGNAASGGAPVCRIPLGEGVRIRCGSNNAGGTLAG
P.falciparum        NNN-----------------------------------------EEKLLITKTNNCGGILAG
                                                                    *  **   *

Synechocystis       ISNGEPIIMRIAFKPTATIGQEQKTVSNIG-EETTLAAKGRHDPCVLPRAVPMVEAMAAL
S.lycopersicum      ISNGEVINMRIGFKPTSTISRKQQTVTRDK-HETELIARGRHDPCVVPRAVPMVEAMVAL
S.cerevisiae        ISNGENIYFSVPFKSVATISQEQKTATYDG-EEGILAAKGRHDPAVTPRAIPIVEAMTAL
N.crassa            ISNGAPIYFRVGFKPAATIGQEQTTATYDGTSEGVLAAKGRHDPSVVPRAVPIVEAMAAL
H.influenza         ISSGQPIIATIALKPTSSITIPGRSINLNG-EAVEVVTKGRHDPCVGIRAVPIAEAMVAI
T.gondii            ITSGENIFFRVAFKPVSSIGLEQETADFAG-EMNQLAVKGRHDPCVLPRAPPLVESMAAL
P.falciparum        ISTGNNIVFRSAIKPVSSIQIEKETSDFYG-NMCNLKVQGRHDSCILPRLPPIIEASSSM
                    *  *  *     *    *                      ****     *  *  *

Synechocystis       VLCDHLLRFQAQCKTL-----------------------------
S.lycopersicum      VLVDQLMAQYSQCMMFPINPELQEPLQSSPESAEVTL-----------
S.cerevisiae        VLADALLIQKARDFSRSVVH------------------------
N.crassa            VIMDAVLAHEARVTAKSLLPPLKQTINSGKDTVGNGVSENVQESDLAQ
H.influenza         VLLDHLLRFKAQCK-------------------------------
T.gondii            VIGDLCLRQRAREGPHPLLVLPQHSGCPSC---------------
P.falciparum        VIGDLILRQISKYGDKKLPTLFRNM--------------------
                    *  *
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Figure 5.5 B Comparison of the deduced amino acid sequence of *T.gondii* chorismate synthase with chorismate synthase from other organisms. The deduced amino acid sequence has considerable identity with chorismate synthases of diverse species: *Plasmodium falciparum* (49.7%); *Synechocystis* (51.4%); *Saccharomyces cerevisiae* (49.6%); *Solanum lycopersicum* (47.2%); *Neurospora crassa* (45.0%) and *Haemophilus influenza* (44.5%). * indicates an amino acid which is conserved in all 6 species.

The Alternative Pathway of Respiration

In vitro experiments

Toxicity assays

SHAM was found to be non-toxic to HFF at doses of 3.12μg/ml and below, ($p>0.375$), (Figure 5.6a). Ethanol was also non-toxic at this dilution, ($p>0.375$).

8-HQ was non toxic to HFF at a concentration of 0.5μg/ml and below, ($p>0.225$), (Figure 5.7A). Serial dilutions of SHAM (0.19 to 0.78μg/ml) and 8-HQ (0.25 μg/ml and 0.12μg/ml) were then used in the parasite growth inhibition assays.

Parasite growth inhibition assays

SHAM inhibited the growth of *T.gondii* tachyzoites in a dose dependent manner (0.78μg/ml, $p<0.0001$), (Figure 5.6B). Similarly inhibition of parasite growth was visualised in giemsa stained preparations. Parasites were not seen in SHAM treated cultures and the fibroblast monolayer was intact and appeared normal. In contrast, control cultures showed extensive destruction of the monolayer and remaining groups of cells infected with tachyzoites (Figure 5.6C).

8-HQ also inhibited parasite growth in a dose dependent manner (0.25μg/ml, $p<0.005$), (Figure 5.7B).

Synergy assays

SHAM was found to act synergistically with NPMG to restrict the growth of *T.gondii in vitro* (Table 5.1). Thus at a concentration of 3.25mM NPMG and 0.19μg/ml SHAM inhibited parasite growth by 60% and 32% respectively. In combination they

were able to inhibit 88% of parasite growth which was greater than the 73% predicted if the effect was purely additive.

In vivo survival experiments

The survival of mice treated with SHAM was not significantly enhanced compared with that of untreated control mice (Figure 5.8). Control mice treated with high dose sulphadiazine all survived.

Demonstration of alternative oxidase by SDS-PAGE and western blotting

All five antibodies to the alternative oxidase recognised a protein in *T.gondii* of approximately 66kD, the same molecular weight as *T.bruceii* and Voodoo lily alternative oxidases (Figure 5.9). Furthermore, under strong reducing conditions two bands of 66 and 33 kD were recognised (Figure 5.9). This suggests that similar to alternative oxidase in *T.bruceii* and Voodoo lily the presumed alternative oxidase protein in *T.gondii* is a 66kD dimer composed of two 33kD monomers.

These bands were also recognised in the mitochondrial enriched fraction of *T.gondii*. although it was not of increased intensity. This is probably in part due to the lower protein concentration and lack of purity of this fraction.

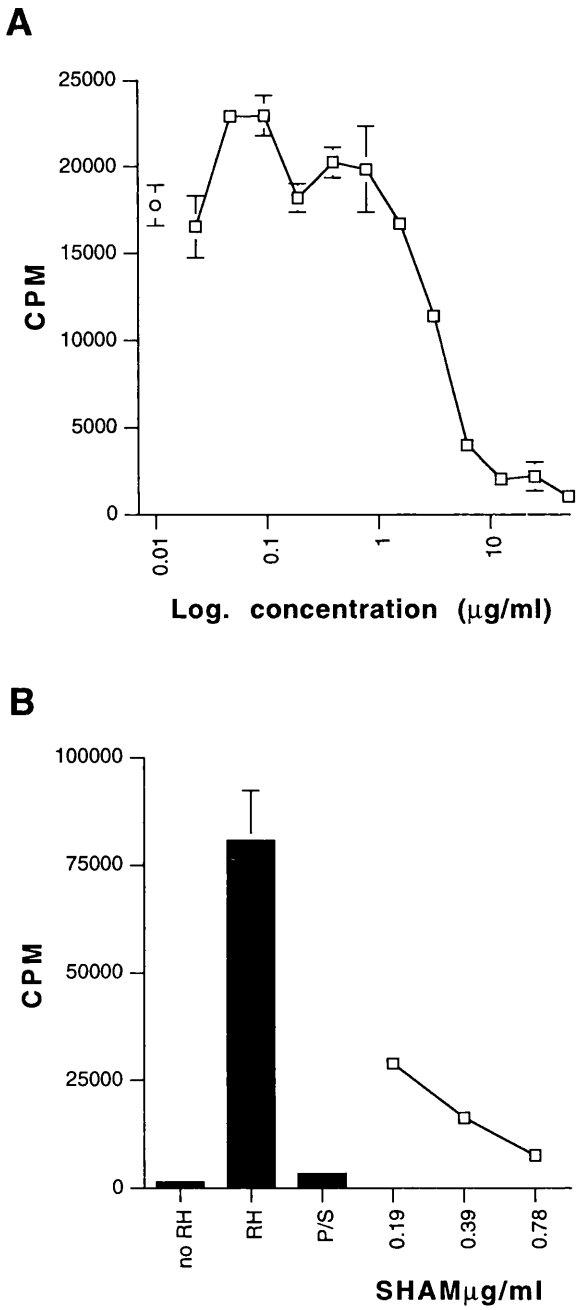

Figure 5.6 A The toxicity of SHAM to HFF as measured by uptake of tritiated thymidine. Concentrations of 3.12μg/ml and below were non-toxic to the fibroblast monolayer (p>0.375). (○, control cultures;□, SHAM treated cultures). B The effect of SHAM on the growth of *T.gondii* in HFF as measured by uptake of tritiated uracil. At a concentration of 0.78μg/ml SHAM inhibited over 90% of parasite growth (p<0.0001). Abbreviations: No RH, uninfected control cultures; RH, control cultures infected with RH; P/S infected control cultures treated with pyrimethamine and sulphadiazine.

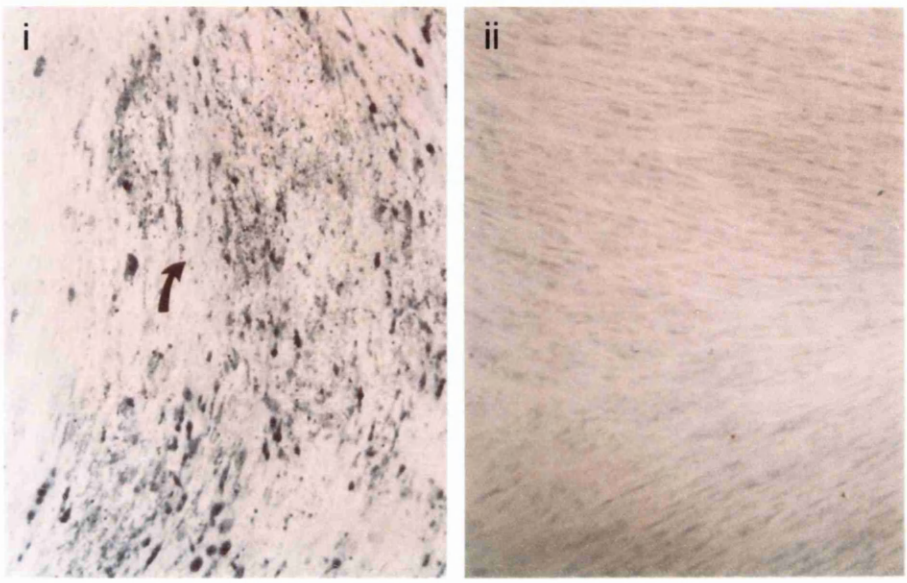

Figure 5.6 ***continued*** **C The effect of SHAM on the growth of *T.gondii* in HFF. (i) Control cultures showing large groups of parasites, destruction of the fibroblast monolayer and numerous extracellular parasites (arrow). (ii) Cultures treated with SHAM (0.8μg/ml). No parasites are identified and the fibroblast monolayer is intact. (Giemsa, x 100).**

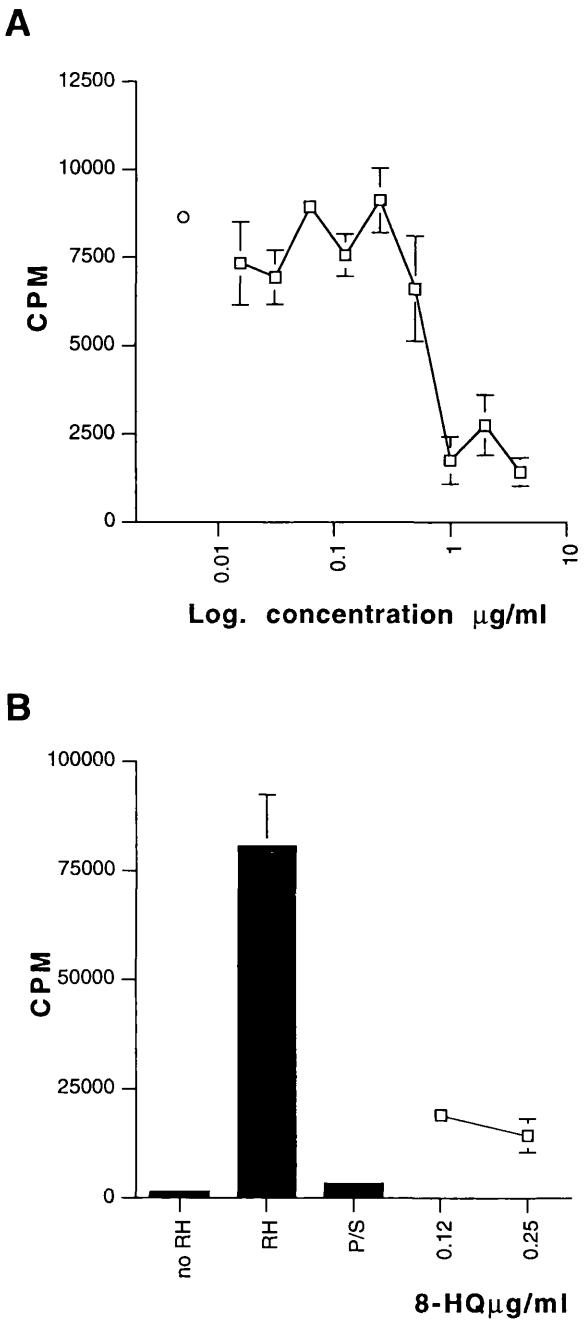

Figure 5.7 **A The toxicity of 8-HQ to HFF as measured by uptake of tritiated thymidine. Concentrations of 0.5µg/ml and below were non-toxic to the fibroblast monolayer (p>0.225). (○, control cultures;□, 8-HQ treated cultures). B The effect of 8-HQ on the growth of *T.gondii* in HFF as measured by uptake of tritiated uracil. At a concentration of 0.25µg/ml 8-HQ inhibited over 80% of parasite growth (p<0.005). Abbreviations: No RH, uninfected control cultures; RH, control cultures infected with RH; P/S infected control cultures treated with pyrimethamine and sulphadiazine.**

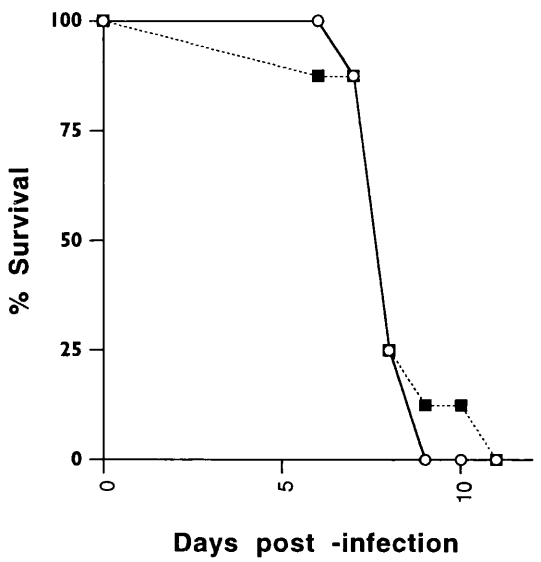

Figure 5.8 ***In vivo*** **activity of SHAM against** ***T.gondii*** **infection in mice. Treatment with SHAM (■) was not sufficient to prevent death, compared with control mice (○).**

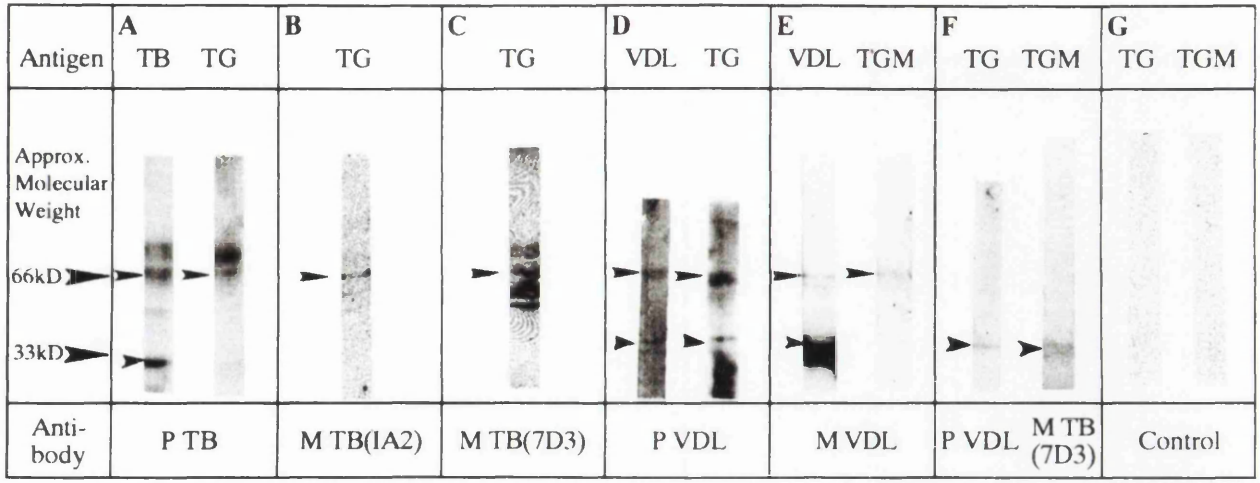

Figure 5.9 Western blot analysis of *T.gondii*, *T.bruceii* and Voodoo lily proteins with antibodies to alternative oxidase. **A** Murine polyclonal antibody to *T.bruceii* alternative oxidase (PTB) recognises a group of bands of approximately 66kD in electrophoresed *T.bruceii* mitochondria (TB) and *T.gondii*, RH strain, whole organisms (TG). A 33 kD band is also recognised in TB. **B** Monoclonal antibody IA2 to *T.bruceii* alternative oxidase recognises a band of approximately 66 kD in TG. **C** Monoclonal antibody 7D3 to *T.bruceii* alternative oxidase recognises a group of bands of approximately 66kD in TG. **D** Murine polyclonal antibody to Voodoo lily alternative oxidase (P VDL) recognises two groups of bands of approximately 66kD and 33 kD in electrophoresed Voodoo lily mitochondria (VDL) and TG. **E** Monoclonal antibody to Voodoo lily alternative oxidase (M VDL) recognises two groups of bands of 66kD and 33 kD in VDL and a band of 66kD in electrophoresed *T.gondii* mitochondrial enriched fraction (TGM) (protein concentration in mitochondrial enriched fraction is less than 10μg). **F** Under rigorous reducing conditions a band of 33kD is identified in TG and TGM with PVDL and M TB (7D3). **G** Representative negative controls incubated with secondary antibody only.

5.5 Discussion

These studies provide evidence for two previously undescribed biochemical pathways in *T. gondii*. These pathways, the shikimate pathway and the alternative pathway of respiration appear to be essential for the replication of *T. gondii*. Thus inhibition of either pathway is able to substantially restrict parasite growth. Since neither pathway is present in mammals they may prove to be excellent targets for new antimicrobial agents.

In bacteria, fungi, algae and higher plants the shikimate pathway is essential for the biosynthesis of aromatic compounds including folate, ubiquinone and the aromatic amino acids (Bentley, 1990). The herbicide, glyphosate (NPMG), which inhibits the action of EPSP synthase and thus the shikimate pathway, has been used with great success to kill higher plants (Haslam, 1993; Kishore & Shah, 1988). These studies demonstrate that inhibition of EPSP synthase by NPMG is able to restrict the growth of intracellular *T. gondii*. In addition, collaborative studies have shown that NPMG was able to restrict the growth of two other medically important Apicomplexans, *P.falciparum* and *C. parvum* (Appendix 1). Furthermore, NPMG was equally effective *in vitro* against both pyrimethamine sensitive and pyrimethamine resistant *P.falciparum* strains. This further emphasises the potential therapeutic value of targeting alternative enzymes of the folate biosynthetic pathway.

In these studies it was found that simultaneous administration of PABA to NPMG treated *T. gondii* cultures antagonised the inhibitory effect of this compound. Similarly, collaborative studies have shown that PABA can antagonise the inhibitory

effect of NPMG on *P. falciparum* (Appendix 1). This suggests that the shikimate pathway plays an essential role in the supply of folates for these parasites.

The role of the shikimate pathway in supplying chorismate for the synthesis of ubiquinone and aromatic amino acids in these parasites remains to be determined. *T.gondii* is certainly incapable of synthesising the aromatic amino acid, tryptophan (Pfefferkorn, 1984). However, tryptophan is not essential for the survival of *P.falciparum* for which tyrosine is an essential aromatic amino acid (Divo *et al.*, 1985). The synthesis of these two amino acids occurs by divergent enzymatic pathways from chorismate (Bentley, 1990). Interestingly, the addition of PABA did not ablate the effects of NPMG on the growth of *C. parvum*. The reason for this apparent difference between closely related parasites is uncertain but may reflect poor transport of PABA into the parasite. Alternatively, it may be that in *C. parvum*, the shikimate pathway is an absolute requirement for the supply of ubiquinone or aromatic amino acids. These observations imply that only certain pathways originating from chorismate, the end product of the shikimate pathway, may be conserved in different Apicomplexans. This may reflect the different evolutionary pressures acting on phylogenetically related parasites living in diverse environments.

These studies also provide the first cDNA and protein sequence of an Apicomplexan shikimate pathway enzyme, chorismate synthase. This information has already proven useful as it was used to find an homologous *P. falciparum* EST which has also now been sequenced. The deduced amino acid sequence of the *P.falciparum* chorismate synthase gene is included in the multiple sequence alignments shown in Figure 5.5b. Based on the gene sequences, the putative *T.gondii* and *P. falciparum* chorismate

synthase proteins differ from other known chorismate synthases in length. This is mainly due to a number of insertions which on the basis of their amino acid composition appear to form exposed loops. The function of these insertions remain to be determined.

In addition collaborative studies have detected four shikimate pathway enzymes in extracts of *T.gondii*. The enzymes detected were 3-dehydroquinate dehydratase, shikimate kinase, EPSP synthase, and chorismate synthase. These represent the third, fifth, sixth and seventh enzymes in the pathway, respectively (Figure 2.4). Furthermore the inhibitory effect of NPMG on *T.gondii* EPSP synthase was confirmed in these enzyme assays. The details of these experiments are summarised in Appendix 2. In addition, very low levels of three shikimate pathway enzymes have previously been reported in crude extracts of *P.falciparum*, albeit from the use of a rather non-specific colorimetric assay (Dieckmann & Jung, 1986). The enzymes identified were 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, shikimate dehydrogenase and shikimate kinase, the first, fourth and fifth enzymes in the shikimate pathway, respectively. Thus, there is evidence for all of the shikimate pathway enzymes, except 3-dehydroquinate synthase, in Apicomplexan parasites.

The discovery of the shikimate pathway in *T.gondii* therefore provides new opportunities for the development of drugs against this parasites. The ability of NPMG to act in synergy with conventional antifolate drugs *in vitro* further emphasises the potential benefits of targeting this pathway. The inhibitor used in these studies (NPMG) may be a valuable lead compound in the development of new drugs. The fact that NPMG, albeit in combination with pyrimethamine, was active against

T.gondii infection *in vivo* is encouraging from this point of view. A variety of derivatives of NPMG are currently being used to elucidate structure function relationships for inhibitors of plant EPSP synthases (Marzabadi *et al.*, 1996). A similar approach could be useful for characterising the active site of the parasite enzyme. Inhibitors of chorismate synthase (Davies *et al.*, 1994) and other enzymes within the shikimate pathway are also being developed in the search for new herbicides and drugs against bacterial and fungal pathogens.

These studies also provide compelling evidence for the presence of an alternative pathway of respiration in *T.gondii*. Thus, the growth of *T.gondii* was inhibited by SHAM and 8HQ, both well characterised inhibitors of the alternative oxidase. Similarly, inhibitor studies were performed by collaborators on the asexual erythrocytic forms of *P.falciparum* (pyrimethamine sensitive and pyrimethamine resistant strains) and *C.parvum* oocysts. SHAM and 8-HQ were able to inhibit the growth of these parasites (Appendix 3). The ability of 5 distinct antibodies, against 2 different alternative oxidases, to recognise a 66 kD *T.gondii* protein, further supports the presence of a *T.gondii* alternative oxidase. Pre-immune serum was not available as a control for the two polyclonal antibodies. However, non-specific binding by 5 separate antibodies seems unlikely. Furthermore, the two *T.bruceii* monoclonal antibodies are known to recognise different epitopes or isoforms of the alternative oxidase. Moreover, under rigorous reducing conditions the putative *T.gondii* alternative oxidase (66kD) also appears to be composed of two 33 kD monomers, similar to the *T.bruceii* alternative oxidase (Chaudhuri *et al.*, 1995).

Since performing these studies, Murphy *et al.*, (1997) also have found strong evidence for the presence of an alternative oxidase in *P.falciparum*. In these experiments parasite oxygen consumption was measured in the absence and presence of cyanide. As cyanide inhibits conventional mitochondrial but not alternative pathway respiration, oxygen consumption which occurs in the presence of cyanide is, by implication, alternative pathway respiration. Addition of SHAM inhibited cyanide insensitive oxygen consumption by *P.falciparum*. Surprisingly, these workers found no evidence for cyanide insensitive oxygen consumption by *T.gondii* tachyzoites (RH). However, the tachyzoites used in these assays were extracellular whereas all the studies described in this thesis used intracellular organisms. There may be significant differences in oxygen metabolism between intracellular and extracellular tachyzoites. Indeed a reduction in mitochondrial membrane potential is only evident after tachyzoites have invaded the cell (Tanabe & Murakami, 1984). This suggests that conventional mitochondrial respiration may be essential for the high energy demands of extracellular survival and host cell invasion. Intracellular tachyzoites may not require as much energy and may therefore utilise alternative oxidase. This hypothesis could be tested by examining cyanide insensitive respiration in intracellular tachyzoites, grown in a mitochondrial-deficient cell line. This would ensure that parasite and not host cells respiration was measured.

The role of the alternative oxidase in *T.gondii* is unclear although *in vitro* inhibition of intracellular growth by SHAM suggests that it is fundamental to parasite survival. A survey of the botanical literature provides a few clues as to the possible role of this pathway. There is evidence in plants and other organisms that alternative pathway respiration is an adaptive response. The alternative oxidase production of heat in the

thermogenic blooms of the voodoo lily appears to be a very specific purpose, unique to the Araceae (Meeuse, 1975). In other plants and organisms, alternative oxidase is induced or expressed under conditions of 'stress'. For example, low temperature, wounding, pathogen attack, elevated carbohydrate status, cell culture stage, addition of ethylene, ripening, and elevation of salicylic acid and pyruvate levels all increase alternative pathway respiration in plants (Day & Wiskich, 1995; Hoefnagel *et al.*, 1995; Moore & Siedow, 1991; Rhoads & McIntosh, 1992).

It is now widely acknowledged that stress, for example, heat, pH or immunological, is a key factor in the life-cycle of *T.gondii* and is responsible for tachyzoite to bradyzoite transformation (Soete *et al.*, 1994). A well-defined, physiologically relevant and immunologically important molecule known to induce stage conversion is nitric oxide (Bohne *et al.*, 1994). Notably nitric oxide has been demonstrated to inhibit conventional cytochrome oxidase but not alternative oxidases in plants (Millar & Day, 1996). The alternative oxidase is expressed in the blood stream trypomastigote forms and not the insect forms of *T.bruceii* (Chaudhuri *et al.*, 1995). The reason for this remains to be determined. However, in common with *T.gondii* tachyzoites and the asexual erythrocytic stages of *P.falciparum*, the blood stream trypomastigote of *T.bruceii* must survive the effects of nitric oxide produced by the host's immune system. The alternative oxidase therefore may be a common mechanism used by these 3 parasites to escape the inhibitory effects of nitric oxide on conventional mitochondrial respiration. This 'escape' mechanism for the tachyzoite may induce conversion to the bradyzoite stage.

The penalty for using alternative pathway respiration instead of conventional mitochondrial respiration is the loss of the ability to phosphorylate two ADPs. This may be of little consequence to these parasite stages as they are all in a relatively high glucose environment and presumably could obtain sufficient energy through glycolysis. Under these circumstances the advantage of using the alternative oxidase would be the ability to maintain a functional citric acid cycle. The citric acid cycle would provide a means of reducing pyruvate levels and perhaps more importantly, supply essential precursors for amino acid synthesis.

Whatever the role of the alternative oxidase in *T.gondii*, its presence provides new opportunities for the development of drugs against *T.gondii*. The inability of SHAM to prolong survival in mice infected with RH was disappointing. However, it is possible that SHAM may not have reached adequate therapeutic concentrations in tissues of these mice. There are no easy means of measuring serum or tissue concentrations of this compound. Its effects *in vitro* are unequivocal and SHAM may therefore prove a useful lead compound for development of *in vivo* drugs. Indeed, additional agents which appear to target alternative pathway respiration have recently been described. For example, ascofluranone, an isoprenoid antibiotic, specifically inhibits the blood stream trypomastigote stage of *T.bruceii in vitro* apparently by inhibiting alternative pathway respiration (Minagawa *et al.*, 1997).

The two compounds, NPMG and SHAM, used in these studies acted in synergy to inhibit *T.gondii* growth. Drugs may act in a synergistic fashion for a variety of reasons, but this phenomenon is most usually attributed to them targeting different points of the same pathway (Krogstad & Moellering, 1986). This suggests that the

shikimate pathway is important for the generation of ubiquinone in *T.gondii*. If this is the case NPMG, in common with SHAM, may affect parasite mitochondrial respiration (Figure 5.10) and ultimately the control of stage conversion. More importantly these studies suggest that simultaneously targeting the shikimate pathway and alternative pathway respiration may be a useful therapeutic strategy for disease caused by Apicomplexan parasites.

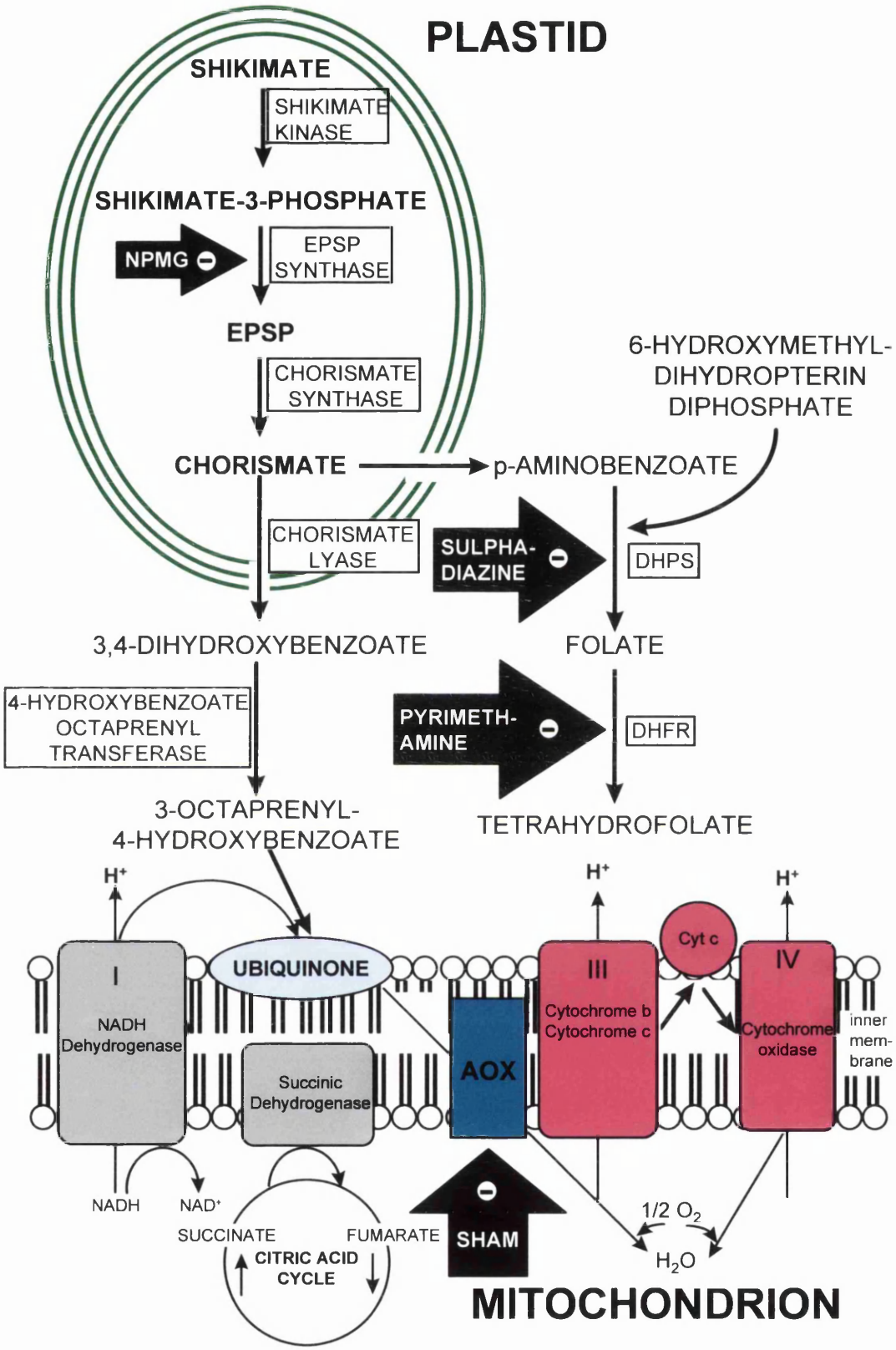

Figure 5.10 **Diagram showing the connection between the shikimate pathway and the alternative pathway of respiration in plants. The observed synergy between NPMG and SHAM suggests that these pathways may also be connected in *T.gondii*.**

CHAPTER 6

FINAL DISCUSSION

In earlier chapters, I have discussed the morphological features of congenital ocular toxoplasmosis in the fetus and infant and evidence supporting the presence of the shikimate pathway and alternative pathway of respiration in *T.gondii.* In this final chapter, I am therefore confining this discussion to the overall implications of my studies and their contributions to clinical aspects and potential treatment of toxoplasmosis. In particular, I will discuss (i) the morphological lesions of the earliest stages of congenital ocular toxoplasmosis and their significance to current strategies for prenatal diagnosis and management; (ii) novel therapeutic strategies arising from the description of two plant like biochemical pathways in *T.gondii* and; (iii) suggestions for future work.

Although ocular toxoplasmosis is an important cause of ocular disease in both immunosuppressed and immunocompetent individuals the histopathological features have not been extensively studied. One possible explanation for this may be that ocular toxoplasmosis itself is not life threatening and consequently infected eyes are rarely available for these studies. Nonetheless, the histopathological features of recurrent retinochoroiditis have previously been described in a number of small series and case reports (Bamatter, 1947; Binkhorst, 1948; Heath & Zuelzer, 1944; Hogan, 1958; Koch *et al.*, 1943; Rao & Font, 1977). A review of such case reports by Hogan (1951) described the histopathological findings in 13 infant eyes (ages ranging from term to 5 and a half weeks). At that time he stated that 'there have as yet been no descriptions of the earliest stages of toxoplasmic chorioretinitis'. Since then, the early stages of ocular toxoplasmosis during acute infection of the fetus had only been described in a study of 4 fetuses between the ages of 22 and 27.5 weeks (Brezin *et al.*,

1994). In two of these fetuses the eyes were normal, however the other two fetuses showed retinal necrosis, neovascularisation and marked chorioretinal inflammation.

The first part of this thesis examined the very early lesions of ocular toxoplasmosis in the fetus. This study, the largest of its kind to date, was made possible by collaborations between institutions in France and Chicago. The main findings in the fetal eyes were of retinitis, retinal necrosis, disruption of the RPE with marked choroidal inflammation and congestion. The two infants in the study showed severe ocular disease with retinal necrosis, retinal detachment and large subretinal exudates. Many of the histopathological findings in the fetuses were similar to those previously described in infants, children and adults. The features represent an earlier stage of the disease than that seen in the eyes from the 2 year old child. This study emphasises that extensive irreversible ocular damage can occur *in utero.*

Several features which may contribute to our understanding of the pathogenesis of ocular toxoplasmosis were also identified. Focal retinal dysplasia identified in the eye of the 32 week old fetus suggests that, like other infectious agents, *T.gondii* can have a teratogenic effect on the developing eye. The mechanism may involve disrupting the normal relationship of tissues in the eye such as the close association of the retina and RPE (Fulton *et al.*, 1978; Godel *et al.*, 1981).

The frequent presence of optic neuritis or predilection for the posterior pole and macular area *in utero* may be a contributing factors to the often poor visual acuity in surviving patients with congenital toxoplasmosis. Optic neuritis may, in part, reflect the presence of meningoencephalitis in these fetuses. Montoya & Remington, (1996)

found that the visual outcome of patients with acute acquired toxoplasmic retinochoroiditis is better than for those with recrudescent congenital disease. This may in part reflect the tendency of acquired disease to be unilateral. However, none of these patients had clinical symptoms of optic neuritis and only 2 patients had macular involvement (Montoya & Remington, 1996).

An inflammatory infiltrate dominated by CD4+ T cells in these eyes demonstrates that the fetus and infant are capable of mounting an immune response to *T.gondii* infection. However, the presence of numerous extracellular forms of *T.gondii* and lack of tissue cysts suggests that this immune response is less effective than that of the immunocompetent adult or child. The fetuses in this study were known to be severely affected with evidence of cerebral involvement and they may have experienced an overwhelming infection. Less severely affected fetuses, who are more likely to survive may go on to develop effective immunity and *T.gondii* cysts. They may then experience reactivations of disease in later life.

The documentation of the early morphological features of ocular toxoplasmosis has raised some interesting points with regard to implementation and timing of screening programs. Infection with *T.gondii* can cause extensive irreversible ocular damage early in gestation. This is important information for an expectant mother regarding the risks of this infection to her baby. Furthermore, for a pregnant woman who becomes infected with *T.gondii* it provides advice as to the likely severity of disease in her baby. This advice will allow a more informed decision regarding continuation or termination of a pregnancy in cases where the fetus is infected. Overall, this study presents a strong argument in favour of prenatal screening and treatment of congenital

toxoplasmosis. If fetuses are infected early in gestation extensive damage occurs to the eye in most cases. Thus, to improve the ophthalmic outcome from congenital toxoplasmosis treatment would ideally be initiated as soon as possible after infection of the fetus had occurred. At birth much of the damage has already occurred making complete resolution of symptoms and signs unlikely even with early postnatal diagnosis and treatment.

In France prenatal screening for *T.gondii* infection has been routinely carried out since 1978 (reviewed, Hall, 1992). The sensitivity of amniotic fluid PCR for the *T.gondii B1* gene now approaches 100% making rapid diagnosis of fetal infection possible (Hohlfeld *et al.*, 1994). Following a positive diagnosis of infection in the fetus the mother may be treated with sulphadiazine and pyrimethamine or may consider termination of the pregnancy if her fetus is severely affected (Couvreur *et al.*, 1993). Studies suggest that in the short term *in utero* treatment decreases the severity of congenital toxoplasmosis (Berrebi & Kobuch, 1994b; Hohlfeld *et al.*, 1989). In countries where prenatal screening is not routine, early postnatal diagnosis allows treatment to be initiated soon after birth and treatment is continued throughout the first year of life. Infants who became infected in the first trimester usually have severe involvement in the postnatal period and thus early diagnosis is possible (McAuley *et al.*, 1994). Infants who are infected later in gestation may appear to be normal in the postnatal period and without routine screening the diagnosis is often missed (Guerina *et al.*, 1994). Untreated, more that 90% of these individuals develop ophthalmological disease by adolescence (Koppe *et al.*, 1974; Wilson *et al.*, 1980).

However, my histopathological findings, although important, do not address the main clinical problem associated with ocular toxoplasmosis in that it is a recurring, progressively destructive disease due to the limitations of current therapies. Even with prompt *in utero* or early postnatal diagnosis, the treatment of congenital toxoplasmosis is associated with significant problems.

Currently the most effective therapy includes the antifolate agents, pyrimethamine and sulphadiazine (Remington *et al.*, 1995). These are both associated with significant adverse reactions. Pyrimethamine targets DHFR, an enzyme also present in human cells and is consequently, often associated with bone marrow toxicity (Williams, 1996a). This can in part be counteracted by simultaneous administration of folinic acid (Giles, 1971). Nonetheless, folate is critical to the developing nervous system and the use of pyrimethamine during pregnancy or in infants is not without risk. Sulphadiazine is commonly poorly tolerated resulting in problems with compliance and many patients develop allergy. Furthermore, therapy is required for long periods of time. Pregnant woman are treated until the birth of their child (Couvreur *et al.*, 1993). Congenitally infected infants receive treatment throughout the first year of life (McAuley *et al.*, 1994). Recurrent ocular disease requires treatment for approximately 6 weeks (Mets *et al.*, 1996; Rothova *et al.*, 1993). Prolonged and repeated therapy is required as there are no drugs which can eliminate the quiescent bradyzoite form of the parasite. Recent advances in therapy include spiramycin therapy, to reduce the risk of *in utero* transmission (Couvreur *et al.*, 1988) and clindamycin (Behbahani *et al.*, 1995) or atovaquone therapy (Haile *et al.*, 1993), in conjunction with pyrimethamine, in cases of sulphadiazine intolerance. However, these alternative therapies do not address the problems of bone marrow toxicity associated with pyrimethamine therapy

nor do they eliminate encysted parasites. Identification of new drug targets and ultimately new therapies is therefore desirable.

To this end, the plastid organelle has recently received considerable attention as a potential drug target in Apicomplexan parasites. Many research studies have been based on determining functions based on the residual plastid genome. Indeed, prokaryotic ribosomes in the plastid may be the target of the macrolide antibiotics including clindamycin, roxithromycin and azithromycin (Fichera & Roos, 1997). In addition, these studies on the plastid have emphasised several other so-called 'plant-like' properties of *T.gondii* (McFadden *et al.*, 1996). Recognition of these properties proffers alternative strategies for drug targeting.

The identification of potential new drug targets has been the focus of the research described in the remainder of this thesis. In Chapter 5, I have provided evidence for the existence of two previously undescribed biochemical pathways in *T.gondii* and outlined their potential as drug targets. Adopting a more lateral approach to the question of plastid function, the herbicide glyphosate (NPMG) was used in an attempt to disrupt a key point in the shikimate pathway which occurs in the plastid of higher plants. Glyphosate (NPMG) was able to inhibit the growth of *T.gondii* both *in vitro* and in combination with pyrimethamine *in vivo*.

The cloning and sequencing of a *T.gondii* chorismate synthase gene and the measurement of shikimate pathway enzyme activity in crude extracts of *T.gondii* by collaborators provides direct evidence for this pathway in *T.gondii* (Appendix 2) . Similarly, compounds which target the alternative oxidase of higher plants were also

able to inhibit the growth of *T.gondii in vitro*. Antibodies to two alternative oxidases were able to bind to a *T.gondii* protein of approximately 66 kD. Furthermore, NPMG was able to act in synergy with conventional antifolate agents as well as with SHAM suggesting a potential role for related compounds in combined therapy for toxoplasmosis.

Thus, both the shikimate pathway and the alternative pathway of respiration are potential new drug targets in *T.gondii* which are not present in mammalian hosts. It is an intriguing possibility that other plant-like or plastid associated pathways may also be present in *T.gondii*. For example, the biosynthetic pathways for porphyrins, are confined to the plastids of plants rather than mitochondria as in animals (Kannagara *et al.*, 1988). There may therefore be many enzyme targets in *T.gondii*, which are not present in animals, and which could provide potential drug targets.

The biochemical studies in *T.gondii* also shed light on possible biochemical processes in other Apicomplexans. Similar to *T.gondii* many of these parasites cause diseases which lead to considerable morbidity, mortality and economic losses. There are also no ideal antimicrobial agents available for treatment. For example, three to five million children and adults die each year from malaria (Krogstad, 1995). Effective treatment of malaria is hindered by drug intolerance and continual emergence of drug resistant parasites (Krogstad, 1995). Cryptosporidiosis is a common opportunistic pathogen in patients with AIDS but may also occur in epidemics (Ungar, 1995). There is currently no effective treatment for this parasite. Studies by collaborators have shown that NPMG and SHAM are able to inhibit the growth of *P.falciparum* and *C.parvum in vitro*. The chorismate synthase gene has been cloned from *P.falciparum*. Furthermore,

a recent study by (Murphy *et al.*, 1997) has identified cyanide insensitive respiration in *P.falciparum* suggesting it has an alternative pathway of respiration. There is therefore gathering evidence for the presence of the shikimate pathway and alternative oxidase in other pathogenic Apicomplexan parasites.

Therefore, antimicrobial agents developed to target these pathways in *T.gondii* may be useful against other pathogenic Apicomplexans. Indeed, other microbes that cause opportunistic infections in AIDS patients, including *Pneumocystis carinii* (Banerji *et al.*, 1995) and *Mycobacterium tuberculosis* (Garbe *et al.*, 1991) also have the shikimate pathway. Recent studies suggest that *P.carinii* may utilise alternative pathway respiration (Ittarat *et al.*, 1995). Thus, there is the exciting possibility that a single compound could be useful against several opportunistic pathogens simultaneously infecting a single individual.

6.1 Summary

In summary the work described in this thesis provides important knowledge in two main areas of toxoplasmosis research. First, it describes the morphological features of the earliest stages of ocular disease and the inflammatory cells present. This work has important implications for the implementation and timing of any proposed screening programs for congenital toxoplasmosis. It also provides some insight into the pathogenesis of this disease in the fetus. This work forms a basis for further studies into the local immune response in both human fetal tissues and in an animal model.

The devastating findings in the eyes of the fetuses and infants described in the first part of this thesis confirms the need for effective antimicrobial agents which will cure

this disease. The second part of this thesis describes two new pathways present in *T.gondii*. These pathways are associated with the so-called 'plant-like' properties of *T.gondii* and are therefore not present in human cells. They are thus ideal candidates for potential new drug targets. In addition extrapolation of both these pathways in plants suggests that they are linked to parasite mitochondrial respiration (conventional and alternative). Thus, agents targeting these pathways may be able to eliminate bradyzoites. They may therefore be of considerable importance in preventing recrudescent disease in immunocompromised patients and those with congenital toxoplasmosis.

6.2 Suggestions for Future Work

Studies on human tissues

The human ocular tissues studied in this thesis represents the largest collection of its kind to date. The collaborations and studies which made it possible to obtain these tissues are ongoing. Therefore, there will be opportunities to expand this collection when more ocular tissues become available. The initial immunohistochemical studies on these eyes have shown that the major infiltrating inflammatory cell is the CD4+ T cell. These cells can be classified according to the pattern of cytokines they produce (Mosmann *et al.*, 1986). For example, *T.gondii* infection is usually associated with a T helper 1 pattern of cytokines which includes IFN-γ and IL-2 production (reviewed, Subauste & Remington, 1993). Previous studies have demonstrated that infants with congenital toxoplasmosis often have diminished lymphocyte blastogenic responses to *T.gondii* antigen and production of the pro-inflammatory cytokines IFN-γ and IL-2 is reduced (McLeod *et al.*, 1990). It would therefore be of interest to study local

cytokine production in these eyes either by immunohistochemistry or *in situ* hybridisation. A predominance of Th2 cytokines would suggest that congenital infections are more severe due to a Th2 response in the fetus. The observed anergy of infants lymphocytes in *T.gondii* antigen may also be related to a lack of co-stimulatory molecules in the fetus. In particular B-7 and CD28 co-stimulatory molecules are required to effectively stimulate Th1 cytokine production and proliferation of lymphocytes (Khan *et al.*, 1996b). Murine studies have demonstrated that T cell non-responsiveness can be due to excessive nitric oxide (Candolfi *et al.*, 1994), IFN-γ (Candolfi *et al.*, 1995), IL-10 (Khan *et al.*, 1995) or IL-12 (Hunter *et al.*, 1995b), production. More significantly lack of IL-2 (Haque *et al.*, 1994) or down regulation of the co-stimulatory molecule, CD28 (Khan *et al.*, 1996b) have also been noted in these circumstances. Future studies will determine the presence or absence of these molecules in infected and uninfected fetal eyes by immunohistochemistry.

In addition to studies on fetal eyes, I have collected other tissues from these fetuses and infants. Similar histopathological and immunohistochemical studies on brain, liver, lung and placenta will provide insight into the local and systemic immune response to this disease in the fetus. It may provide information about the organ predilection of congenital toxoplasmosis and whether this relates to the site specific immune responses.

Studies using an animal model

Studies on human tissues are not without limitations. It is almost impossible to document the kinetics of *T.gondii* infection *in utero*. The clonal derivation of the

parasite is varied, the infecting life-cycle stage and dose have not been defined. Thus comparison of cases of human congenital ocular toxoplasmosis always carry this caveat. Until recently experimental studies of ocular disease have been hampered due to the lack of an easily manipulatable experimental model. However, recently Gazzinelli *et al.*, (1994) demonstrated that C57/BL6 mice infected intraperitoneally with an avirulent strain of *T.gondii* develop toxoplasmic retinochoroiditis. I have been able to reproduce this model and will use it to characterise this disease and immunological factors which control disease progression in the eye.

Further studies on the shikimate pathway and alternative oxidase

Further characterisation of the two pathways described in Chapter 5 is needed. For the shikimate pathway several questions should be addressed. The site of this pathway in the parasite should be definitively demonstrated. Although, usually associated with the plastid of higher plants and algae, under certain circumstances, this pathway can occur outside the plastid (Bentley, 1990). One approach to this would require production of antibodies to a key enzyme in the pathway and the site of this enzyme could then be localised by immunogold electron microscopy.

The synergistic actions of NPMG and SHAM suggest that the shikimate pathway may be important for the generation of ubiquinone precursors. Further studies to identify other pathways which branch from the shikimate pathway would be of interest. In particular confirmation of this mechanism of ubiquinone synthesis in *T.gondii* may allow specific targeting of mitochondrial respiration. A detailed study of other enzymes in this pathway may facilitate the design of more suitable inhibitors.

Further characterisation of the alternative oxidase is also required. Murphy *et al.*, (1997) found no cyanide insensitive respiration in extracellular tachyzoites. It would therefore be of interest to compare cyanide insensitive respiration in extracellular and intracellular tachyzoites. To circumvent the problems of host cell toxicity these studies would be performed with parasites grown in a mitochondrial deficient cell line. Identification of an alternative oxidase gene has proved more difficult than for the shikimate pathway as no obvious EST sequences have been found. Complementation and degenerative primer based PCR techniques using conserved regions in the plant or trypanosome genes have not yet yielded a candidate sequence. An alternative approach would be to utilise the available alternative oxidase antibodies to screen a cDNA library after protein expression. Again, this technique has been tried, but with limited success due to the large volumes of antibodies required for such studies. Identification and sequencing of a gene would allow assessment of differences in expression of this gene between extracellular and intracellular tachyzoites and bradyzoites. It would also assist in outlining a potential role in parasite stage conversion. Similar to the shikimate pathway the use of more specific inhibitors of the alternative oxidase may allow characterisation of the active enzyme site and the design of more suitable inhibitory agents.

Finally, NPMG and SHAM may act as useful lead compounds in the design of new drugs to treat toxoplasmosis. To assess the ability of these drugs to treat recurrent eye disease it may be useful to test new compounds using the mouse model of ocular disease.

APPENDICES

APPENDIX 1
THE EFFECT OF NPMG ON THE GROWTH OF *P.FALCIPARUM AND C.PARVUM*

A1.1 Introduction

To investigate the presence of the shikimate pathway in other Apicomplexan parasites the effect of NPMG on the *in vitro* growth of *P.falciparum* and *C.parvum* was assessed. In addition, these *in vitro* assays were performed in the presence of exogenous PABA.

The experiments on *P.falciparum* were performed by D.E.. Kyle and W.K.Milhous of the Walter Reed Army Institute of Research, Washington DC, USA. The experiments on *C.parvum* were performed by S.Tzipori, Tufts University School of Veterinary Medicine, Boston, USA.

A1.2 Materials and Methods

P.falciparum growth inhibition studies

The effect of NPMG on *P.falciparum* was measured using a modification of the semi-automated microdilution technique for assessing anti-folates (Milhous *et al.*, 1985). Two clones of *P.falciparum* were used for these tests; the W2 clone is susceptible to mefloquine, but resistant to chloroquine, pyrimethamine , sulphadoxine and quinine; the D6 clone is susceptible to chloroquine, pyrimethamine and sulphadoxine but is resistant to mefloquine (Oduola *et al.*, 1988). Briefly, NPMG was dissolved in DMSO and diluted 400 fold into RPMI 1640 media supplemented with 10% Albumax 1 (Gibco). Serial dilutions of NPMG were added to parasites in culture. The cultures were incubated at 37°C in 5% O_2, 5% CO_2 and 90% N_2 for 48hours. [^{3}H]-hypoxanthine incorporation was measured as described previously (Milhous *et al.*, 1985). The IC_{50} and IC_{90} for each clone was determined.

The parasite growth inhibition assay was performed in the presence of exogenous PABA and folic acid. NPMG was used at a concentration of 1.08mM with serial dilutions of PABA or folic acid.

C.Parvum growth inhibition studies

To assess the effect of NPMG on *C.parvum*, oocysts were added at 50,000/well to confluent MDBK F5D2 cell monolayers in 96 well tissue culture plates, as described by Tzipori, 1998. These were incubated at 37°C under air with 8% CO_2 in medium containing NPMG. After 48 hours the level of infection was determine by an immunofluorescence assay. Briefly *C.parvum* asexual forms were detected by addition of rabbit anti-*C.parvum* serum, followed by fluorescein-conjugated goat anti-rabbit antibody. Sixteen fields were counted at x100 magnification and mean parasite count per field determined. The effect of NPMG was compared with that of paromomycin (2000μg/ml), the standard positive control drug used in these *in vitro* assays. This assay was also performed in the presence of exogenous folate.

A1.3 Results

NPMG was equally effective *in vitro* against both pyrimethamine-sensitive (W2) and pyrimethamine resistant *P.falciparum* clones. The IC_{50} and IC_{90} for each clone is shown in Table A1.1. These results are the mean +/- standard deviation of 5 replicate experiments. The inhibitory concentrations are comparable to that required to inhibit the growth of *T.gondii in vitro* and are similar to the *in vitro* activity of antimalarial drugs in current clinical use (*e.g.* proguanil). NPMG was equally effective against pyrimethamine-sensitive (D6) and pyrimethamine-resistant (W2) clones. This

emphasises the potential therapeutic value of targeting alternative enzymes of folate biosynthesis. Addition of both PABA and folic acid were able to ablate the inhibitory effect of NPMG (Figure A1.1), suggesting that the shikimate pathway is important for the supply of folate in *P.falciparum*.

Table A1.1 The concentration of NPMG required to inhibit the growth of pyrimethamine-resistant (W2) and pyrimethamine-sensitive (D6), *P.falciparum* clones

P.falciparum clone	IC_{50} (mM)	IC_{90}(mM)
W2	1.1+/-0.04	2.1+/-0.09
D6	0.5+/-0.04	1.6+/-0.1

NPMG was also able to inhibit the growth of *C.parvum, in vitro* in a dose dependent manner and was non-toxic to the MDBK F5D2 monolayer at the concentrations used, (4.5, 6, 8, and 9mM). The results are shown in Figure A1.2. However, in this case, exogenous PABA was not able to ablate *C.parvum* growth inhibition by NPMG. This may reflect poor transport of exogenous PABA into the parasite. Alternatively, the shikimate pathway may be required for synthesis of other compounds *e.g.* ubiquinone or aromatic amino acids, in *C.parvum*.

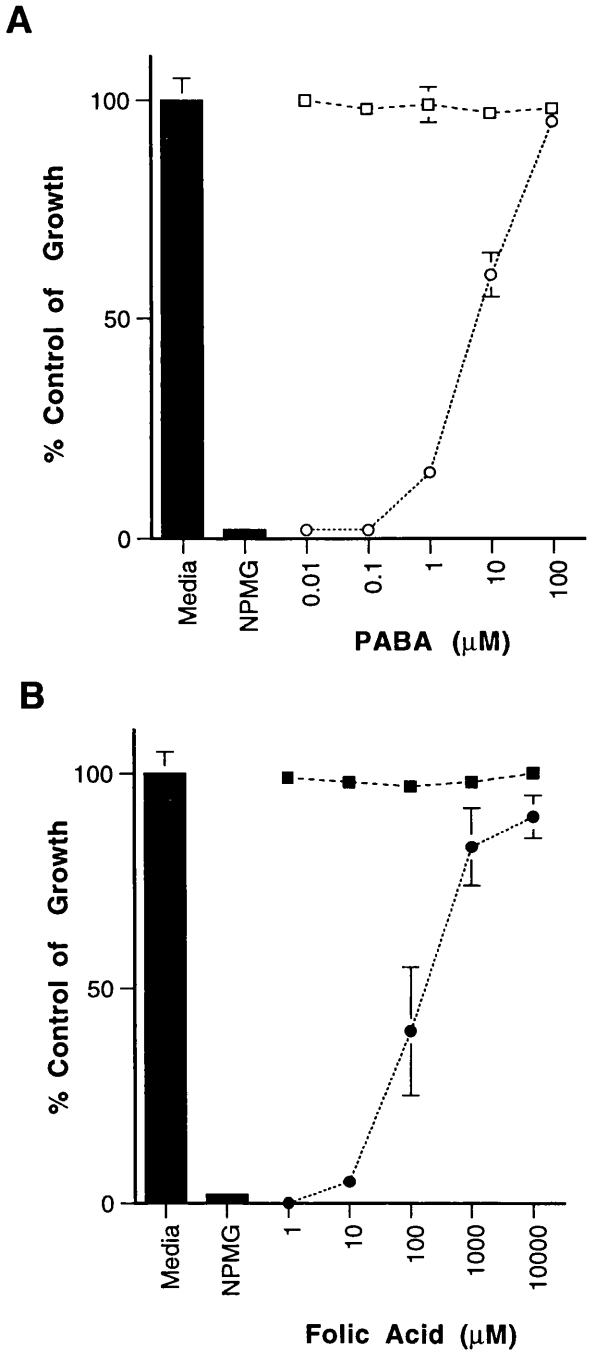

Figure A1.1 Product rescue of the inhibitory effect of NPMG on the growth of the asexual erythrocytic forms of *P.falciparum*. In **A**, addition of PABA reduced the effects of NPMG in a dose dependent manner. Similarly, in **B**, addition of folic acid reduced the effects of NPMG in a dose dependent manner. Key: PABA, (□); PABA+ NPMG, (○); Folic Acid, (■); Folic acid + NPMG, (●); Media, control cultures infected with *P.falciparum*; NPMG, infected cultures treated with NPMG (1.08mM).

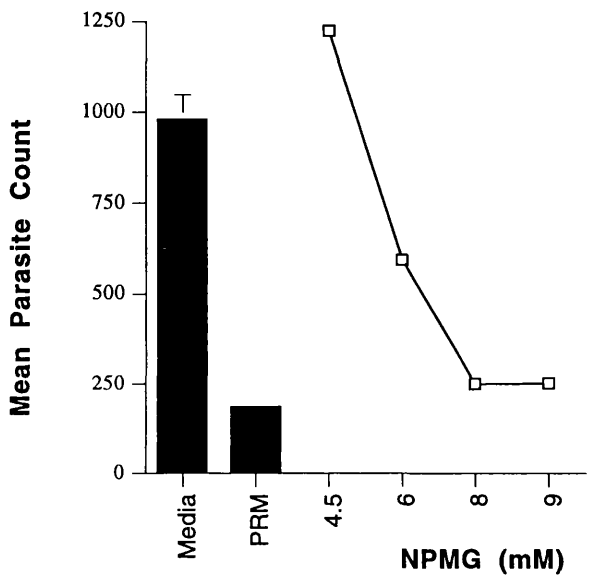

Figure A1.2 The effect of NPMG on the growth of *C.parvum.* At a concentration of 8mM NPMG inhibited 75% of parasite growth. Key: Media, control cultures infected with *C.parvum*; PRM, infected control cultures treated with paromomycin.

APPENDIX 2
MEASUREMENT OF SHIKIMATE PATHWAY ENZYMES IN *T.GONDII*

A2.1 Introduction

Further confirmation for the presence of the shikimate pathway in *T.gondii* was obtained by directly measuring enzyme activities in parasite lysates. These experiments were performed by Dr. J. Coggins, Dr. T. Krell of Glasgow University and Dr. C.W. Roberts of University of Strathclyde, Glasgow.

A2.2 Materials and Methods

Parasite extracts were produced at 4°C by suspension of pure tachyzoites in extraction buffer (50nM Tris.HCl, pH7.5), containing complete TM protease inhibitor cocktail (Boehringer Mannheim, 1 tablet per 50ml buffer). This was followed by sonication and centrifugation. The resulting supernatant was diluted 6-fold with extraction buffer and loaded onto a Resource Q column (1ml, Pharmacia) equilibrated with extraction buffer. The bound protein was eluted in a single step using extraction buffer containing 500 mM KCl. The eluted material was used for enzyme assays.

The third enzyme, 3-dehydroquinate dehydratase was detected using a direct spectrophotometric assay (Coggins *et al.*, 1987). The fifth enzyme of the pathway, shikimate kinase was detected by measuring directly the formation of shikimate 3-phosphate from shikimate using a high performance liquid chromatography (hplc) based assay (Mousdale & Coggins, 1985). The identity of this product was then confirmed by observing its conversion to EPSP after the addition of phosphoenol pyruvate and purified *Escherichia coli* EPSP synthase (Duggan *et al.*, 1995). Detection of the sixth enzyme, EPSP synthase entailed monitoring the generation of EPSP using hplc. The identity of the product was confirmed by comparing its

retention time and UV spectrum with authentic samples of EPSP generated by biotransformation (Coggins *et al.*, 1987). This assay was also performed in the presence of 1mM NPMG. Chorismate synthase, the seventh enzyme, was detected using an anaerobic assay (Ramjee *et al.*, 1994).

A2.3 Results

The results are shown in Table A2.1 and Figure A2.1.

Table A2.1 Enzyme activity detected in *T.gondii* extracts

Enzyme	Amount present in *T.gondii* extracts
Dehydroquinate synthase	3.0mU/mg protein
Shikimate kinase	1.6mU/mg protein
EPSP synthase	0.34mU/mg protein
Chorismate synthase	Low levels

The enzyme activities detected were dehydroquinate synthase (3.0mU/mg protein), shikimate kinase (1.6mU/mg protein), EPSP synthase (0.34mU/mg protein). Chorismate synthase was detected at low levels in repeated experiments. A precise activity could not be accurately determined. EPSP synthase activity was inhibited by the addition of NPMG, 1mM.

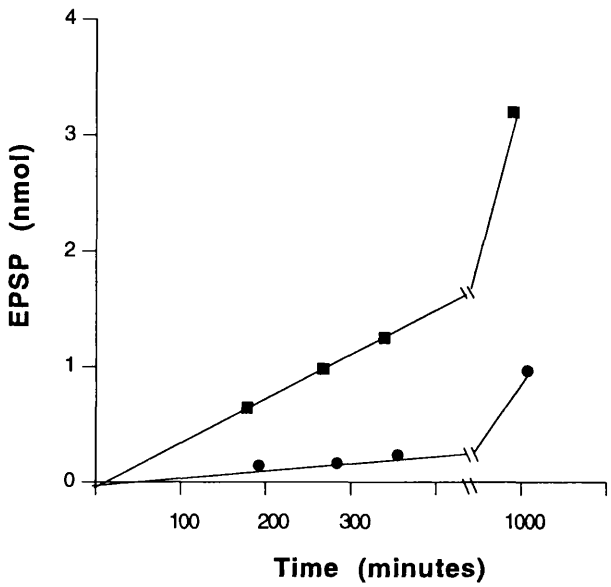

Figure A2.1 Inhibition of EPSP synthase activity by 1mM NPMG. Key: Control EPSP synthase activity (■); EPSP synthase activity in the presence of 1mM NPMG (●).

APPENDIX 3
THE EFFECT OF SHAM AND 8-HQ ON THE GROWTH OF *P.FALCIPARUM* AND *C.PARVUM*

A3.1 Introduction

To investigate the presence of the alternative oxidase in other Apicomplexan parasites the effect of SHAM and 8-HQ on the *in vitro* growth of *P.falciparum* and *C.parvum* was assessed.

The experiments on *P.falciparum* were performed by D.E.. Kyle and W.K.Milhous of the Walter Reed Army Institute of Research, Washington DC, USA. The experiments on C.parvum were performed by S.Tzipori, Tufts University School of Veterinary Medicine, Boston, USA

A3.2 Materials and Methods

The ability of SHAM and 8-HQ to inhibit *in vitro* growth of *P.falciparum* and *C.parvum* was assessed as described in Appendix 1.

A3.3 Results

Both SHAM and 8-HQ inhibited the growth of *P.falciparum*. The IC_{50} and IC_{90}, the means of 5 replicate experiments, are shown in Table A3.1. SHAM and 8-HQ also inhibited the growth of *C.parvum* in a dose dependent manner. At a concentration of 10mg/ml both SHAM and 8-HQ inhibited over 50% of parasite growth. SHAM and 8-HQ were non-toxic to the MDBK F5D2 monolayer at a concentration of 10μg/ml. At 100μg/ml these compounds showed mild toxicity to the monolayer. The results are shown in Figure A3.1

Table A3.1 The concentration of SHAM and 8-HQ required to inhibit the growth of pyrimethamine-resistant (W2) and pyrimethamine-sensitive (D6), *P.falciparum* clones

P.falciparum clone	Inhibitor	IC_{50} (μg/ml)	IC_{90} (μg/ml)
W2	SHAM	5.7	43
	8-HQ	1.6	4.5
D6	SHAM	6.2	25
	8-HQ	1.2	1.9

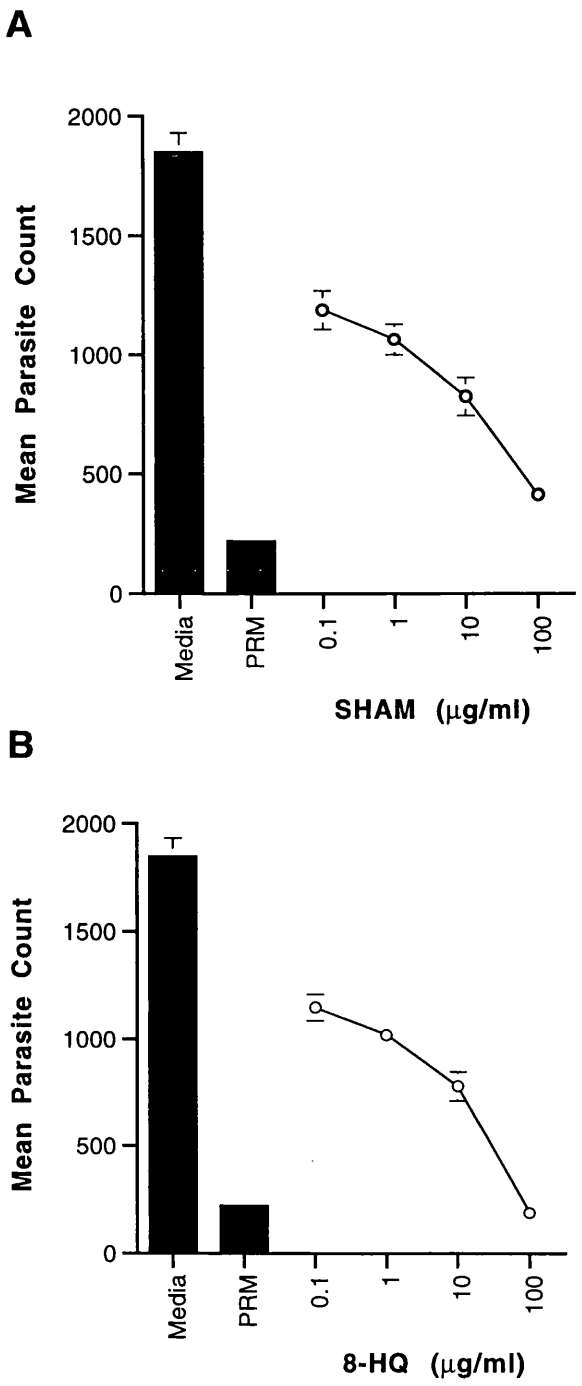

Figure A3.1 **A The effect of SHAM on the growth of *C.parvum*. At a concentration of 10µg/ml SHAM inhibited over 50% of parasite growth. B The effect of 8-HQ on the growth of *C.parvum*. At a concentration of 10µg/ml 8-HQ inhibited over 50% of parasite growth. Key: Media, control cultures infected with *C.parvum*; PRM infected control cultures treated with paromomycin.**

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