Immunological control of *Toxoplasma gondii* infection.

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Abstract

Studies using B10 H-2 congenic and recombinant mice indicated that control of brain cyst number in *T. gondii* infected mice mapped to the D region of the MHC complex. Further studies using BALB MHC congenic mice demonstrated that a drop in cyst number coincided with a dramatic change in the ratio of splenic CD4: CD8 T lymphocytes in favour of the latter. Adoptive transfer and selective depletion of T lymphocyte populations demonstrated a role for both CD8+ and CD4+ T cell populations in the acute phase of infection while CD8+ T cells play the dominant role in the control of cyst numbers.

The *Lsh* gene was found to have a route of infection dependent influence on early mortality in congenic Lsh^r and Lsh^s BALB/c and C57BL/10ScSn mice. Mice homozygous for the *beige* allele were more susceptible to *T. gondii* infection as demonstrated by high rates of mortality and increased cyst burdens. Mice carrying the Lps^d allele were found also to have increased susceptibility to acute mortality, but developed fewer cysts in their brains.

The use of the polymerase chain reaction revealed the presence of transcripts for TNF-α, IL-6, MIP-1β, IL-4, IL-2, and IFN-γ in the brains of C57BL/10ScSn mice, with progressive meningoencephalitis. Analysis of the kinetics of cytokine production revealed temporal differences in their production which were related to neuropathological effects.

The development of a murine model of congenital toxoplasmosis facilitated the testing of a vaccine preparation consisting of non ionic surfactant vesicles (NISV) and the soluble fraction of a tachyzoite lysate (STAg). Vaccination was found to result in fewer brain cysts, prevent foetal death and reduce congenital transmission.

Acknowledgements

I would like to thank my supervisor, Dr. J. Alexander for introducing me to such an exciting area of research and for his support and guidance throughout this project. Thanks also to Dr. S.J. Parker formerly of The University of Strathclyde, who taught me basic laboratory techniques in handling *T. gondii* and provided guidance in the initial stages of this project. Mr. J. Keys looked after the animals used in this project and provided expert technical help in setting up the murine model of congenital toxoplasmosis.

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Finally, I would like to thank my family and Fiona for their encouragement throughout this project.

'If a little knowledge is dangerous,

where is the man who has so much as to be out of danger ?'

T.H. Huxley On Elementary Instruction in Physiology (1877)

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CHAPTER 1

Introduction

1.1. History

Toxoplasma gondii was discovered by Nicolle and Manceaux (1908) in the spleen of the north African rodent, the gondi (*Ctenodactylus gundi*) and later in a Brazilian rabbit by Splendore (1908). For the first year Nicolle and Manceaux believed the parasite to be a member of the genus *Leishmania*, calling it *Leishmania gondii* but in 1909 it was recognised as a separate genus and renamed *Toxoplasma gondii* (Nicolle and Manceaux, 1909). It is now recognised within the subkingdom Protozoa as a member of the *Apicomplexa* in the subclass *Coccidia* and order *Eucoccidiida* .

1.2. Life-cycle

For around 60 years after its discovery, the life cycle of *T. gondii* remained unknown. It had been suggested that undercooked meat could be a source of infection (Weinman and Chandeler ,1954) and this prediction was supported in 1965(b) by Desmonts *et al.*, who observed that in a Parisian tuberculosis hospital raw meat fed to patients seemed to be responsible for transmitting the disease. This hypothesis could not however account for the presence of the disease in herbivorous animals, including rabbits and sheep or vegetarian humans, all of which were known to be infected by this parasite (Splendore, 1908, Hartley & Marshall, 1957 and Rawal, 1959). Although the cyst in animal tissues was finally demonstrated as an infective agent in 1960 by Jacobs *et al.*. the question as to how herbivores and vegetarians became infected remained unanswered.

Although Hutchison, (1965) demonstrated that cat faeces were capable of transmitting the disease to laboratory mice, he believed that *Toxocara cati* ova were necessary to act as transport hosts. Hutchison *et al.*, (1969) and Frenkel *et al.*, (1970) later demonstrated that the presence of *T. cati* in cat faeces was not necessary for transmission of *T. gondii* through this route, and in doing so discovered the oocyst (Hutchison called this the 'new cyst', since the coccidian nature of the parasite had yet

to be proven).

The parasite was confirmed as a member of the *coccidia* when Hutchison *et al.*, (1970), observed schizogonic and gametogonic stages in the posterior portion of the small intestine in specific pathogen free cats fed 5 days previously with aseptic mouse brains containing *T. gondii* tissue cysts (Hutchison *et al.*, 1970 and 1971).

The life cycle (Figure 1.1) is now known to consist of an extraintestinal cycle, taking place in any warm blooded host (Table 1.1) and an enteroepithelial cycle which may only occur in the posterior small intestine of the *Felidae* (Miller *et al.*, 1972). All three stages, the tachyzoite, the bradyzoite and the sporulated oocyst are known to be infective to homologous and heterologous hosts, although the tissue cyst seems to be most effective in initiating the enteroepithelial cycle in the cat (reviewed by Jackson and Hutchison, 1989).

The extraintestinal cycle

The extraintestinal stage of the life cycle involves the rapid asexual multiplication of tachyzoites in the reticuloendothelial cells of the host. The tachyzoite enters the host cell by a combination of phagocytosis and an active mechanism involving the use of apical organelles called rhoptries. These structures secrete an active component that is able to reduce the surface viscosity of the host cell membrane and possibly even transiently disintegrate it at the point of entry (reviewed Werk, 1985). Host cell surface components such as laminin react with parasite laminin binding proteins on the tachyzoites surface facilitating attachment during this process (Joiner *et al* , 1989).

The tachyzoite undergoes repeated cycles of endodyogeny in the parasitophorous vacuole where it is able to resist host defence mechanisms by preventing lysosomal fusion and acidification of the vacuole (reviewed McLeod, *et al.*, 1991). Ultimately, the host cell eventually lyses releasing tachyzoites to infect adjacent cells. This process of rapid multiplication in the mononuclear phagocytes of the host continues until a

Table 1.1. Animals known to permit the extra-intestinal growth of *T. gondii* in their tissues. (includes animals found to be naturally infected and those experimentally infected).

Animal	Author
Gondi	Nicolle & Manceaux (1908)
Rabbit	Splendore (1908)
Hare	Christiansen & Siim (1951)
Man	Janku, (1923)
House mouse	Lubroth *et al.*, (1982)
White footed mouse	Lubroth *et al.*, (1982)
Cotton rat	Lubroth *et al.*, (1982)
Norway rat	Lubroth *et al.*, (1982)
Oppossum	Lubroth *et al.*, (1982)
Brown rat	Hay, *et al.*, (1983)
Hamster	Frenkel, (1951)
Guinea pig	Lainson (1958)
Sheep	Olafson & Monlux, (1942)
Goat	Munday & Mason (1979)
Pig	Work, (1967); Durfee, *et al.*, (1974)
Cattle	Work, (1967); Munday, (1978)
Horse	Dubey & Desmonts (1987)
Red deer	Williamson *et al.*, (1980)
Red tailed hawk	Lindsay, *et al.*, (1991)
Sparrows	Ruiz & Frenkel, *et al.*, (1980)
Feral chickens	Ruiz & Frenkel, *et al.*, (1980)
Rough-legged hawk	Miller *et al.*, (1972)
Sparrow hawk	Miller *et al.*, (1972)
Great-horned owls	Miller *et al.*, (1972)
Barn-owls	Kirkpatrick *et al.*, (1990)
Domestic chicken	Wallace (1973)
Domestic pigeon	Wallace (1973)
Barred dove	Wallace (1973)
Spotted dove	Wallace (1973)
Brazillian Cardinal	Wallace (1973)
Chimpanzee	Draper *et al.*, 1971)
Aoutos monkey	Escajadillo & Frenkel (1991)
Dog	Olafson & Monlux (1942)
Red fox	Reed & Turek (1985)
Ring-tailed lemurs	Dubey *et al.*, (1985)
Racoon	Lubroth *et al.*, (1982)
West indian manatee	Buergelt (1983)
Bank vole	Hay, *et al.*, (1983)

continued:-

Fur seal	Holshuh, *et al.,* (1985)
Sea lion	Migaki, *et al.,* (1985)
Polar bear	Sekla, *et al.,* (1981)

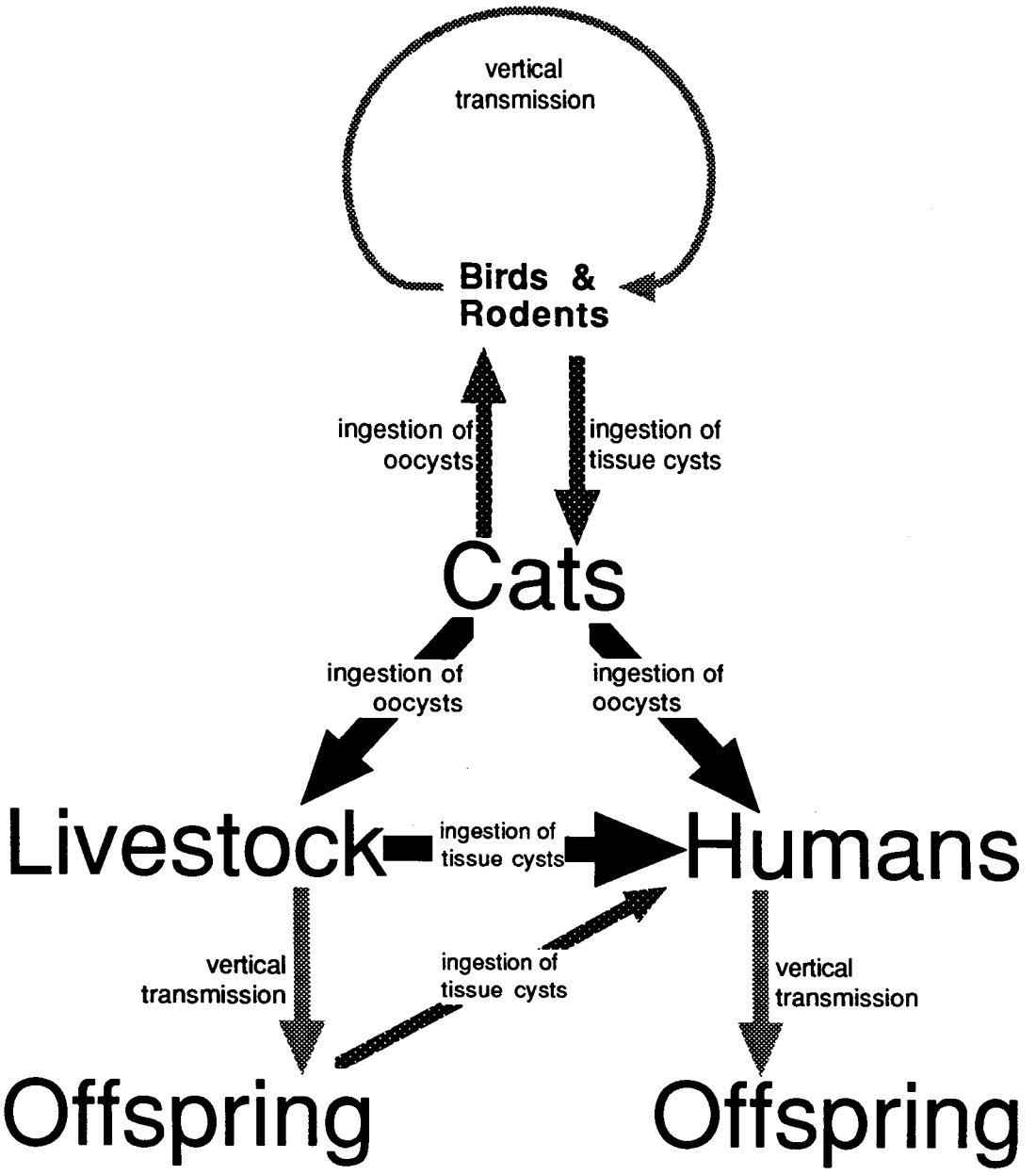

Figure 1.1. The life-cycle of *Toxoplasma gondii.*
The sexual cycle is initiated in the ileum of the cat most effectively upon ingestion of tissue cysts (but can also be initiated by oocysts) resulting in the release of oocysts in the faeces which are infective to the heterologous host after sporulation. After ingestion by the heterologous host (Humans, Livestock, birds and rodents) the sporocysts transform to tachyzoites and undergo a brief period of rapid multiplication in the reticuloendothelial system before forming tissue cysts containing many infective bradyzoites. These cysts are infective to both homologous and heterologous hosts upon ingestion. Vertical transmission can occur in the heterologous host and occasionally in the homologous host. (see text for detailed description of life-cycle)

period that coincides with the onset of immunity (10-14 days in the human or mouse) : after this time the predominant form of the parasite is the bradyzoite contained in the tissue cyst. Tissue cysts are found in almost any organ within the host although the CNS, skeletal and cardiac muscle are preferred sites (Jacobs *et al.*, 1960 and Remington and Cavanaugh, 1965). Tissue cysts have been shown to develop in the host tissues as early as 3 days post infection (Dubey & Frenkel, 1976). The bradyzoites contained within these cysts continue to divide but at a much slower rate than the tachyzoites, giving rise to as many as 3000 organisms in any one cyst. Cysts have been described as occurring either intracellularly (Ferguson and Hutchison, 1987), or extracellularly (Pavesio, *et al.*, 1992). These cysts containing a mixture of viable and degenerating organisms, can rupture during the life-span of the host, apparently releasing both tachyzoites and bradyzoites in their contents (Pavesio, *et al.*, 1992). Tachyzoites released from tissue cysts are responsible for recrudescent patent parasitaemias commonly observed in the immunocompromised host.

The enteroepithelial cycle

The enteroepithelial cycle can be initiated in the gut of the cat by any of the infective stages mentioned above, although most effectively by the tissue cyst (Jackson & Hutchison, 1989). In the epithelial cells at the tip of the villi in the ileum, repeated cycles of endopologeny (a modification of schizogony) and gametogony take place giving rise to merozoites or microgametes and macrogametes. Both these processes, sexual and asexual can occur at the same time, a merozoite being able to differentiate sexually on the reinvasion of a cell. Microgametes fertilise macrogametes giving rise to the infective oocyst containing two sporocysts which each contain 8 sporozoites. The oocysts are not immediately infective but within 5 days they have undergone sporulation and are thus capable of initiating an infection. Oocysts are shed in the faeces for around 17 days, starting 3-5 days post-infection and as many as ten million may be shed per day (Hutchison, 1973).

Congenital transmission

Congenital transmission of the parasite, most commonly detected in the heterologous host although documented in at least one cat (Dubey and Johnstone, 1982), is facilitated through the extraintestinal cycle, the infective stage being the tachyzoite which invades the placenta and eventually the foetus.

1.3. Clinical disease

Toxoplasma gondii was not associated with human disease until 1923 when congenital toxoplasmosis was identified as the cause of choroidoretinitis in a Czechoslovakian infant (Janku, 1923). Congenital transmission has since been confirmed in humans (Wolf and Cowen, 1937; Wolf *et al.*, 1939; reviewed Cook, 1990). The first fatal case of toxoplasmosis in humans was described in 1940 by Pinkerton and Weinman in a patient who was found to have enlarged lymph nodes and areas of necrosis in a variety of organs at post mortem examination. The world wide prevalence of *T. gondii* infection as deduced from serology, is estimated at 33% although prevalences can vary tremendously geographically or within groups studied, (Table 1.2), (reviewed, Jackson & Hutchison, 1989). A recent longitudinal study in South Yorkshire, using samples obtained between 1988-1990 found that the seroprevalence of *T. gondii* antibodies in individuals of 16-45 years of age was 11% (Walker *et al.*, 1992). This study also demonstrated that the prevalence of the disease was lowest in the young at just over 3% in children under 5 years of age, but rose steadily with age to around 50% in people 80 years of age or older (Figure 1.2). Comparative analysis of serum obtained from 1969-1985 revealed a slight decrease in overall prevalence between these dates. The incidence of congenital infection is difficult to estimate due to lack of adequate testing in many countries, but reports world wide vary between 0.07 and 10 per 1000 births (Table 1.3). However, the consensus is that these are gross under estimates, as the incidence of toxoplasmic uveitis, a condition believed to be almost entirely due to congenital infection can be 10 times higher than the reported incidence of congenital infection (reviewed, Jackson &

Table 1.2. Sero-prevalence of *T. gondii* antibodies in various groups of people (Modified from Jackson & Hutchison, 1989).

Group	Region	Prevalence(%)	Author
Women of childbearing age	Mali	34	Maiga *et al.*, (1984)
Pregnant women	Paris	84	Desmonts and Couvreur (1974)
	Glasgow	13.4	Williams *et al.*, (1978)
	Brussels	53	Foulon *et al.*, (1984)
	England	14.9	Jackson *et al.*, (1987)
	Southern Finland	20.3	Lappalainen *et al.*, (1992)
	Slovenia	50.9	Logar *et al.*, (1992)
Blood Donors	Kenya	42	Griffin & Williams (1983)
	Thailand	1.2-4.6	Morakote *et al.*, (1984)
	Scotland	7.6-7.8	Jackson *et al.*, (1987)
Veterinarians	California	43.7	Behymer *et al.*, (1973)
	New York	8.3	Sengbusch and Sengbusch (1976)
Abattoir workers	Brazil	60-92	Riemann *et al.*, (1975)
Military recruits	USA	3-20	Feldman (1965)
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)
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.*, (1977)
	Brazil(tropics)	54	Ferraroni & Marzochi (1988)

continued:-

Remote population	Alaska (arctic)	28	Peterson *et al.*, (1974)
Medical outpatients	England	35.7	Jackson *et al.*, (1987)
Travelling people	Scotland	28	Jackson *et al.*, (1987)
Aids patients	Zaire	24-33	De Clercq *et al.*, (1986)
Asymptomatic HIV infected	UK	27	Holliman (1990)

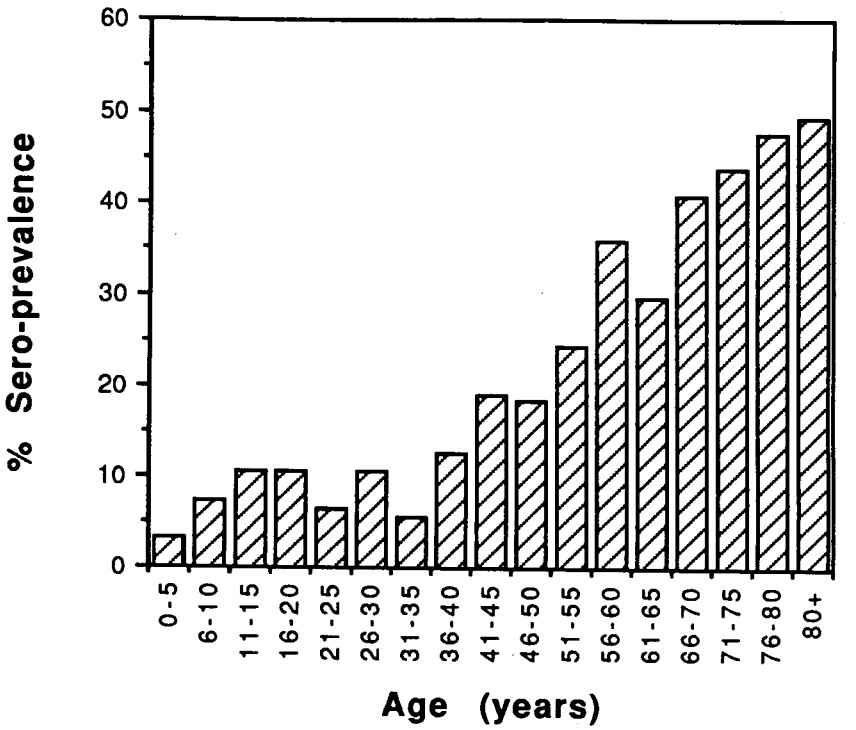

Figure 1.2. Sero-prevalence of *T. gondii* with respect to age in South Yorkshire, 1988-90. (compiled from data published by Walker *et al.*, 1992)

Hutchison; 1989; Cook, 1991).

Post-natally acquired disease

Toxoplasmosis in the immunocompetent host is frequently asymptomatic although a mild febrile illness is common at the onset of infection (reviewed, Hughes, 1985). However, individuals that are immunocompromised due to chemotherapy for malignancies or post transplant surgery as well as those suffering from AIDS, may experience disease manifestations such as encephalitis, myocarditis, enteritis, peritonitis or pneumonia (reviewed, Curry, *et al.,* 1991). Toxoplasmosis in the immunocompromised can be due to a recently acquired infection but most commonly follows the reactivation of a latent chronic infection (Zangerle *et al.,* 1991). A recent study found that almost 50% of the AIDS patients sero-positive for *T. gondii* developed toxoplasmic encephalitis before death or by the end of the study (Zangerle *et al.,* 1991). At present chemotherapy for toxoplasmic encephalitis involves treatment with pyrimethamine and sulfadiazine, which synergistically block folic acid metabolism and are effective against the tachyzoite stage, but not the bradyzoites. Due to its inability to kill bradyzoites within cysts, this combination of drugs does not eliminate the disease and consequently upon cessation of chemotherapy, 80% of cases relapse (Luft & Remington,1988). An experimental drug, hydroxynaphthoquinone 566C80, appears to be effective in killing bradyzoites within cysts *in vitro* (Huskinson-Mark *et al.*, 1991) and can reduce the number of cysts in the brains of infected mice (Araujo, *et al.,* 1991), although its clinical potential awaits determination.

Congenitally acquired disease

Congenital infection occurs if a woman is infected for the first time while pregnant. A prior infection appears to prevent vertical disease transmission although immunodepressed mothers may not be able to give their foetus such protection (Desmonts, *et al.*, 1990). The time during pregnancy when the mother is infected is important in determining both the likelihood of vertical disease transmission and the severity of the disease. While the greatest risk of foetal infection is in mothers infected

Table 1.3. Estimates of the incidence of human congenital toxoplasmosis (Updated from Jackson & Hutchison, 1989).

Region	Author	Incidence per 1000
Germany	Kraubig (1966)	5.3
Mexico City	Roch and Varela (1966)	2
New York	Kimball *et al.*, (1971)	0.7
London	Ruoss and Bourne (1972)	0
Vienna	Thalhammer (1973)	6 - 7
The Netherlands	Koppe (1974)	6.5
Paris	Desmonts and Couvrier (1974)	1 0
UK	Fleck (1974)	0.07-0.25
Birmingham, Alabama	Alford *et al.*, (1974)	1.3
Austria	Thalhammer and Heller-Szollosy (1973)	8.6
Glasgow	Williams *et al.*, (1981)	>0.5
Europe	Williams *et al.*, (1981)	3-6[a]
USA	Williams *et al.*, (1981)	1-2[a]
Britian	Henderson *et al.*, (1984)	0.9
Brussels	Foulon *et al.*, (1984)	>2
USA	McCabe and Remington (1989)	1 - 8
Slovenia	Logar *et al.*, (1992)	3
Southern Finland	Lappalainen *et al.*, (1992)	0.96

[a] Computed averages

during the third trimester, the disease is most severe in those infections initiated in the first trimester and may result in intracranial calcification, hydrocephalus or abortion (reviewed, Buxton, 1990). The incidence of infection in children born to mothers infected in the first trimester is 25% while infection rates are 54% and 65% in mothers infected in the second and third trimester respectively. The incidence of vertical transmission can be significantly reduced by chemotherapy during pregnancy resulting in lower incidences of 8%, 19% and 25% respectively (Figure 1.3), although the severity of disease is unchanged (reviewed, McCabe *et al.*, 1987). Neurological and ophthalmological lesions, common consequences of congenital disease in the second and third trimester are not always apparent at birth and retinochoroiditis and neurological sequelae may develop in childhood or early adulthood (Desmonts & Couvrier, 1974).

1.4. Veterinary Importance

T. gondii is known to infect many domestic animals including sheep, swine, cattle and goats (Table 1.1). The world wide prevalence of *T. gondii* infection in veterinary animals is estimated at about 30% for sheep, 25% for cattle and 29% for pigs. (reviewed, Jackson & Hutchison,1989). Post-natally acquired infection in sheep is usually asymptomatic although infection during pregnancy can cause foetal death, abortion or foetal congenital disease (Hartley & Marshall, 1957; Buxton, 1983; Dubey & Taylor, 1986). Toxoplasmosis was found over a 3 year period from 1975-1978, to be the second most important cause of ovine abortion in south east Scotland, accounting for over 30% of all diagnosed abortions (Linklater & Dyson, 1979). Since sheep are herbivorous it must be assumed that the most likely route of infection is by the oral ingestion of oocysts on pasture or foodstuffs and the control of cat populations in the vicinity of farms could reduce the incidence of ovine infection. The level of protection afforded against congenital transmission by a new commercially available live veterinary vaccine "Toxovax" (Buxton, *et al.*, 1991) is encouraging and it is hoped that this will effectively reduce the frequency of *T. gondii* induced abortion in ewes.

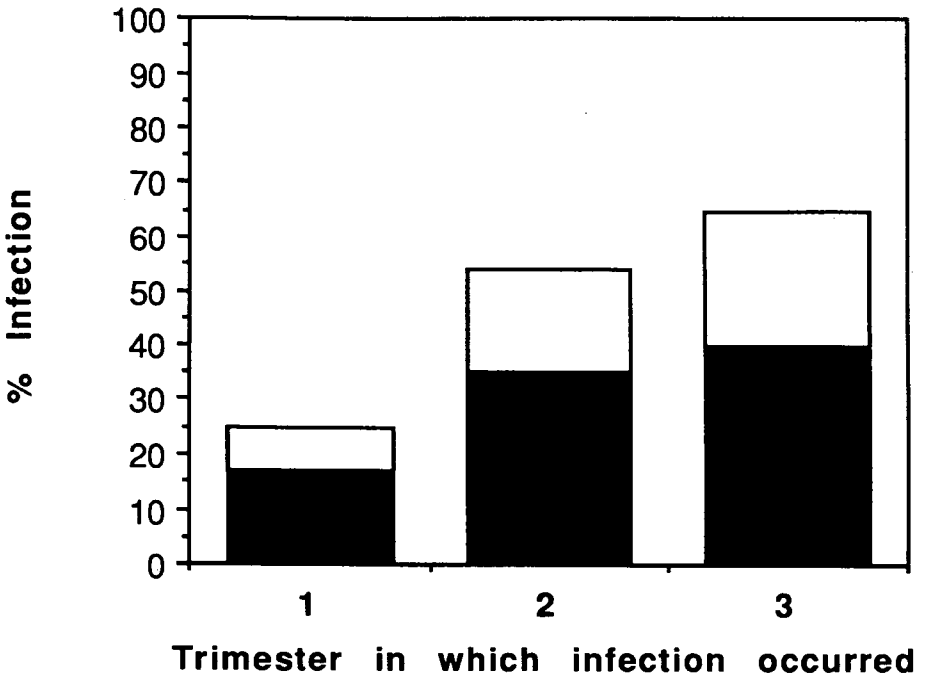

Figure 1.3. The incidence of congenital toxoplasmosis in the children of mothers infected during the first, second or third trimester of pregnancy. Filled area shows the reduction acheived by chemotherapy (compiled from information published by McCabe, *et al.*, 1987).

1.5. Genetic control

The variation in the severity of infection and the spectrum of clinical manifestations displayed by individuals infected with *T. gondii* (reviewed, McCabe *et al.*, 1987) indicate that the disease in man is subject to a number of genetic controls operating through the immune system. Studies using inbred mice and their congenic derivatives have indicated that the progress of a *T. gondii* infection is subject to a number of genetic controls which can influence either mortality and long term cyst burden. The major genetic influences that have been identified are summarised below.

H-2

Cyst burden

The ability of mice to survive an infection or limit the severity of the disease is partially under the control of genes within the H-2 complex (Jones & Erb, 1985; McLeod *et al.*, 1989b; Brown and McLeod, 1990). Looking at brain cyst numbers in mice challenged intraperitoneally, Jones and Erb, (1985), mapped resistance to the D end of the H-2 complex, finding resistance associated with 'd' and 's' haplotypes and susceptibility associated with the 'b' haplotype. Brown and McLeod (1990), demonstrated that regulation of brain cyst numbers in mice infected by the natural oral route, also cosegregated with H-2D or L, mice carrying D^b and L^b alleles developed a large cyst burden and mice with D^d and L^d alleles developed relatively few cysts. These results are consistent with functional studies demonstrating that $CD8^+$ cells have an important role to play in the control of brain cyst numbers (Parker *et al.*, 1991; Brown and McLeod,1990; Gazzinelli *et al.*, 1992b) as this is the region of H-2 that encodes for the class I molecules. Brown and McLeod (1990), confirmed the importance of this region for the regulation of cyst numbers by demonstrating that the mutant mice, B10.D2-H-2^{dm1}, that have a gain-loss mutation in D^d and L^d genes and, BALB/c-H-2^{dm2}, that have a deletion of the L^d gene, are both susceptible to the formation of large cyst numbers. A further *in vitro* study confirmed the importance of this region of the H-2 complex for the killing of infected or antigen pulsed cells. Using cells from

congenic mice in a chromium release cytotoxicty assay, Hakim *et al.*, (1991), demonstrated that the ability of CD8+ effector cells to kill target cells was dependent on both populations being H-2D/L^{d}. Thus, effector cells from BALB/c mice (H-2D^{d}), had decreased cytotoxicity against target cells derived from the mutants, B10.D2-H-2^{dm1} and BALB/c-H-2^{dm2}.

Toxoplasmic encephalitis

Although toxoplasmic encephalitis (TE) is normally associated with immunodepression in humans, it may occur in the apparently immunocompetent (McCabe *et al.*, 1987) suggesting that certain individuals may have a genetic predisposition to this complication.

In a mouse model, using the intraperitoneal route of infection, Suzuki, *et al.*, (1991) demonstrated genetic control of toxoplasmic encephalitis was governed by genes in the H-2D region. H-2D^{b} and H-2D^{k} mice were susceptible to toxoplasmic encephalitis whereas, H-2D^{d} mice were resistant. Thus, resistance to both large cyst burdens and toxoplasmic encephalitis would appear to map to the same area in the H-2 complex, a fact also confirmed by Suzuki, *et al.*, (1991) in the above study. Using this model, Freund, *et al.*, (1992) demonstrated by northern blot analysis, RNA transcripts for TNF-α in chronically infected mice carrying the D^{b} or D^{k} allele, but not those carrying the D^{d} allele. Further analysis, demonstrated a restriction fragment length polymorphism (RFLP) of the promoter sequence of the TNF-α gene in mice bearing the D^{b} or D^{k} allele (Freund, *et al.*, 1992). These findings led Freund *et al.*, (1992) to propose a link between this RFLP, TNF-α production and toxoplasmic encephalitis. However, although this study examined recombinant haplotype mice which eliminated the possibility that the HSP70 genes in the H-2D region were important in the control of toxoplasmic encephalitis, mice with differing TNF and D alleles were not examined. Interestingly, an earlier study (Brown and McLeod, 1990), demonstrated that mice with a point mutation in the D and L genes were susceptible to large cyst burdens in

their brains. Although, these mice were not examined for the presence of toxoplasmic encephalitis, current evidence suggests a 100% correlation with mice susceptible to toxoplasmic encephalitis and the formation of large cyst burdens (Suzuki *et al.*, 1991; Freund *et al.*, 1992). Hence, the relative contribution played by the TNF genes and the class I genes (D and L) to the prevention of toxoplasmic encephalitis remains unresolved.

Mortality

Williams *et al.*, (1978), were the first to demonstrate a role for the H-2 complex, in determining the relative resistance of mice to *T. gondii* challenge as judged by survival rates, although other genes were also implicated in protection. In this study, susceptibility to intraperitoneal infection was found to be associated with the $H\text{-}2^a$ and $H\text{-}2^b$ haplotypes while resistance was associated with the $H\text{-}2^d$ and $H\text{-}2^k$ haplotypes. The ability of mice to survive an oral challenge with *T. gondii* for the first 30 days is also under polygeneic control and is regulated by a minimum of 5 genes, one of which maps to the H-2 region (McLeod *et al.*, 1989b). There does not appear to be any correlation between early survival and the number of cysts found in the brains of infected mice 30 days post infection (McLeod *et al.*, 1989b), a result that is consistent with the combination of results reported above.

The ability of inbred mice to survive infection with *T. gondii* can also be influenced dramatically by the route of infection (Johnson, 1984). In this study, C57BL/6 mice were found to be susceptible to oral but resistant to intraperitoneal infection. In contrast, LACA mice exhibited the reverse characteristics while BALB/c mice were resistant to both the oral and intraperitoneal route of challenge. It is not known if this is a H-2 dependent phenomenon.

H-13

By using recombinant inbred mice originating from BALB/c x C57BL/6J, the ability of mice to survive an intraperitoneal challenge was found to be influenced by H-13 genes.

Of the seven recombinant lines obtained, the three most susceptible were found to contain the H-13 allele of the susceptible C57BL/6J parental strain, while the four most resistant strains had the H-13 allele of the resistant BALB/c parent (Williams *et. al.*, 1978).

Lsh

The *Lsh* gene, which is most probably identical to those designated *Ity* and *Bcg* , has been implicated in the resistance of mice to many macrophage parasites including *Salmonella typhimurium* and *Mycobacterium bovis* . The *Lsh* gene has been associated with an increase in macrophage production of TNF-α and IL-1 and the upregulation of class II expression after stimulation with IFN-γ or LPS (Blackwell *et al.*, 1989; Kaye & Blackwell, 1989). Surprisingly the *Lsh* gene has not been implicated in a protective capacity in murine toxoplasmosis. McLeod, *et al.*, (1989b) found that the *Lsh* gene on the B10(H-2^b) background had no significant effect on early survival when compared with their congenic counterparts and in fact, the presence of this gene resulted in increased cyst numbers in the brains of infected mice.

1.6. Immunological control

B cells and antibodies

The intracellular localisation of *T. gondii* for the vast majority of the life-cycle does not suggest an important role for antibodies in the control of the disease. Nonetheless, murine anti-*T. gondii* antibodies are capable of killing extracellular tachyzoites *in vitro via* the classical complement pathway (Schreiber & Feldman, 1980; Suzuki & Kobayashi, 1985) and *T. gondii* specific rabbit antibodies can enhance the ability of human myeloid cells (monocytes, macrophages neutrophils and eosinophils) to kill extracellular tachyzoites in culture (Erb *et al.*, 1991). Furthermore, immunoglobulin μ-suppressed mice are not able to control *T. gondii* infection as well as their immunocompetent counterparts (Frenkel & Taylor, 1982). However, the removal of

B cells, which in addition to antibody production comprise an important population of antigen presenting cells could have profound effects on the immune system as a whole unrelated to the production of antibodies. This may explain why the passive transfer of anti-*T. gondii* sera can only partially protect μ-suppressed mice (Frenkel & Taylor, 1982). In addition, the passive transfer of immune sera into immunocompetent mice has no effect on the outcome of an infection (Chinchilla & Frenkel, 1978) and does not increase the efficacy of adoptively transferred lymphocytes (Frenkel, 1967). Nevertheless, the transfer of immune B cells and serum has been reported to induce resistance to a fatal challenge with RH strain *T. gondii* in guinea pigs (Pavia *et al.*, 1992) and passive transfer of IgE has been found to mediate some degree of protection in rats (Ridel *et al.*, 1988).

A reduction in the infectivity of tachyzoites pre-incubated with monoclonal antibodies to certain *T. gondii* antigens has been observed (Johnson *et al,* 1983) and F3G3, a monoclonal antibody to a cytoplasmic component of *T. gondii* can prevent death in infected mice (Sharma *et al.*, 1984a). Other monoclonal antibodies have been shown *in vitro* to enhance, phagocytosis of tachyzoites by macrophages and render them susceptible to intracellular destruction (Hauser and Remington, 1981). If antibodies do play a protective role against *T. gondii* infection, they probably do so by interfering with the parasite's interaction with the host cell. For example, Grimwood and Smith, (1992) have shown that a monoclonal antibody to P30, a major tachyzoite surface protein, could interfere with cell invasion, while Sibley, (1985), demonstrated that the ability of tachyzoites to prevent acidification of the phagosome, as first reported by Jones & Hirsch, (1972), could be impaired if the tachyzoites were opsonised with *T. gondii*- specific antisera.

Macrophages

Non-specifically as well as specifically activated macrophages have an enhanced ability to kill intracellular *T. gondii* tachyzoites (Remington *et al.*, 1972). This ability to kill internalised tachyzoites appears to be due to IFN-γ and TNF-α acting in synergy

(Sibley, *et al.*, 1991). These are thought to induce reactive nitrogen intermediates that inhibit *T. gondii* proliferation (Langermans, *et al.*, 1992).

Eisenhauer *et al.*, (1988) demonstrated that activation of macrophages in pregnant mice (by intravenous injection of *Propionibacterium acnes*) could reduce the incidence of congenital disease. The activation of macrophages observed in mice with acute toxoplasmosis (Remington *et al.*, 1972) may be due to p30, a major component of the tachyzoite surface membrane which has been shown to activate macrophages *in vitro* (Makioka & Kobayashi, 1991). In this way the macrophage may play a significant role in protecting the host, and possibly the foetus, by reducing the number of viable tachyzoites which can ultimately form cysts or invade the foetus *via* the placenta (Eisenhauer, *et al.*, 1988).

Macrophage killing mechanisms associated with T. gondii

Jones and Hirsch (1972), showed that about 50% of tachyzoites of *T. gondii* survive in macrophages by preventing the fusion of lysosomes with the parasitophorous vacuole, the remaining tachyzoites being killed in the hostile environment of the phagolysosome. The mechanism(s) preventing fusion were operative locally as both parasitophorous vacuoles that had, and parasitophorous vacuoles that had not prevented lysosome fusion were apparent within any one cell. Furthermore, *T. gondii* can prevent phagosome acidification, although opsonisation with specific antibodies facilitates rapid acidification of the phagosome (Sibley *et al.*, 1985). Macrophages that have been activated by specific or non-specific means have increased ability to mediate *T. gondii* killing by both oxygen-dependent (reviewed, Hughes, 1988) and L-arginine-dependent mechanisms (Adams *et al.*, 1990). In order to elicit either of these mechanisms it is believed that the macrophages have to be primed and triggered. Cytokines such as IFN-γ are capable of priming macrophages but a second trigger signal is also required for the activation of microbicidal mechanisms. TNF-α or certain constituents from bacterial cell walls, such as LPS or MDP, probably through the induction of TNF-α, can act as this trigger signal for macrophage activation.

Oxygen-dependent killing

Macrophages can be induced to produce an array of toxic molecules that are dependent on oxygen including O_2^- , H_2O_2 , $^1O_2$ and -OH. These molecules are short lived and non-specific in their toxicity although different parasites have different susceptibilities to them. Tachyzoites of *T. gondii* are relatively resistant to H_2O_2 and O_2^-, but susceptible to $^1O_2$ and -OH, compared with *L. donovani* which is susceptible to H_2O_2 though resistant to O_2^-, $^1O_2$ and ·OH (reviewed, Hughes, 1988). These differences are most likely due to the differences in the levels of oxygen scavengers present in these parasites. *T. gondii* has high levels of superoxide dismutase (SOD), an effective scavenger of O_2^- as well as high levels of glutathione peroxidase (GPO) and catalase which can counteract the effects of H_2O_2. In contrast, *L. donovani* has very low GPO or catalase levels although it does have similar levels of SOD to *T. gondii*.

L-arginine-dependent killing

Nitric oxide is generated by a broad spectrum of cell types and has many varied functions throughout the body including that of a neurotransmitter and a toxic molecule involved in the killing of pathogens or tumour cells. Recent reports have demonstrated a substantial role for nitric oxide and its products in the killing of protozoan parasites, notably *Leishmania major* (Green *et al.*, 1990), *Plasmodium yoelii* (Nussler, *et al.*, 1991), *Plasmodium falciparum* (Rockett *et al.*, 1991) and *T. gondii* (Adams *et al.*, 1990). The ability of cells to produce nitric oxide is dependent on L-arginine while its production can be down regulated by the competitive inhibitor N^Gmonomethyl-L-arginine, (N^GMMA). Adams *et al.*, (1990), demonstrated that the ability of macrophages activated by IFN-γ and LPS to prevent multiplication of intracellular tachyzoites of *T. gondii* could be reversed by the addition of N^GMMA into the culture medium. The ability of IFN-γ to induce nitric oxide and so prevent *T. gondii* multiplication, has since been shown to be dependent on endogenous TNF-α

production, but not IL-1α or IL1-β (Langermans *et al.*, 1992). IFN-γ mediated NO killing of *T. gondii* can be ablated by the addition of IL-10 (Gazzinelli *et al.*, 1992a).

T cells

Initial studies in the nude mouse demonstrated a crucial role for T cells in mediating protection against overwhelming fatal toxoplasmosis (Lindberg & Frenkel, 1977). In recent years the role of the various T cell subsets and how they interact to mediate protection have been comprehensively studied by a number of workers.

CD4+ T cells

Mortality studies

Adoptively transferred spleen cells from mice vaccinated with the temperature sensitive mutant (ts-4), (Suzuki & Remington, 1988; Gazzinelli *et al.*, 1991) or chronically infected with the RRA strain of *T. gondii* , (Parker *et al.*, 1991) can reduce mortality in mice challenged intraperitoneally with tachyzoites of the C56 *T. gondii* strain (Suzuki & Remington, 1988), or the RH strain (Gazzinelli *et al.*, 1991) or tissue cysts of the RRA strain (Parker *et al.*, 1991). Since protection against fatal toxoplasmosis in all three of these studies was reduced by pretreating the cells with an anti-CD4+ monoclonal antibody (GK 1.5) and complement, a protective role for this CD4+ T cells could be deduced. In addition to this, Gazzinelli *et al.*, (1991), found that CD4+ T cells from ts-4 immunised mice produced IL-2 and IFN-γ when stimulated *in vitro* with crude *T. gondii* antigen, implying the protection observed was due to cells of the TH1 CD4+ subset.

Regulation of cyst number

Parker *et al.*, (1991) demonstrated that the adoptive transfer of T cells from BALB/c mice chronically infected with the RRA strain of *T. gondii* , resulted in the development of fewer cysts in the brains of mice subsequently infected with the same

strain, an effect that was unaffected by the depletion of CD4+ cells. However, Araujo (1991) found that depletion of CD4+ cells *in vivo* (by treatment with GK1.5 and complement) in BALB/c, C57BL/6 or CBA/Ca prior to infection with the Me49 strain resulted in increased cyst numbers in the brains of all three strains of mice.

CD8+ T cells

Mortality studies

Adoptively transferred T cells from immune donors can protect syngeneic recipients from fatal toxoplasmosis an effect that has been shown to be partially dependent on CD8+ cells (Suzuki & Remington, 1988; Parker *et al.*, 1991). The ability of adoptively transferred CD8+ cells to protect mice from fatal toxoplasmosis could be ablated by the administration of an anti-IFN-γ neutralising monoclonal antibody (Suzuki & Remington, 1990). Vaccination with recombinant p30 using Quil A as an adjuvant protects mice from an oral challenge with the Me49 strain, an effect that can be transferred to syngeneic recipients with CD8+ T cells. T cells from these vaccinated mice were also directly parasiticidal and proliferated *in vitro* in response to p30 producing IL-2 and IFN-γ (Khan, *et al.*, 1991).

Cyst numbers

Parker *et al.*, (1991) demonstrated that CD8+ T cells from chronically infected immune mice, were able to adoptively transfer resistance which significantly reduced cyst burdens in the brains of recipients . In this study, the importance of CD8+ T cells in the control of cyst numbers was confirmed by depleting this sub-population prior to transfer (Parker *et al.*, 1991). Further evidence for their importance has been demonstrated by injecting mice with anti-CD8 monoclonal antibodies prior to oral (Brown & McLeod, 1991), or intraperitoneal infection (Gazzinelli *et al.*, 1992b) with the Me49 *T. gondii* strain. This resulted in increased brain cyst number 30 days post infection.

Cytotoxic CD8+, MHC class I restricted, T cells from infected humans (Yano *et al.*, 1989) and mice (Hakim *et al.*, 1991; Subauste *et al.*, 1991) can lyse infected target cells *in vitro* . The predominate phenotype of antigen specific T cell clone isolated from recently infected and therefore symptomatic individuals is CD8+ (Sklenar *et al.*, 1986) implying that CD8+ T cells may play an important role early in infection. As well as lysing target cells, CD8+ T cells derived from mice vaccinated with recombinant p30 can also kill extracellular tachyzoites *in vitro,* by a mechanism independent of antibody or MHC control. NK cells are not involved as the functional population is insensitive to treatment with anti-asialo-GM1 and complement (Khan, *et al.*, 1988b; Khan, *et al.*, 1991).

These functional studies, demonstrating an important role for CD8+ cells in resistance to infection are in agreement with immunogenetic studies, demonstrating the importance of class I genes in the regulation of brain cyst numbers (Jones & Erb, 1985; McLeod *et al.*, 1989b; Brown and McLeod, 1990) and survival (Williams *et al.*, 1978) (discussed above).

The importance of CD4+ and CD8+ T cells in the prevention of reactivation of chronic disease

Reactivation of a chronic *T. gondii* infection is usually associated with the immunodepressed, most notably AIDS patients or patients receiving chemotherapy after organ transplantation or for the treatment of malignancies. Degeneration of CD4+ T cell function in AIDS patients has been suggested as being responsible for the reactivation of chronic toxoplasmosis (Fauci *et al.*, 1984). However, studies in mice have produced a series of conflicting observations. While an early *in vivo* depletion experiment in C3H/HeN mice revealed that the depletion of CD4+ T cells, using the monoclonal antibody GK 1.5, reactivated a chronic infection (Vollmer et al, 1987), a later study indicated that this treatment actually reduced inflammation in the brains of

chronically infected mice (Israelski *et al,* 1989). A further study found that in C57BL/6N mice the depletion of both CD4+ and CD8+ T cells was necessary to reactivate a chronic infection; the depletion of CD4+ cells alone, did not reduce inflammation or induce encephalitis (Gazzinelli, *et al.,* 1992b). Nevertheless, Gazzinelli, *et al.,* (1992b) noted that chronically infected mice depleted *in vivo* of their CD4+ T cells for 30 days had more cysts in their brains at necroscopy when compared with control mice or those depleted of CD8+ T cells. The increase in cyst number in these mice would suggest that reactivation of the disease had in fact occurred, although not to the same extent observed by Vollmer *et al.,* (1987). In contrast, mice simultaneously depleted of both CD4+ and CD8+ T cells or administered neutralising IFN-γ monoclonal antibody, demonstrated encephalitis, had free tachyzoites visible in their brain, as well as increased cyst numbers. Both CD4+ and CD8+ T cells were able to produce IFN-γ on stimulation with *T. gondii* antigen *in vitro*, although only CD4+ T cells were capable of producing IL-2. Both IL-2 (Sharma, 1985) and IFN-γ (reviewed, Subauste & Remington, 1991) have been implicated in host protection during acute toxoplasmosis and together with the above observation would suggest a synergistic role for cells of the CD4+ TH1 subset and CD8+ T cells in the prevention of reactivation of chronic infections. It may therefore be significant that human CD4+ T cell clones that secrete IFN-γ and IL-2 in response to *T. gondii* soluble antigen have also been obtained from asymptomatic infected individuals (Saavedra & Herion, 1991). It is likely that CD4+ TH1 cells have a role in maintaining a balance in the host parasite relationship through the release of cytokines such as IFN-γ and IL-2, which may activate NK cells, macrophages and CD8+ T cells to effect parasite killing.

Natural Killer cells

Murine NK cells can kill *T. gondii* infected cells or extracellular tachyzoites *in vitro* and may play an important role in host defence from as early as 3 days post infection

(Hauser & Tsai, 1986). It seems likely that they may also act as a source of IFN-γ (Djeu, 1983), the effects of which are discussed in detail below. Cytoplasmic and membrane antigen fractions from *T. gondii* have been shown to enhance the activity of human NK cells *in vitro* , an effect associated with a concomitant rise in IFN-γ , but not IL-2 levels in the culture medium (Sharma *et al,* 1984b). Using mouse cells *in vitro* similar results have been obtained, namely, increased NK cell activity with a concomitant rise in IFN-γ levels, although in this study accompanied with a decrease in IL-2 levels (Diez *et al,* 1991). The recent observation that NK cells can function as antigen presenting cells (Roncarolo *et al,* 1991), raises the possibility that this population of cells, may have an important role to play during the early stages of infection. In the light of new evidence it has recently been postulated that NK cells by producing IFN-γ, may be instrumental in directing the immune response towards a predominantly TH1 $CD4^+$ T cell response (Romagnani, 1992).

Cytokines

Cytokines derived from the cell types described above, undoubtedly play an important role in the host defence system against *T. gondii.* However, in certain circumstances they may also be involved in the suppression of protective immunity or even in mediating severe immunopathological reactions.

IFN-γ

IFN-γ is by far the most studied cytokine to date and arguably the most important in the induction of a protective immune response against *T. gondii* (reviewed, Subauste & Remington, 1991). It has been demonstrated that spleen cells from mice infected with *T. gondii* produce IFN-γ in response to *T. gondii* antigen (Shirahata & Shimizu, 1986). Supernatants from immune lymphocytes cultured *in vitro* with *T. gondii* lysate antigen have also been shown to activate macrophages and inhibit parasite growth, a phenomenon that has been attributed to IFN-γ (Borges & Johnson, 1975, Anderson *et al,* 1976). *In vivo* recombinant IFN-γ administration has been shown to prolong the time to death and decrease overall mortality in mice challenged

with *T. gondii* (McCabe *et al.*, 1984). IFN-γ is also required to prevent cyst rupture *in vitro* (Jones, *et al.*, 1986) and *in vivo* (Suzuki *et al.*, 1989b), thus preventing reactivation and the ensuing encephalitis in chronically infected mice. The ability of adoptively transferred $CD8^+$ cytotoxic T cells to prevent mortality in BALB/c mice subsequently infected can be ablated by the administration of an anti-IFN-γ monoclonal antibody (Suzuki & Remington, 1990). Recombinant IFN-γ administered to nude mice can protect them from acute infection, preventing proliferation of organisms in the brain, lungs, heart, liver and spleen, an effect shown to be independent of TNF-α (Suzuki, *et. al.*, 1991). However, Sibley *et al.*, (1991) have demonstrated a TNF-α dependent IFN-γ mediated mechanism *in vitro* where TNF-α was required to trigger IFN-γ primed macrophages for killing. In addition Langermans *et al.*, (1992) have demonstrated that a neutralising antibody to TNF-α can ablate the protective effect of IFN-γ.

The ways in which IFN-γ elicits its protective effects appear to be many fold, but include the activation of the macrophage to kill or inhibit growth of the parasite by oxygen dependent and L-arginine dependent mechanisms (discussed in detail above). In addition, IFN-γ has also been shown to be parasitostatic *in vitro,* a process which appears to involve starving the parasite of tryptophan (Pfefferkorn *et al.*, 1984).

Crude *T. gondii* antigen (Saavedra & Herion, 1991; Kelly, *et al.*, 1987; Kelly, *et al.*, 1989; Sklenar, *et al.*, 1986; Canessa, *et al.*, 1988) and p30 (Khan, *et al.*, 1988a), can induce IFN-γ production when incubated with lymphocytes (Kelly, *et al.*, 1987; Kelly, *et al.*, 1989;) or lymphocyte clones (Saavedra & Herion, 1991; Sklenar, *et al.*, 1986; Canessa, *et al.*, 1988), derived from chronically infected individuals. In contrast, lymphocyte clones derived from individuals with acute symptomatic infections produce significantly less IFN-γ in response to crude *T. gondii* antigen (Sklenar, *et al.*, 1986). This suggests that the ability to recognise *T. gondii* antigen and respond with IFN-γ production is important in protective immunity. These facts, combined with the apparent necessity of IFN-γ to prevent toxoplasmic encephalitis in

mice (Suzuki, *et al.*, 1989b; Suzuki & Remington, 1990), has led to speculation that toxoplasmic encephalitis in AIDS patients may be due to an inability to produce this cytokine. Murray *et al.*, (1984), examined the ability of lymphocytes from AIDS patients to produce IFN-γ in response to *in vitro* stimulation with *T. gondii* antigen or mitogen. In this study all but one of the fourteen patients examined were incapable of producing IFN-γ in response to *T. gondii* antigen and eleven out of sixteen patients had impaired IFN-γ secretion after stimulation with mitogen. These results provide a possible explanation for the high incidence of reactivation of chronic *T. gondii* infections in people with AIDS.

IFN-α/β

Diez *et al.*, (1989), measured IFN-α and IFN-β in the serum of mice infected with the C56 strain of *T. gondii,* finding a gradual increase throughout the 9 day duration of the experiment. In contrast, IFN-γ could not be detected in the serum. *In vitro* stimulation of spleen cells from infected mice with mitogen induced production of IFN-α and IFN-β but not IFN-γ. Recombinant IFN-β can reduce mortality in mice infected with the C56 strain of *T. gondii* an effect that appears to be dependent on the presence or the production of IFN-γ, as neutralisation of this cytokine abrogated the protective effect (Orellana *et al.*, 1991). IFN-β may also act indirectly through its ability to activate NK cells (Ortaldo and Heberman, 1986) or induce TNF production (Pelus *et al.*, 1988).

TNF-α/β

The role of TNF, is far less clear cut than IFN-γ and is somewhat controversial. TNF-α has been reported not only as being beneficial in protecting mice from infection (Chang *et al.*, 1990) but also detrimental as its administration to mice with acute infections can shorten their time to death (Black *et al.*, 1989). However, a recent study indicated that endogenous TNF-α could be protective as *in vivo* neutralisation using anti-TNF-α antibodies increased mortality in mice subsequently infected with *T. gondii* (Johnson, 1992). Nevertheless, a further study found that mice with

acute toxoplasmosis had high serum TNF levels which could be reduced by an enforced swimming exercise program hastening their recovery (Chao, *et al.*, 1992b). It is of further interest that genetic susceptibility to toxoplasmic encephalitis and increased brain cyst number has been reported to be associated with a restriction fragment length polymorphism in the promoter region of the TNF-α gene (Freund *et al*, 1992). In this study TNF-α transcripts were detectable by Northern blot analysis in the brains of *T. gondii* infected mice with this polymorphism, but not in resistant haplotype recombinant mice.

The way in which TNF-α acts to control *T. gondii* infection under certain circumstances is not clear although it may act in synergy with IFN-γ to activate macrophages (Sibley *et al.*, 1991, Langermans, *et al.*, 1992). *In vitro* studies would suggest that TNF-α is not directly cytotoxic to extracellular parasites (Black *et al.*, 1990, Black *et al.*, 1989 and DeTitto *et al.*, 1986). Its ability to hasten time to death in other circumstances is probably due to its over-production and well documented side effects; inflammation, nephritis, anaemia, hypoglycaemia and hypotension (reviewed, Cerami & Beutler, 1988).

GM-CSF

Recombinant human GM-CSF has been shown to activate human macrophages *in vitro* to inhibit the growth of *Leishmania donovani*, by oxygen-dependent mechanisms (Nicola & Vadas, 1984), while recombinant murine GM-CSF has been shown to protect mice against a *Listeria monocytogenes* challenge (Magee & Wing, 1989). However, no anti-*T. gondii* inhibitory effect was observed when GM-CSF was used to activate murine macrophages in culture and recombinant murine GM-CSF administered to BALB/c mice with a virulent RH strain infection did not prolong survival or reduce mortality (Hughes, *et al.*, 1987).

IL-2

The administration of recombinant IL-2 can significantly reduce mortality in mice subsequently infected with *T. gondii*, as well as reducing the number of cysts present

in the brains of surviving mice. This enhanced immunity was found to be due to increased NK cell activity and was not associated with increased macrophage killing (Sharma, *et al.,* 1985). It may be significant that IL-2 can induce TNF-α production (Economou, *et al.,* 1989) and may contribute to protection indirectly.

IL-6

Murray *et al.,* (1990), examined the aqueous humour from 8 patients with toxoplasmic uveitis finding IL-6 in five of them. The presence of IL-6 was not confined to the toxoplasma uveitis patients as it was also detected in a similar proportion of patients with Fuchs' heterochromic cyclitis. It is therefore possible that IL-6 is simply diagnostic of damage to the eye. IL-6 has also been detected by PCR in the brains of C57BL/10 mice with fatal toxoplasmic encephalitis, though not in control uninfected mice (Hunter *et al.,* 1992a&b), again suggesting a role for IL-6 in inflammation. IL-6 has been associated with lethal *Escherichia coli* infection in mice, a condition that can be reversed with the administration of an anti-IL-6 neutralising antibodies (Starnes *et al.,* 1990). In contrast, IL-6 was found to inhibit intrahepatic development of *Plasmodium yoelii* (Pied, *et al.,* 1991).

IL-10

IL-10 is capable of inhibiting IFN-γ production by a number of different cell types including CD4+ TH1 cells, NK cells, and CD8+ T cells as well as directly inhibiting the effects of IFN-γ (Waal Malefyt, *et al.,* 1991) The addition of IL-10 to cultures can reduce the ability of IFN-γ treated inflammatory macrophages to kill *T. gondii* (Gazzinelli*et al.,* 1992a). IL-10 can be produced by a wide range of cells including CD4+ cells of the TH2 subclass (Fiorentino *et al.,* 1989), Ly-1 B cells (O'Garra *et al.,* 1990) and mast cells (Moore *et al.,* 1990) and while it would appear likely that it would have a detrimental effect and exacerbate *T. gondii* infections *in vivo* , this remains to be confirmed.

1.7. Vaccine Development

Past research indicates that the prospects for developing effective vaccines against *T. gondii* are very good. The recent commercial availability of an apparently successful live veterinary vaccine 'Toxovax', highlights this potential. While a live vaccine for use in humans is unlikely and undesirable, the prospects for a sub-unit vaccine must be good. The definitive host (Cats), as well as the intermediate hosts (man and domesticated animals) or both could be targeted by vaccination. Protecting the cat population would have obvious advantages although accessing the feral population poses problems. Vaccination of intermediate hosts could prevent congenital transmission as well as protecting the adult. In fact, it is a possibility that a vaccine could be effective in preventing vertical disease transmission without inducing sterile immunity in the adult.

Most vaccination studies in the laboratory have concentrated on the post-natally acquired disease using murine models, whereas the majority of studies in the field using sheep and goats have been concerned with the prevention of congenital transmission. Until recently there was no well characterised laboratory model of congenital toxoplasmosis. However, the recent description of both a rat (Dubey & Shen, 1991), and a murine (Roberts and Alexander, 1992; Chapter 9) model, indicate it is now possible to examine a wide range of vaccine formulations in the laboratory for efficacy in preventing vertical disease transmission.

Studies in Cats

Effective vaccination of cats to prevent oocyst shedding would protect people and livestock that live in close proximity. The feasibility of such a vaccine is demostrated by the fact that approximately 90% of kittens infected naturally develop immunity to experimental challenge (Frenkel & Smith, 1983). Frenkel, *et al.*, (1991) have since vaccinated kittens with bradyzoites of a mutagenized clone that had lost the ability to cause oocyst shedding, successfully preventing 84% of them shedding oocysts on

challenge with a complete strain. However, this mutagenized clone was still able to form cysts in mice and there remains the possibility that cysts could form in the vaccinated cat. The extraintestinal cycle has been documented in the cat (Dubey & Johnstone, 1982). Also, administration of this vaccine would be by infected tissue (in the above case infected mice were used) which combined with the inherent risks of live vaccines and the less than 100% success rate, would not encourage people to have their pets vaccinated.

Studies in mice and rats

Early studies examined the use of killed or sub-lethally irradiated parasites (Mas Bakal & In't Veld, 1979; Chhabra, *et al.,* 1979; Krahenbuhl, *et al.,* 1972) and had varying degrees of success in reducing mortality or increasing the time to death of challenged mice. A temperature sensitive mutant ts-4 (Pfefferkorn & Pfefferkorn, 1976) has been successful in reducing the severity of disease, as measured by decreased mortality, in adult mice, (Waldeland & Frenkel, 1983) as well as hamsters (Elwell & Frenkel, 1984). Intra-intestinal vaccination of dams with ts-4 before pregnancy can reduce the incidence of congenital infection in their offspring when challenged on day 11 of pregnancy (McLeod *et al.,* 1988). This temperature sensitive mutant has proven useful as a model to examine the mechanisms involved in eliciting effective immunity against the adult disease (Gazzinelli *et al.* 1991; Araujo, 1991; Subauste *et al.,* 1991). However, due to its persistence for up to 2 months after vaccination (Waldeland *et al.,* 1983) and its ability to cause foetal death and congenital infection in *Aotus* monkeys (Escajadillo & Frenkel, 1991), it is unlikely to be considered as a commercial vaccine. Defined antigen fractions purified from the parasite or made through recombinant techniques have also been tested against the post-natally acquired disease. The major surface protein p30, which has been recently sequenced and cloned (Burg *et al.,* 1988), has been used in a number of vaccine studies. Surprisingly, vaccination with p30 can both exacerbate (Kasper, *et al.,* 1985) and protect (Bulow & Boothroyd, 1992, Khan, *et al.,* 1991) against acute infection. These different results could be attributable to the choice of adjuvant used in the formulation; whereas Kasper, *et al.,*

(1985) used FCA, Khan, *et al.*, (1991) used Quil A and Bulow & Boothroyd, (1992) used liposomes. The ability of different adjuvants when formulated with the same antigen to protect or exacerbate disease has been reported previously for gp63 the highly conserved surface glycoprotein of *Leishmania* : incorporation of this antigen into liposomes resulted in protection whereas when it was formulated with Freunds Complete Adjuvant (FCA), exacerbated lesion growth was observed following infection (Russell & Alexander, 1988).

Further *T. gondii* antigens have been examined for their protective potential. For example an antigen purified by affinity purification from the cytoplasmic fraction of *T. gondii* using a MAb (F3G3) has been shown to protect mice from fatal toxoplasmosis (Sharma, *et al.*, 1984a). Antigens of 14 and 35 Kd have also been implicated as potentially protective in a study by Araujo and Remington (1984). In addition, the excreted/secreted antigen p24 has also been studied as a vaccine candidate and in its recombinant form can induce protection in rats and synthetic T cell epitopes derived from the primary structure of this molecule, predicted using the algorithm described by Rothbard and Taylor (1988), have also been used to successfully vaccinate rats (Duquesne *et al*, 1991). Whether these antigens will be of use against the acquisition of congenital toxoplasmosis awaits further study.

Studies in sheep and goats

Beverley and Watson (1971), demonstrated that experimental infection with *T. gondii* in ewes was capable of preventing congenital transmission if the ewes were re-infected during pregnancy. This simply demonstrated that protective immunity could prevent vertical disease transmission in sheep and so demonstrated the potential for finding an effective vaccine. Later that year Beverley *et al.*, (1971) vaccinated sheep with a freeze/thawed preparation that was able to prevent foetal death but not foetal infection in sheep subsequently infected with live *T. gondii*.. The closely related coccidian *Hammondia hammondia* was also partially effective as a live vaccine, reducing both the incidence of foetal death and infection in the litters of vaccinated goats (Dubey,

1981). A live tachyzoite vaccine (Toxovax) has now been shown to prevent parasite induced abortion in sheep (Buxton *et al.*, 1991). Although this is a major breakthrough and "Toxovax" has now been licensed as a veterinary vaccine, it is unlikely that a live vaccine would be used in humans. Another concern in the use of this vaccine is its inability to prevent foetal infection. In the above study, almost two thirds of the viable lambs born to the vaccinated ewes were found to be infected. The use of this vaccine while reducing the number of lambs lost due to abortion may increase the number of lambs carrying the disease and in doing so also increase the public health hazard. The search for a fully effective, defined safe sub-unit, or recombinant vaccine must continue.

1.8. Objectives

The importance of toxoplasmosis in terms of congenital transmission and potential killer of the immunocomprimised, alone make this disease worthy of study. However, the impact of toxoplasmosis on agriculture as regards economic loss and the potential risk of infected meat products to the public reinforce the need for research in this area.

Characterisation of the immune response of different inbred mouse strains, exposed to disease would not only give an insight into host parameters important in the control of disease, but would also prove useful in the selection of animals for the study of different aspects of disease. A clear understanding of immunological control of toxoplasmosis, would allow the rational design of treatment or the design of vaccines. This thesis will examine immunol0gical control of disease using congenic and recombinant inbred mouse strains, relating differences in the immune response of these animals to the progress of disease. In this way, the parameters that mediate resistance or susceptibility to disease will be studied and then analysed further within particular inbred strains of mice. These studies, as well as providing useful laboratory models of disease, will give an insight into the parameters that mediate protective immunity. Using these well characterised models of disease putative vaccine

formulations will be tested for their capability to mediate protection against adult acquired disease and prevent vertical disease transmission.

CHAPTER 2

General Materials and Methods

Toxoplasma gondii

The RRA (Beverley) strain of *Toxoplasma gondii* was used for experimental infection in mice. This cyst forming strain of moderate virulence, originally isolated from an apparently healthy rabbit, has been maintained in this laboratory by the continual passage of infective brain homogenate in outbred, in-house bred Strathclyde Albino mice (AA).

The highly virulent RH strain of *T. gondii*, originally isolated from the brain of a child with toxoplasmic encephalitis by Sabin (1941), was used as a source of antigen for ELISAs and vaccination studies. Tachyzoites were obtained from the peritoneal exudate of cotton rats infected intraperitoneally with 5 x 10^7 viable tachyzoites 3 days previously. Tachyzoites were washed 3 times in 0.9% saline and stored at -20°C until used.

Infections

The brains from passage mice infected 17-21 weeks previously with *T. gondii* (RRA strain) were removed, placed in 2ml of saline, and passed through a number 21 gauge needle 6 times. Thirty microlitres of the brain homogenate were placed on a glass slide and mounted with a coverslip. The entire brain preparation was scanned microscopically at x100 magnification and the total number of cysts counted. From this number the total number of cysts from each brain could be calculated. Mice were infected with 10 or 20 cysts subcutaneously intraperitoneally or orally as stated.

Enzyme linked Immunosorbant Assay (ELISA).

Plasma samples were obtained by collecting blood into a heparinised capillary tube *via* the tail vein and separating the plasma portion by centrifugation.

The ELISA was performed by a modification of the procedure described by Voller *et al.* (1976). Each well of a 96-well microtitre plate was coated with 1ug of STAg in 0.02M Tris-HCl buffer (pH 9.0). After an incubation of 1 h at 37°C with test plasma (1:2000 dilution), plates were washed 3 times and rat anti-mouse IgG horseradish peroxidase conjugate (Jackson Laboratories, Stratech Scientific Ltd.) was applied (1:10000 dilution). After a further 1 hr incubation at 37°C and 3 washes, binding was visualised with tetramethyl benzidine in a sodium acetate buffer (pH 5.5), containing H_2O_2. The reaction was stopped with 10% H_2SO_4 after 20 min and the absorbance read at 450nm on a Titertek Multiscan plate reader.

Preparation of soluble tachyzoite antigens (STAg)

Soluble tachyzoite antigen (STAg) was prepared from tachyzoites of the RH strain as follows. Tachyzoites were washed 3 times and resuspended in hypotonic buffer consisting of 10mM Tris-HCl, 2mM EDTA, pH 7.8, with 50uM *N-p-* tosyl-L-lysine chlormethylketone (TLCK) and 15uM leupeptin (Sigma). After a 15 minute incubation on ice, the tachyzoites were disrupted in a Braun homogeniser and centrifuged at 10 000g for 60 min at 4°C. The supernatant which comprised the STAg, was collected and after extensive dialysis against phosphate buffered saline (pH 7.4), the

protein concentration was determined using a Bradford Assay (Bradford, 1976). This preparation was stored at -20°C until used.

CHAPTER 3

Influence of genes within the MHC on mortality and brain cyst development in mice infected with *Toxoplasma gondii:* Kinetics of immune regulation in BALB *H-2* congenic mice.

3.1. Abstract

Previous work has shown that genes within the major histocompatibility complex (MHC) of the mouse influence resistance and susceptibility to *Toxoplasma gondii* infection. Initial studies presented here using B10 *H-2* congenic and recombinant haplotype mice inoculated *via* the oral route with the low virulence Beverley strain of *T. gondii* confirm the D region localisation of MHC-linked control of brain cyst number. All B10 mice were, however exquisitely sensitive to minor changes in virulence of the parasite inoculum resulting in high mortality during the early phase of infection. Further experiments examining mortality and brain cyst number in BALB MHC congenic mice inoculated *via* different routes indicated that the BALB background would provide a more favourable genetic environment in which to analyse kinetics of MHC controlled immune regulation following infection *via* the natural (oral) route. In studies comparing d and k haplotype mice a dramatic inverse relationship between splenic CD4:CD8 T cell ratios and brain cyst number was observed, particularly in the strain (BALB/K; H-2^k) most susceptible to high brain cyst numbers and subsequent toxoplasmic encephalitis. Of particular interest was the observation that splenomegaly and relative increase in the splenic CD8 T cell population preceded and accompanied the very dramatic and rapid increase in brain cyst formation. The results suggest that the too rapid development of a potent anti-parasite response in the viscera may drive the parasite to encyst in the brain.

3.2. Introduction

The ability of genes within the H-2 complex to mediate protection as measured by survival, the ability to control the development of cysts in the brain and prevent toxoplasmic encephalitis is extensively reviewed in the main introduction. The fact that genes at the D/L end of H-2 are important in regulating cyst numbers (Jones and Erb, 1985; Brown and McLeod, 1990; Suzuki *et al.*, 1992), early mortality (Williams *et al.*, 1978) and toxoplasmic encephalitis (Suzuki *et al.*, 1992; Freund *et al.*, 1992) is consistent with the reports that $CD8^+$ cells are important in the regulation of cyst numbers (Parker *et al.*, 1991; Chapter 6; Brown and McLeod, 1991) and survival (Suzuki and Remington, 1989; Parker *et al.*, 1991; Chapter 6). However, the work of several laboratories all point towards a dual/synergistic role for $CD4^+$ and $CD8^+$ T cells in the regulation of cyst numbers and survival (Vollmer *et al.*, 1987; Suzuki and Remington, 1988; Gazzinelli *et al.*, 1991; Parker *et al.*, 1991; Chapter 6). In this study, we have examined MHC regulated responses to *T. gondii* infection on different genetic backgrounds and have found that BALB mice provide a desirable model in which to study the kinetics of brain cyst formation and associated T cell regulation.

3.3. Materials and Methods

Mice.

C57BL/10ScSn (B10), and its H-2 congenic and recombinant haplotype derivatives B10.BR, B10.D2/n, B10.A, B10.A(2R), B10.A(3R), B10.A(4R) and B10.A(5R) mice, were purchased from Harlan and Olac Ltd., Oxon, England and Bantin and Kingman Ltd., Hull, England. H-2 congenic BALB/c, BALB/B and BALB/K mice were bred in-house at the University of Strathclyde from mice originally purchased from Harlan Olac Ltd. Mice were used when 8-10 weeks old and groups comprised 4-8 mice per group as indicated.

Parasites & infection protocol

Mice were infected with 10 or 20 cysts of the RRA (Beverley) strain of *T. gondii* subcutaneously, intraperitoneally or orally as described in Chapter 2.

Monitoring infection.

Mortality was recorded daily, antibody levels were measured using an ELISA and cyst numbers in the brains of mice determined as described above.

Enzyme linked Immunosorbant Assay (ELISA).

Details of ELISA are in chapter 2.

Cytofluorometric assays.

Spleen weights were recorded and the spleens were gently teased apart in PBS containing 0.1% sodium azide and 0.3% BSA. Red blood cells (RBC) were depleted by using Boyle's solution (0.17M Tris, 0.16M Ammonium chloride; BDH Ltd., Dorset, U.K.) and after washing, viable cells, excluding trypan blue were counted in a haemocytometer. Cells were stained using monoclonal antibodies to B220 (pan B cell), Thy-1.2 (pan T cell), Lyt-2 (CD8), and L3T4 (CD4) directly conjugated to FITC or RD1 (Coulter, Luton, Beds.). Cytofluorometric analysis was performed on an EPICS Profile (Coulter Electronics).

3.4. Results

Oral infection in B10 congenic and recombinant haplotype mice.

In initial experiments we examined the influence of MHC on mortality (Figure 3.1) and brain cyst formation (Table 3.1) following oral infection of mice carrying different haplotypes and recombinant haplotypes on a B10 background. In the first experiment two strains, B10.A and B10.A(2R), showed reduced mortality compared with all other strains. Examination of this initial data in relation to MHC haplotype (Table 3.1) provided no simple answer as to localisation of control over mortality within the MHC. However, when brain cyst numbers in surviving mice were compared against haplotype (Table 3.1), results broadly consistent with previous work (Jones & Erb, 1985; Brown & McLeod *et al.*, 1989; Suzuki *et al.*, 1991) localising H-2^d control over low cyst numbers was observed. Unfortunately our control H-2^d (B10.D2/n) mice died early thus precluding verification of low cyst numbers. The dichotomy in brain cyst numbers between H-$2D^d$ and H-$2D^b$ mice was most marked in two low mortality strains which, with >80% survival, provide the most valid brain cyst data. The only genetic difference between these recombinant haplotype strains is at H-2D (Table 3.1). All surviving mice had positive antibody levels confirming infection but not correlating with brain cyst number (data not shown). Overall our results suggest that, while brain cyst number is regulated by genes readily localisable to a specific region within the MHC, mortality is under more complex genetic control possibly involving interaction between class I and class II restricted responses. In a repeat experiment, all B10 congenic strains showed early mortality (data not shown), indicating that genes on the B10 background also confer acute susceptibility to infection which is sensitive to minor changes in virulence of the inoculum and easily over-rides MHC control. Hence, the oral route on a B10 genetic background would not necessarily provide the best model system in which to monitor immune response associated with the MHC control of brain cyst formation even with the low virulence Beverley strain of *T. gondii* we employed.

Table 3.1. H-2 haplotype, mortality and brain cyst numbers in B10 recombinant haplotype mice following oral infection with 20 cysts of *T. gondii* (Beverley strain).

Mouse Strain	H-2 haplotype					% Mortality	Totalcysts/brain
	K	A	E	S	D	at day 30	at day 30
B10	b	b	b	b	b	100	-
B10.BR	k	k	k	k	k	83	760
B10. D2/n	d	d	d	d	d	100	-
B10.A	k	k	k	d	d	17	128±43
B10.A(2R)	k	k	k	d	b	17	3420±1652
B10.A(3R)	b	b	k	d	d	67	140±60
B10.A(4R)	k	k	b	b	b	83	520
B10.A(5R)	b	b	k	d	d	83	0

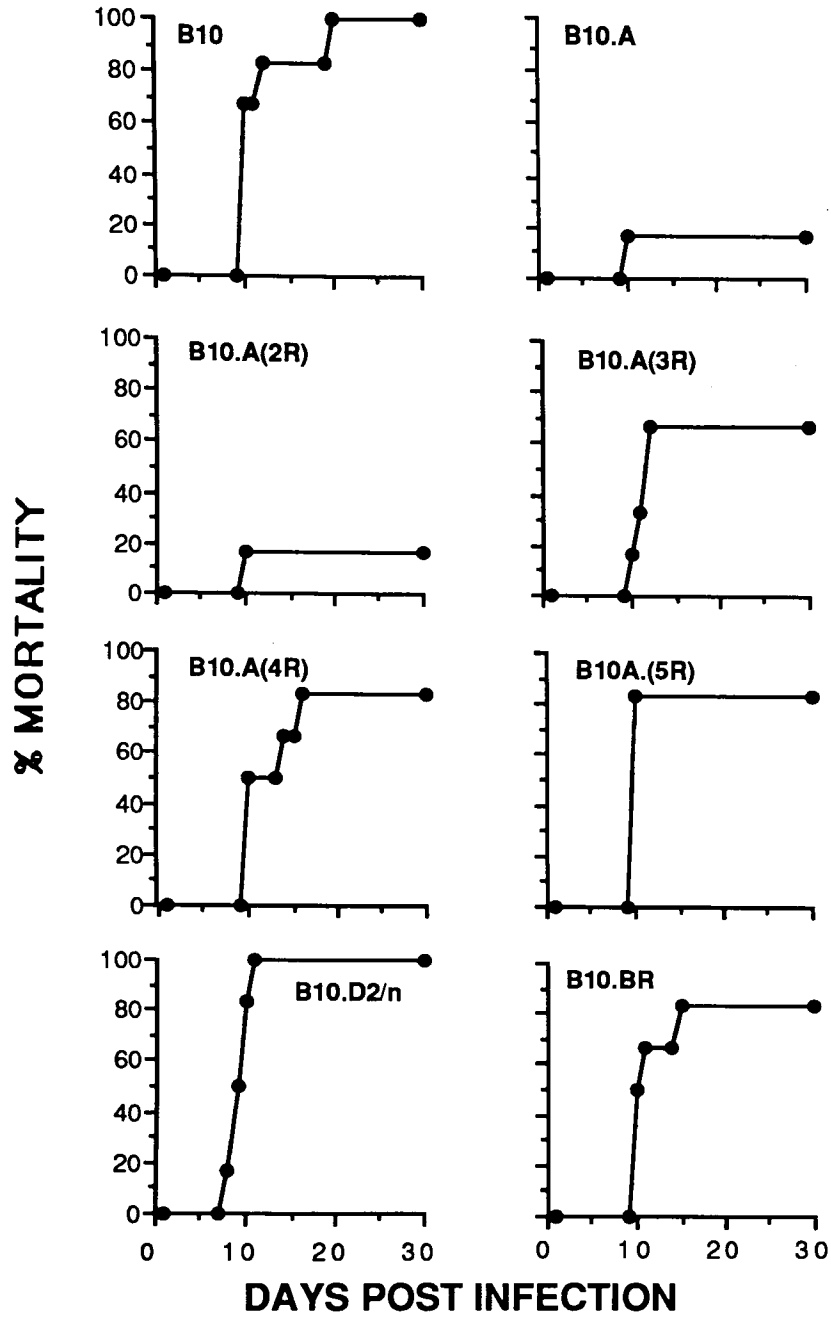

Figure 3.1. Percent mortality over 30 days in B10 H-2 recombinant mice following oral infection with 20 cysts of the Beverley strain of *T. gondii*. MHC haplotypes for these strains are given in Table 1.1. Six mice were examined for each strain.

Comparison of different routes of inoculation in BALB MHC congenic strains.

In a second series of experiments, the influence of MHC on mortality and brain cyst numbers was examined in congenic mice carrying d, b or k haplotypes on a BALB genetic background. In the first experiment three different routes of infection were compared. On this background, the i.p. route gave high mortality (Figure 3.2) in all three strains. Although the mean time to death was delayed, s.c. and oral routes produced high mortality in b, but not in d or k, haplotype mice. For these haplotypes, the BALB background clearly contributes to overall resistance to infection. When cyst counts were examined at 5 weeks in the surviving mice, dramatic differences between d (low cyst numbers:160$\pm$86 oral; 60$\pm$13 s.c.; 280$\pm$106 i.p.) and k (high cyst numbers: 2008$\pm$727 oral; 1743$\pm$373 s.c.) haplotype mice were observed. Again all surviving mice had positive antibody levels but these did not correlate with brain cyst number. In a repeat experiment examining the three haplotypes infected via the oral route, we again observed 50% (5/10) mortality in d haplotype mice and 10% (1/10) in k haplotype mice, this time associated with cyst counts at week 8 of 0 (n=5) and 293$\pm$61 (n=9), respectively. These results indicated that the BALB background might provide a good model for comparison of MHC controlled immune regulation of toxoplasmic encephalitis and brain cyst development using the d and k haplotype mice and the natural (oral) route of infection.

Kinetics of cyst formation and CD4:CD8 immune regulation in d and k haplotype BALB mice.

In all previous reports brain cyst numbers and development of associated toxoplasmic encephalitis has been examined at a single time point, ranging from 30 (McLeod *et al.*, 1989b; Brown & McLeod, 1990), 40-50 (Jones & Erb, 1985) to 70 (Suzuki *et al.*, 1991: Freund *et al.*, 1992) days post infection. In our two initial experiments on the BALB genetic background we observed a 10-fold difference in brain cyst numbers within the MHC haplotypes (e.g. BALB/K mice with 2008$\pm$727; n=6 at week 5 in experiment 1 and 293$\pm$61; n=9 at week 8 in experiment 2) associated with the 3-4 week difference in the time of necropsy, suggesting that there may eventually be resolution of brain cyst numbers even in the susceptible k haplotype mice. An

additional experiment was therefore performed in which 30 mice of each strain were given 20 cysts orally and four mice per strain sacrificed and examined for brain cysts and cytofluorimetric splenic T/B cell composition on each of days 0 (pre-infection control), 14, 21, 28, 35 and 77 days post infection. In this experiment, brain cyst numbers (Figure 3.3A) in the susceptible k haplotype peaked at 21 days post infection, associated with an overall reduction in percentage of T cells (Figure 3.3B) and a maximal decline in the CD4:CD8 T cell ratio (Figure 3.3A). This was preceded by a 4-fold increase in spleen weight at 14 days post infection, which was maintained at 3-fold above background through 21-35 days of infection (Figure 3.3B). Hence, while the percentage of T cells (Figure 3.3B) fell relative to B cells (Figure 3.3D), the absolute number increased with a higher representation of $CD8^+$ cells (Figure 3.3C) in the T cell population associated with peak brain cyst formation. Resolution of brain cyst numbers after day 21 was accompanied by a gradual return towards normal (pre-infection) CD4:CD8 ratios (Figure 3.3A). Antibody response increased as brain cyst numbers declined. In d haplotype mice, a later (35 days post infection) peak in cyst numbers (Figure 3.4A; 6-fold lower in magnitude than k haplotype mice) was observed also associated with a less dramatic reduction in the CD4:CD8 ratio. Kinetics of splenomegaly (Figure 3.4B) was similar to BALB/K mice but reduced in magnitude. The percentage of $CD8^+$ T cells never exceeded $CD4^+$ cells in the more 'resistant' BALB/c strain (Figure 3.4C). Antibody levels (Figure 3.4D) followed the same pattern as BALB/K mice suggesting again that there is no correlation between total anti-parasite IgG levels and the disease severity measured either in terms of mortality or cyst number.

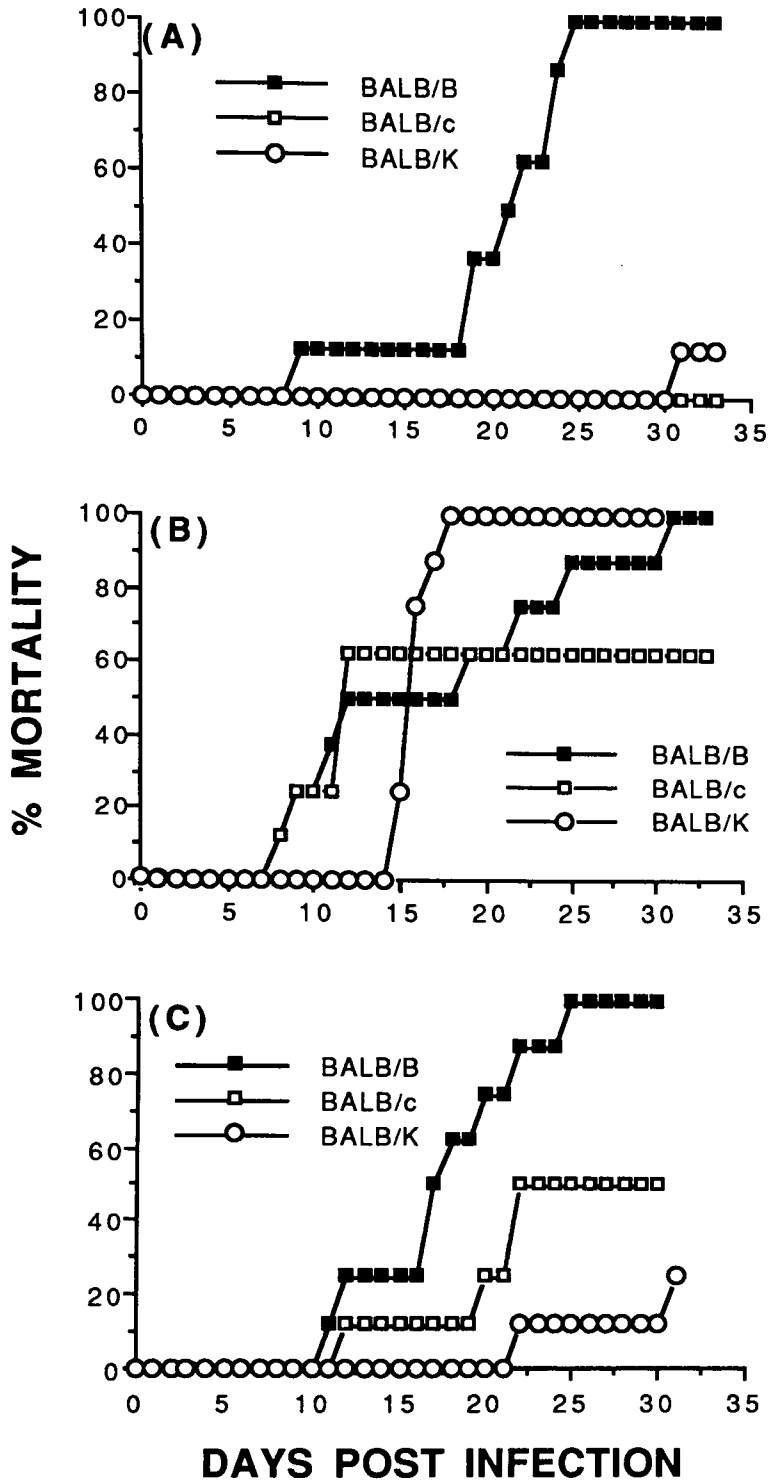

Figure 3.2. Percent mortality over 35 days in BALB H-2 congenic mice following (A) s.c., (B) i.p. or (C) oral infection with 10 cysts of the Beverley strain of *T. gondii*. BALB/c = H-2^d, BALB/B = H-2^b, BALB/K = H-2^k. Eight mice were examined for each strain.

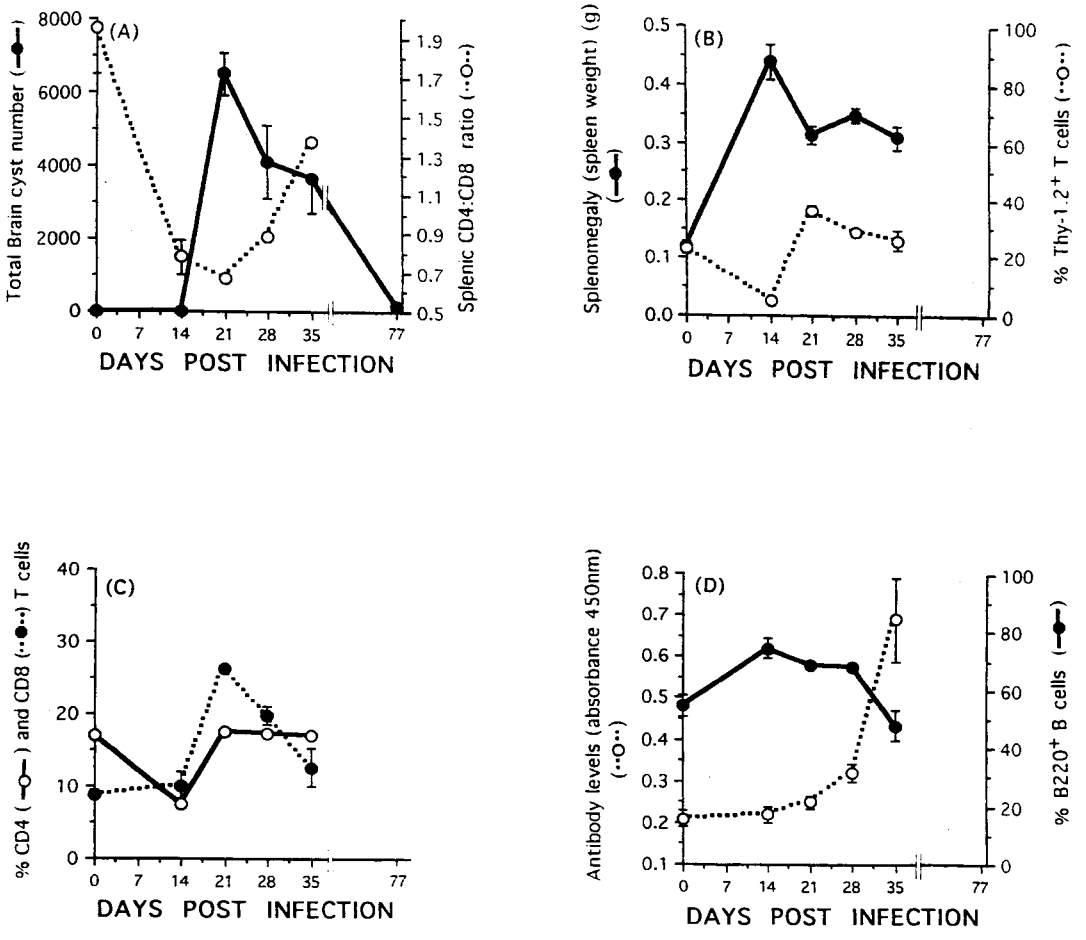

Figure 3.3. Kinetics of infection and immune regulation in BALB/K mice measured as: (A) total brain cyst number and splenic CD4:CD8 ratio; (B) splenomegaly (spleen weight) and percentage Thy 1.2+ T cells; (C) percentage L3T4+ (CD4) and Lyt-2+ (CD8) T cells; (D) antibody levels (absorbance) and percentage B220+ B cells. T/B and CD4/CD8 cells all measured as a percentage of total splenic lymphocytes. A total of 30 mice were inoculated for each strain, with 4 mice per strain sacrificed on each of days 14, 21, 28, 35 and 70 days after oral infection with 20 cysts of the Beverley strain of *T. gondii*. Four preinfection controls were also examined. Immunological data was not available for the 77 day time point.

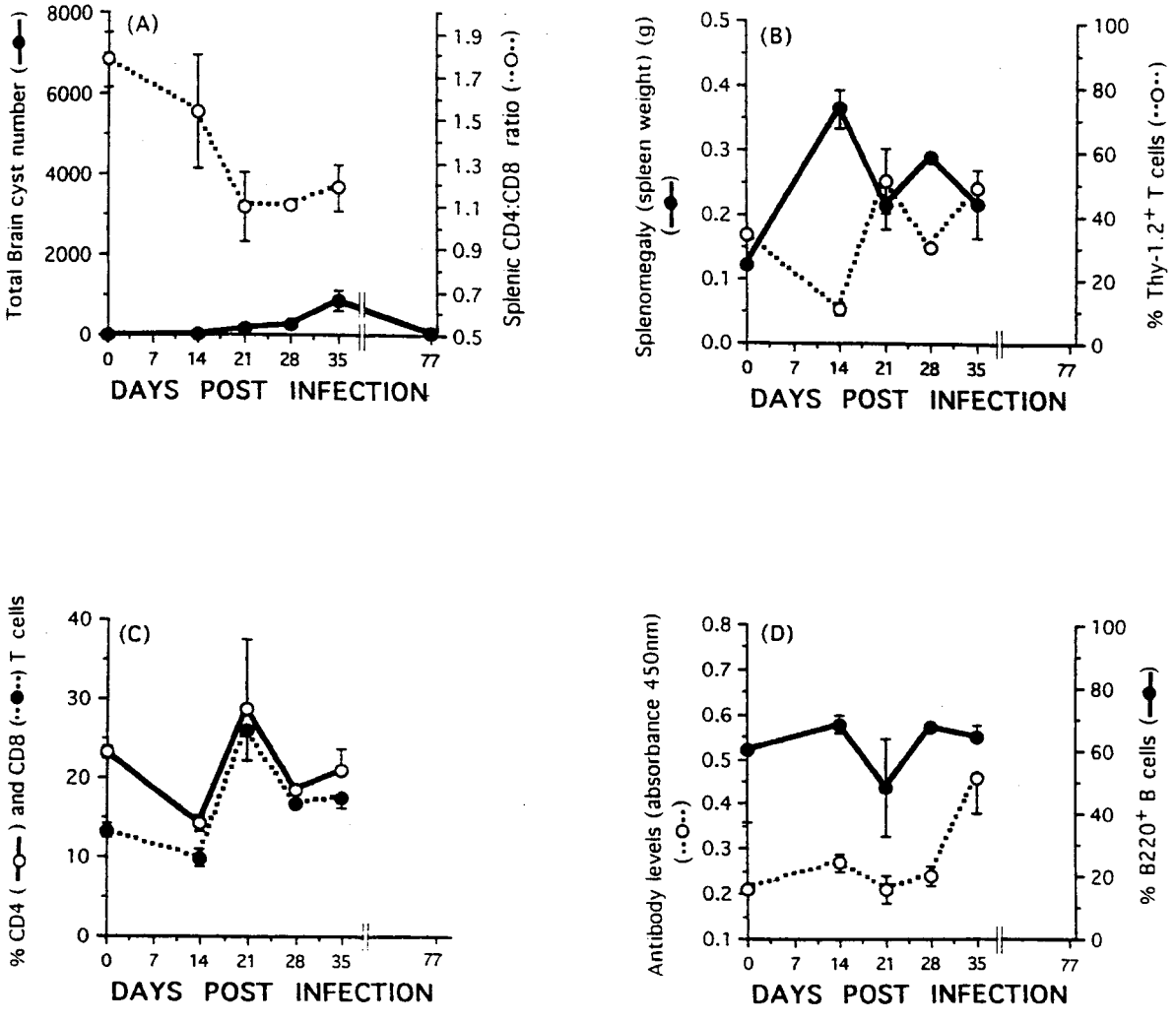

Figure 3.4. Kinetics of infection and immune regulation in BALB/c mice measured as: (A) total brain cyst number and splenic CD4:CD8 ratio; (B) splenomegaly (spleen weight) and percentage Thy 1.2+ T cells; (C) percentage L3T4+ (CD4) and Lyt-2+ (CD8) T cells; (D) antibody levels (absorbance) and percentage B220+ B cells. T/B and CD4/CD8 cells all measured as a percentage of total splenic lymphocytes. A total of 30 mice were inoculated for each strain, with 4 mice per strain sacrificed on each of days 14, 21, 28, 35 and 70 days after oral infection with 20 cysts of the Beverley strain of *T. gondii*. Four preinfection controls were also examined. Immunological data was not available for the 77 day time point.

3.5. Discussion

Initial studies presented here confirmed the D region localisation of MHC-linked control of brain cyst number following *T. gondii* infection. Studies from two laboratories provide conflicting data on localisation within the D region, one group (Brown & McLeod, 1990) favouring localisation to the class I, H-2L genes distal to H-2D, the other (Freund *et al.*, 1992) suggesting that polymorphisms in the promoter region of the TNF-α gene located proximal to H-2D might result in the differential expression of TNF-α seen in the brains of susceptible and resistant strains. Whilst there is growing evidence that TNF-α may play both protective (Chang *et al.*, 1990 & 1992; Sibley *et al.*, 1991; Johnson 1992) and deleterious (Black *et al.*, 1989) roles during the course of *T. gondii* infection, there are many pathways *via* which differential regulation of TNF-α expression might be influenced. Although, Freund and coworkers were able to examine recombinant haplotype mice which excluded the HSP70 genes in the H-2D region control of brain cyst number, there was no recombinant haplotype mouse strains separating the TNF-α and D loci. Hence, despite the elegant demonstration of restriction fragment length and microsatellite variants in the TNF-α gene, the implication that these polymorphisms are involved in the regulation of TNF-α levels and hence brain cyst number and toxoplasmic encephalitis must remain hypothesis. More clear cut was the observation by Brown and McLeod (1990) that mouse strains deleted at L^d were transformed from cyst resistant to cyst susceptible, indicating a direct role for class I (L locus) restricted $CD8^+$ T cells in regulation of *T. gondii* infection. An increase in cyst number in B6.C-*H-* 2^{bm12} mice carrying a class II mutation compared with normal B6 mice also led Brown and McLeod (1990) to postulate that $CD4^+$ T cells are important helper cells for induction of class I-restricted CD8 response. Both lines of evidence are consistent with many functional studies (Suzuki & Remington, 1988; Brown & McLeod, 1990; Gazzinelli *et al.*, 1991; Parker *et al.*, 1991) which point towards dual/synergistic roles for CD4 and CD8 T cell populations in the generation of a protective immune response. The task ahead is to determine precisely how these MHC-controlled responses interact to

generate protection *versus* exacerbation of disease.

In pursuing the functional correlates of immunity, our next series of results indicated that the BALB background might provide a more favourable genetic environment in which to analyse MHC control of the T cell response. The immediate hurdle is to by-pass the acute phase of the T cell response associated with high mortality which, as previous studies have shown (Johnson, 1985), is clearly influenced by the route of inoculation. In our studies even the resistant d haplotype (BALB/c) mice died following infection *via* the i.p. route, a phenomenon which we have independently shown can be hastened by a single i.p. dose of neutralising rabbit anti-mouse TNF-α (see Chapter 4). Presumably the parasites do not reach the right cell population to trigger a protective early burst of TNF-α. The same treatment prior to oral infection has no protective or exacerbative influence on mortality, even on the acutely susceptible B10 background, indicating that mediators other than TNF-α may be stimulated *via* the natural oral route of infection. Clearly the oral (and thus the natural) route is the preferred route for studies relating to induction of T cell responses associated with protection or exacerbation of disease. Hence, the advantage which the relative resistance of the BALB background provides for such studies. At first sight, our data demonstrating the very dramatic inverse correlation between CD4:CD8 ratio and brain cyst number during the course of infection of the strain (BALB/K) more susceptible to cerebral disease suggest a prominent protective role for $CD8^+$ cells preparatory to, and during, the clearance of *T. gondii* infection from the brain. Interestingly, however, our analysis was of splenic lymphocyte populations far removed from the site of the brain cyst formation, with splenomegaly and the relative increase in the CD8 cells *preceding and accompanying* the very dramatic and rapid increase in brain cyst formation in the BALB/K strain. An alternative view is that it is the enhanced immunological activity in the spleen which drives the parasite to encyst. Such a notion is supported by our independent observation that massive encystment in the brains of B10 mice over the equivalent time period is associated with a highly polarised *T. gondii*- specific antibody response in which IgG2a but not IgG1 antibody subclasses are represented (results not shown). This is indicative (Snapper & Paul,

1987) of a TH1 rather than a TH2 response in the periphery. At the same time, transcripts for IL-4 rather than IL-2 are detected in the brain, suggesting that the response within the brain is under TH2 control (Chapter 8; Hunter *et al.*, 1992b). Although the increased immunological activity in the BALB/K spleen appears to protect this strain from the the mortality observed in 50% of the more 'resistant' (in terms of brain cyst number) BALB/c mice through the equivalent time period, the enormous number of cysts which form in the brain clearly predisposes the BALB/K mouse to the encephalitis which accompanies reactivation and clearance of cysts from the brain. Hence, the too rapid development of a potent anti-parasite immune response in the viscera may ultimately prove detrimental to the host. While much current research (e.g. Suzuki *et al.*, 1991; Chang *et al.*, 1992; Hunter *et al.*, 1992a&b) is focussed on events in the brain associated with toxoplasmic encephalitis, our results demonstrate that there is still a need for careful study of events in the periphery and the viscera which precede cerebral disease.

CHAPTER 4

Toxoplasmosis in congenic *Lsh* ^{r} and *Lsh* ^{s} mice.

4.1. Abstract

The influence of the *Lsh* gene on the course of a *T. gondii* infection was examined in *Lsh*^{s} BALB/cπ and C57BL/10ScSn and their respective congenic *Lsh*^{r} counterparts CD2-*Pep*-3 and B10.L. *Lsh*^{r} mice. This gene was found to play a small, but significant role in acute mortality which was found to be route of infection dependent. B10 *Lsh* resistant mice infected via the oral route were more susceptible to death than than their *Lsh* susceptible conterparts, whereas the converse was true when infection was via the intraperitoneal route. No significant difference in mortality was observed between orally infected *Lsh* resistant or susceptible BALB/c mice, although infection by the intraperitoneal route was more severe in *Lsh* resistant than *Lsh* susceptible mice. B10 *Lsh* susceptible mice had greater brain cyst burdens independent of the route of infection. Plasma TNF-α levels were highest in B10 *Lsh* resistant and susceptible mice infected *via* the oral route and although the period of production coincided with death in these animals, administration of rabbit anti-TNF-α serum did not reduce the incidence of fatal infection in these mice. However, rabbit anti-TNF-α administered to BALB/c *Lsh* resistant and susceptible mice infected by the intraperitoneal route, significantly decreased time to death. These results demonstrate that the ability of the *Lsh* gene to influence a *T. gondii* infection is route of infection dependent and is independent of TNF-α production.

4.2. Introduction

The *Lsh* gene has been demonstrated to be important in determining early resistance to *Leishmania donovani* infection in the mouse. This gene is identical to the *Ity* and *Bcg* genes which have been demonstrated to determine the ability of mice to control *Salmonella typhimurium* and *Mycobacterium bovis, Mycobacterium lepraemurium* and *Mycobacterium intracellulare* infection (reviewed, Blackwell, 1989). Although the gene product has not yet been identified, macrophages from mice expressing the Lsh^r allele produce both TNF-α and IL-1 in greater quantities in response to stimulation with IFN-γ or LPS (Blackwell *et al.*, 1989). Stimulation of macrophages in this manner has also been shown to cause a greater increase in the upregulation of class II expression in mice carrying the Lsh^r allele compared with macrophages from mice carrying the Lsh^s allele (Kaye *et al.*, 1988; Kaye & Blackwell, 1989). Furthermore, Denis *et al.*, (1988), demonstrated that splenic macrophages from mice carrying the resistant allele produced greater amounts of reactive oxygen intermediates (H_2O_2 and O_2-). The production of reactive nitrogen intermediates is also influenced by this gene (Roach *et al.*, 1991). In this study, Roach *et al.*, (1991), found that *in vitro* stimulation of peritoneal macrophages from Lsh^r mice resulted in increased production of nitrates and associated *L. donovani* killing, over that observed in Lsh^s mice. In common with *S. typhimurium, M. bovis, M. lepraemurium* and *M. intracellulare, T. gondii* parasitises the macrophage for part of its life-cycle and consequently it would appear likely that the *Lsh* gene may also have some effect on the development of immunity to this parasite. Therefore in this study, we examine the effect of the *Lsh* gene on the course of *T. gondii* infection using C57/BL10ScSn, BALB/cπ and their Lsh^r congenic counterparts B10.L.*Lsh* and CD2-*Pep*-3 respectively.

4.3. Materials and Methods

Mice

Lsh ^{s} BALB/cπ and C57BL/10ScSn mice and their respective congenic *Lsh* ^{r} counterparts CD2-*Pep*- 3 and B10.L.*Lsh* mice, were bred at The London School of Hygiene and Tropical Medicine and maintained at the University of Strathclyde under standard laboratory condition. Mice were used when 8-10 weeks old and groups comprised 6-8 mice as indicated.

Parasites

Mice were infected subcutaneously (s.c.), intraperitoneally (i.p) or orally as indicated with 20 cysts (RRA strain). Described in detail in Chapter 2.

Monitoring infection

Mortality was recorded daily and brain cyst numbers were determined as described in Chapter 2.

Plasma samples

Plasma samples were obtained 0, 2, 4, 6, 8, 10, 12, 14, and 16 days post-infection by collecting blood into a heparinised capillary tube *via* the tail vein and separating the plasma portion by centrifugation. Plasma was stored at -70oC until use.

Cyst numbers

Cyst numbers were counted in the brains of mice as described in Chapter 2 at the time point stated

TNF-α ELISA

TNF-α was measured in the serum samples in a sandwich ELISA using the monoclonal antibody TN3 (10ug/ml) to coat 96 well microtitre plates (Nunc-immunoplate maxisorb, Nunc) and a polyclonal rabbit anti-TNF-α sera (1:200), followed by goat anti-rabbit immunoglobulin G (H and L chains) peroxidase for

detection. Binding was visualised by the addition of 2, 2'-azino-di-(3-ethyl-benz-thiazoline sulfonate) (ABTS) with hydrogen peroxide (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) Plasma samples were assayed at 1 in 10 dilution and the TNF-α concentration calculated using a standard curve (2.5 to 50 U/ml) of rTNF-α on each plate.

Administration of neutralising polyclonal rabbit anti-TNF-α sera

Mice were injected intraperitoneally with polyclonal rabbit anti-TNF-α (2mg/mouse) sera or with normal rabbit sera (2mg/mouse) as controls 24 hours before infection.

Statistical analysis

Statistical analysis of the mortality data was made by the Mann-Whitney U test and Student's t-test was used for analysis of brain cyst numbers.

4.4. Results

Comparison of different routes of infection and the effects of the Lsh gene on mortality and brain cyst numbers in Lsh^s and Lsh^r congenic BALB/c and B10 mice.

The ability of mice to survive an infection with *T. gondii* was found to be dependent on the route of infection (Figure 4.1). BALB/c mice were found to be most susceptible to intraperitoneal infection, exhibiting 100% mortality (6/6) 24 days post infection compared with the oral route of infection which induced only 66.7% mortality (4/6) by day 28 : no further deaths in this group were recorded during the study. In contrast the B10 mice demonstrated greatest susceptibility to the oral route with 100% mortality (6/6) in the first 13 days following infection at which time there were no deaths in the mice infected by the intraperitoneal route. However, 83.3% (5/6) of B10 mice infected by the i.p. route did eventually succumb to death by the end of the study period. Disease progression in mice infected by the s.c. route was less severe than either the oral or i.p. route with both BALB/c and B10 mice demonstrating similar levels of mortality (60%; 3/5) over similar time-spans (BALB/c; day 37, B10; day 43). The influence of the *Lsh* gene on mortality was most noticeable on the B10 background, where its effects were also found to be route dependent. Following oral infection, B10 *Lsh* resistant mice demonstrated significantly ($p \leq 0.05$) more rapid time to 50% mortality compared with B10 *Lsh* susceptible mice. In contrast, B10 *Lsh* resistant mice infected *via* the i.p. route demonstrated lower overall mortality (60%; 3/5) compared with the parental B10 strain (83.3%; 5/6) infected *via* this route and had a significantly ($p \leq 0.05$) longer time to 50% mortality. Conversely, BALB/c *Lsh* susceptible mice infected i.p had a significantly longer ($p \leq 0.05$) time to 50% death than their *Lsh* resistant counterparts, although there was no significant *Lsh* mediated differences recorded by the oral route.

TNF-α levels in the serum of infected mice

Since the *Lsh* gene has been demonstrated to influence the macrophage production of TNF-α in other systems, we decided to examine the plasma of the different strains of mice, infected by different routes for the presence of TNF-α. In this experiment in

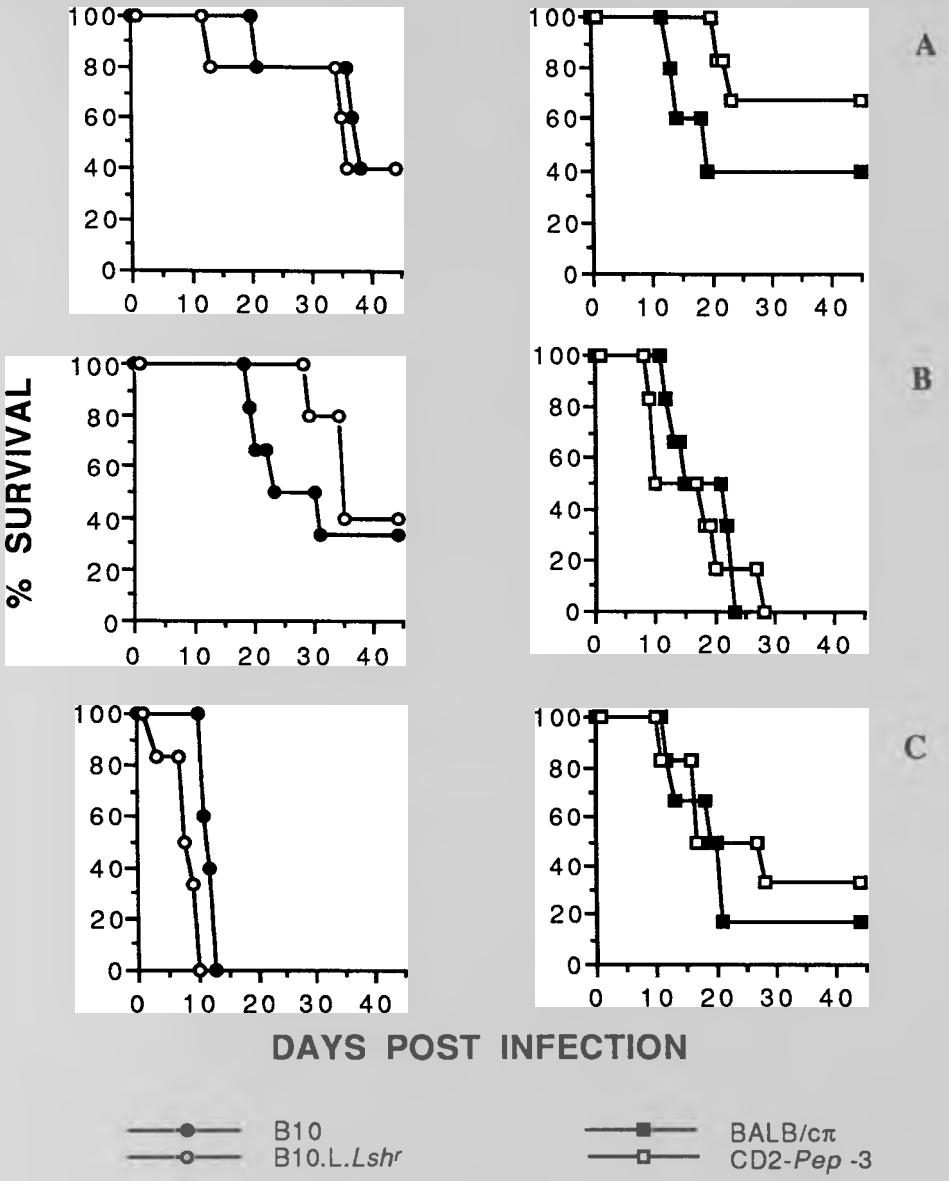

Figure 4.1. Percent survival over 45 days in *Lsh* ^{s} and *Lsh* ^{r} congenic BALB/c and B10 mice following (A) s.c., (B) i.p. or (C) oral infection with 20 cysts of the Beverley strain of *T. gondii*.

agreement with the pevious experiment, B10 mice infected *via* the oral route demonstrated greater levels of acute mortality compared with B10 mice infected *via* the intraperitoneal route (Figure 4.2 & 4.3). The differences observed in mortality between strains of mice in this experiment, although less dramatic, were broadly consistent with the previous experiment (Figures 4.2 & 4.3). Greater quantities of TNF-α was detected in the plasma of B10 and B10.L *Lsh* ^{r} mice infected by the oral route compared with the same strains of mice infected intraperitoneally. In these mice, high levels of TNF-α, sometimes greater than 500U/ml in the plasma of individual mice were detectable in the time period corresponding with high rates of mortality in B10 *Lsh* resistant and susceptible mice infected *via* the oral route. In agreement with the previous experiment, B10.L.*Lsh* ^{r} mice were found to be more susceptible to acute mortality than B10 mice. Due to deaths in each group and the transient nature of the TNF-α levels of individual mice it is difficult to make any firm conclusions as to any differences in TNF-α production between *Lsh* resistant and susceptible B10 mice. However, plasma TNF-α peaked earlier in the *Lsh* resistant (day 10) than the *Lsh* susceptible mice (day 12) although, surviving *Lsh* susceptible mice had higher mean peak levels of circulating TNF-α (260±72 U/ml) than their surviving *Lsh* reistant counterparts (102±35 U/ml) (Figure 4.2). Orally infected BALB/c *Lsh* susceptible mice produced more TNF-α than their *Lsh* resistant counterparts over the first 16 days at which time there were no deaths in either group (Figure 4.2). When mice were infected by the i.p. route plasma TNF-α levels were generally lower (Figure 4.3) than that detected in mice infected by the oral route.

The number of tissue cysts in surviving mice were counted at 35 days post infection. Surviving B10 *Lsh* resistant mice infected s.c., i.p. or orally were found to have less cysts in their brains (3100±458, s.c; 4600±482 i.p; 12640±2525 oral) when compared with surviving *Lsh* susceptible counterparts (13200±8839 s.c; 8866±3541 i.p; 28400±5303 oral), ($p<0.005$ s.c; $p\leq0.1$ i.p; $p\leq0.025$ oral). In contrast, surviving BALB/c *Lsh* resistant mice infected orally but not s.c. or i.p. had significantly more cysts in their brains (1080±370) than *Lsh* susceptible BALB/c mice (293±130),

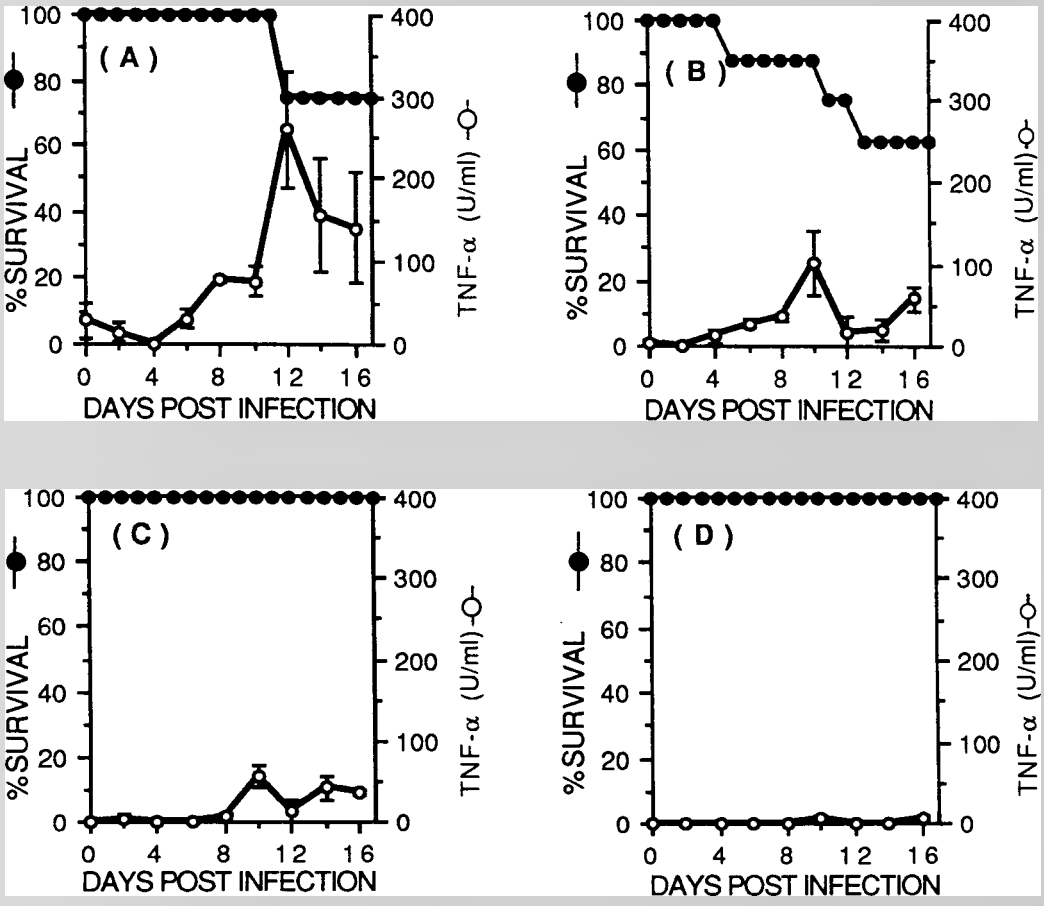

Figure 4.2. Percent survival and plasma TNF-α levels over 16 days in *Lsh*^{s} and *Lsh*^{r} congenic BALB/c and B10 mice following oral infection with 20 cysts of the Beverley strain of *T. gondii*. (A) B10 (*Lsh*^{s}), (B) B10.L. *Lsh* (*Lsh*^{r}), (C) BALB/cπ (*Lsh*^{s}) and (D) CD2-*Pep* - 3 (*Lsh*^{r}) mice.

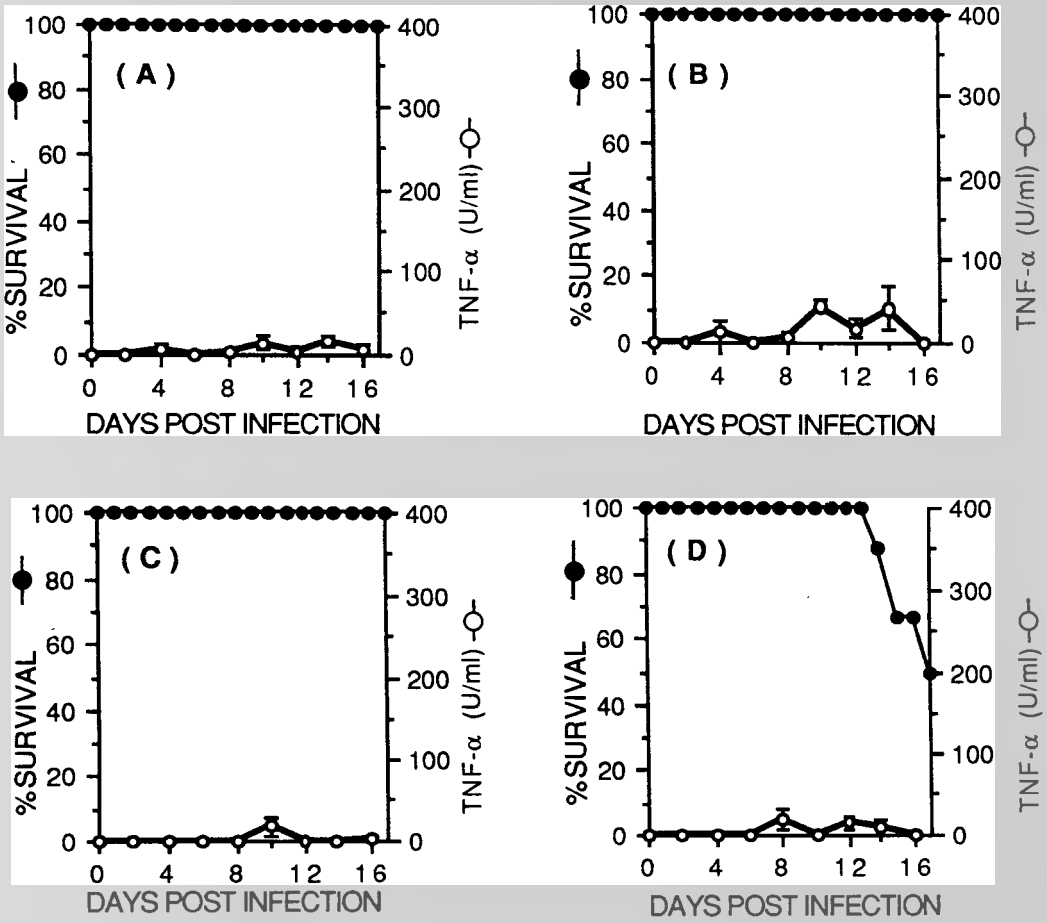

Figure 4.3. Percent survival and plasma TNF-α levels over 16 days in *Lsh*^{s} and *Lsh*^{r} congenic BALB/c and B10 mice following i.p. infection with 20 cysts of the Beverley strain of *T. gondii*. (A) B10 (*Lsh*^{s}), (B) B10.L. *Lsh* (*Lsh*^{r}), (C) BALB/cπ (*Lsh*^{s}) and (D) CD2-*Pep* - 3 (*Lsh*^{r}) mice.

($p \leq 0.025$).

The effect of administration of neutralising anti-TNF-α polyclonal sera on mortality in Lsh ^{s} and Lsh ^{r} congenic BALB/c and B10 mice infected by the oral and intraperitoneal routes.

Administration of TNF-α neutralising polyclonal sera to B10, B10.L.*Lsh* ^{r}, BALB/cπ or CD2-*Pep* -3 mice infected by the oral route had no effect on the rate of, or total mortality. However, administration of this serum to BALB/cπ or CD2-*Pep*- 3 mice infected by the intraperitoneal route made the animals more sensitive to acute mortality, with 100% mortality (6/6) evident by day 10 in both groups of treated mice compared with zero over a similar time span in the non-treated animals. The mortality did rise to 71.4% (5/7) by day 35 in both groups of non-treated mice (Figure 4.4).

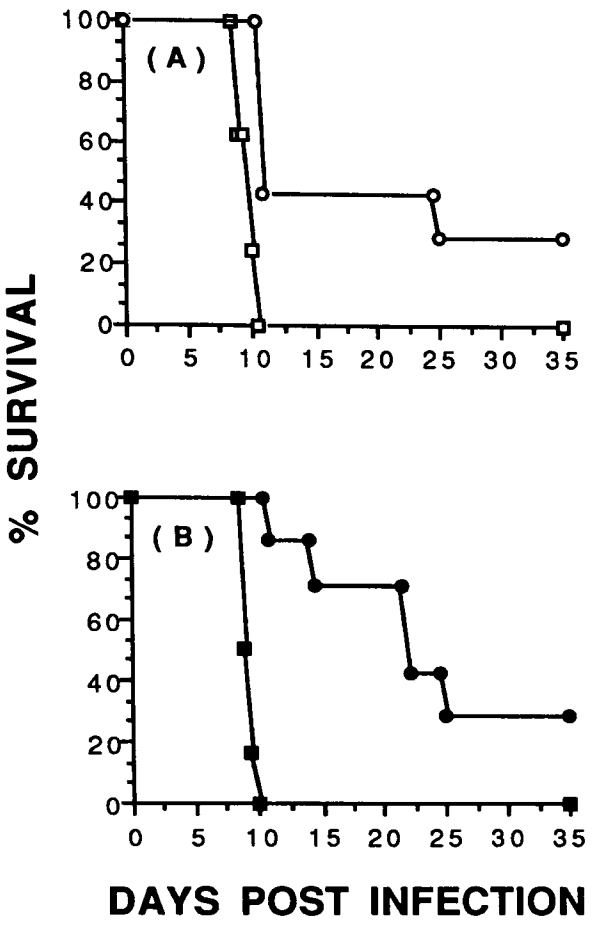

Figure 4.4. Percent survival over 35 days in *Lsh*^{s} and *Lsh*^{r} congenic BALB/c mice pre-treated with rabbit anti-TNF-α serum (□,■) or control rabbit serum (●, ○) (2mg/ml, i.p.) and infected i.p. 24 hours later with with 20 cysts of the Beverley strain of *T. gondi*. (A) BALB/cπ (*Lsh*^{s}) and (B) CD2 *Pep* - 3 mice (*Lsh*^{r}). Administering identical amounts of rabbit anti-TNF-a serum or control rabbit serum and infecting orally 24 hours later with with 20 cysts of the Beverley strain had no significant effect on mortality in B10 (*Lsh*^{s}), B10.L. *Lsh* (*Lsh*^{r}), BALB/cπ (*Lsh*^{s}) or CD2-*Pep* - 3 (*Lsh*^{r}) mice (data not shown).

4.5. Discussion

This study demonstrates that the *Lsh* gene can influence the outcome of infection with *T. gondii* . The effect of this gene is relatively small compared with other genes reported to influence early mortality, H-2 (Williams *et al.,* 1978; McLeod *et al.,* 1989b; Chapter 3) and H-13 (Williams *et al.,* 1978). Consequently the greatest difference in rates of mortality were observed between the two different backgrounds examined. Interestingly, the effects of the *Lsh* gene, with respect to early mortality were found to be route dependent, with B10 mice having a clear survival advantage over B10.L.*Lsh* ^{r} mice *via* the oral route of infection, whereas the opposite was found when infection was *via* the intraperitoneal route. This route dependent effect may be explained by differential expression of the *Lsh* gene in discrete macrophage populations (Crocker *et al,* 1987; Denis *et al.,* 1988).

A previous study, found that the mice carrying the *Lsh* ^{r} allele and infected via the oral route were susceptible to larger cyst burdens than mice carrying the *Lsh* ^{s} allele (McLeod et al., 1989b). However, in the present study B10 mice carrying the *Lsh* ^{r} allele were found to have less cysts than their *Lsh* ^{s} counterparts at necropsy, independent of whether they were infected via the s.c., the i.p. or the oral route. BALB/c *Lsh* resistant mice infected by the oral route were found to have more cysts in the brains than *Lsh* susceptible BALB/c mice. The difference in the results obtained in these two studies is hard to reconcile, but differences in the parasite strain/virulence and the time at which cyst counts are performed may have great bearing in the results obtained. In Chapter 3, the number of cyst in the brains of congenic mice was demonstrated to vary not only between strains but also with time.

The observation that both mortality and plasma TNF-α levels were highest in B10 mice infected *via* the oral route indicated that TNF-α could be responsible for the higher rates of mortality observed. However, the course of the disease in mice infected orally and given anti-TNF-α sera was not significantly altered. Neutralising

anti-TNF-α sera also had no effect in BALB/c mice infected *via* this route. Interestingly, administration of anti-TNF-α to BALB/c and CD2-*Pep* -3 mice infected by the i.p. route, exacerbated disease, resulting in more rapid mortality in these mice. This clearly demonstrates a protective role for endogenous TNF-α in mice infected *via* the intraperitoneal route.

Previous studies have indicated that administration of rTNF-α to infected Swiss Webster mice can both decrease time to death (Black *et al.*, 1989) and significantly protect mice from lethal infection (Chang *et al.*, 1990). Further work has shown that neutralising endogenous TNF-α by administration of antibodies can decrease time to death in the C57BL/6J strain infected by the intraperitoneal or oral route, but have no effect on the overall mortality of CB6F1, (BALB/cBy x C57BL/6J)F1 mice infected by either route. Johnson, (1992) also demonstrated that the most dramatic effects of anti-TNF antibodies in terms of increasing illness or mortality levels was observed in CB6F1 mice infected *via* the i.p. route. This study, in agreement with Johnson (1992), finds the i.p. rather than the oral route of infection liable to disease exacerbation by administration of neutralising antibody. In the present study high levels of TNF-α are found in the circulation of mice infected by the oral route, but only low levels in mice infected by the i.p. route. Consistent with this, Johnson was unable to detect TNF-α in the circulation of intraperitoneally infected mice, but was able to detect TNF-α in the peritoneal exudate of these mice. Other workers, examining the role of cytokines after intravenous or peritoneal LPS challenge found that cytokines produced in the peritoneum of the mouse did not readily diffuse into the circulation. In common with Johnson (1992), the present study administered the anti-TNF-α*via* the i.p. route. It is possible that administrating anti-TNF-α intraperitoneally only effects the local action of this cytokine and consequently the greatest effects are observed when the infection is also *via* this route. An alternative explanation is that, TNF-α plays different roles depending on the route of infection and the different circulating TNF-α levels offer some explanation as to why the outcome of murine *T. gondii* infection is route dependent.

The apparently ambivalent nature of TNF may be explained by differences in the quantity in circulation at any one time. Recent evidence suggest the existence of two distinct receptors on the surface of many cell types. Both the mouse and man have a high affinity receptor (70Kd), which binds TNF preferentially at low concentrations of TNF and is involved in the physiological response including macrophage activation and proliferation of cytotoxic T cells (Tartaglia & Goeddel, 1992). Whereas, the low affinity receptor (55Kd) which is responsible for many of the more severe and often detrimental effects of TNF, would only be activated if TNF concentrations were relatively high (reviewed, Tartaglia & Goeddel, 1992).

One potentially beneficial effect of TNF-α may be its role in macrophage activation. Under certain circumstances TNF-α can act in synergy with IFN-γ to activate macrophages to kill the actively dividing tachyzoites by oxygen or L-arginine dependent mechanisms (Hughs, 1985; Adams *et al.*, 1990). Perhaps ironically, it is this potentially host protective mechanism that has recently been implicated in the cause of the pathology often observed in human or murine malaria (Taverne, 1993). Endogenous IFN-γ has been demonstrated to mediate endotoxic shock (Doherty *et al.*, 1992) and administration of rIFN-γ can induce mortality in mice infected with *Plasmodium vinckei* (Kremsner *et al.*, 1992). Similarly, many of the contradictory observations in the *T. gondii* literature could indicate that TNF-α and IFN-γ may have both beneficial and disease exacerbating effects. For example, although IFN-γ has been demonstrated to be an essential endogenous mediator of resistance to acute (McCabe *et al.*, 1984) and chronic (Suzuki *et al.*, 1989b) toxoplasmosis, it has been found that spleen cells from strains of mice genetically susceptible to acute mortality, stimulated with *T. gondii* antigen *in vitro* produce significantly more IFN-γ than spleen cells from genetically resistant mice (McLeod *et al.*, 1989a). IFN-γ has been implicated in the activation of macrophages to produce ROI and RNI and although previous studies have reported that macrophages can become activated to kill tachyzoite stages by this cytokine alone, more recent studies using stringent low endotoxin conditions have unequivocally demonstrated the need for a second signal (Sibley *et al.*, 1991). This can be supplied by small amounts of either LPS or TNF-α

(Sibley *et al.*, 1991). Therefore, independent of the toxic effects associated with TNF-α, it is likely that overproduction of IFN-γ, in the presence of TNF-α may result in over activation of macrophages resulting in pathological amounts of RNI and ROI.

TNF-α can have pleiotropic effects in many parasitic and auto-immune diseases (reviewed, Jacob, 1992) and indeed, as discussed above it can play both host-protective and pathological roles in murine toxoplasmosis (Black *et al.*, 1989; Chang *et al.*, 1990; Johnson, 1992). The relative contribution of TNF-α to host protection and disease induction may be dependent on the quantity produced, the sites in which it is produced and the length of time it is produced after infection. The TNF-α gene has been shown to be polymorphic in both humans (Nedospasov *et al.*, 1991) and mice (Jongeneel *et al.*, 1990). Freund *et al.*, (1992), have demonstrated restriction length polymorphisms in the promoter region of the TNF-a gene which they have related to susceptibility: high TNF-a levels only being detected in the brains of mice susceptible to toxoplasmic encephalitis. Consistent with this observation, we detected greater amounts of TNF-α in the plasma of susceptible B10 mice which develop larger cyst burdens than from BALB/c mice wich develop small cyst burdens. The increased amounts of this cytokine in both cases could simply reflect the greater parasite load in these mice perhaps due to an inability to kill or prevent parasite multiplication. Alternatively the over production of TNF-α could be genetically predetermined and be responsible for the development of encephalitis.

CHAPTER 5

The influence of the *Lps* and *beige* gene on mortality and brain cyst development in mice infected with *Toxoplasma gondii.*

5.1. Abstract

The ability of the mutant genes, *beige* and Lps^d to influence the course of a *T. gondii* infection in mice was examined. Mortality was significantly higher in C3H/HeJ (Lps^d), compared with C3H/He mice (Lps^n) independent of whether the mice were infected by the oral or subcutaneous route of infection. However, subcutaneous infection induced an overall greater mortality than oral infection in both C3H/HeJ mice (Lps^d) and C3H/He mice (Lps^n). Orally, but not subcutaneously ($p<0.375$) infected C3H/He mice (Lps^n) developed significantly more cysts in their brains compared with C3H/HeJ mice (Lps^d) ($p<0.05$).

Mortality levels for C57BL/6-BGL mice infected with *T. gondii* by the subcutaneous route were higher than for C57BL/6J mice infected by this route. Intraperitoneal infection, however, induced greater levels of mortality in C57BL/6J mice compared with C57BL/6-BGL mice while oral infection induced only low levels of mortality in both strains. Surviving C57BL/6J mice infected by the subcutaneous route were found to have significantly more cysts in their brains ($p<0.05$) than those infected intraperitoneally. Subcutaneous or intraperitoneal infection of C57BL/6J mice produced significantly less cysts than C57BL/6-BGL mice infected by the same route ($p<0.005$ and $p<0.05$, respectively).

The results demonstrate a role for both these genes in the development of an anti-*T. gondii* protective response and contribute further evidence for the role of NK cells and the activated macrophage in controlling *T. gondii* infection.

5.2. Introduction

This chapter examines the ability of mice carrying the *beige* or *Lps*^{d} mutation to survive an infection with *T. gondii* and their ability to control brain cyst formation. The*beige* mutation in the C57BL/6 mouse occurred spontaneously (Roder & Duwe, 1979) and mice that are homozygous (bg/bg) for this autosomal recessive allele have a complete impairment in the ability of their NK cells to lyse YAC cells. In contrast, their heterozygous littermates (+/bg) are able to kill target YAC cells as efficiently as the parental strain (+/+). Nevertheless the ability of lymphocytes from these homozygous mice to kill P815 cells was not impaired, suggesting that the impaired function was restricted to NK cells. Further studies have however, cast doubt on this observation as the ability of T cells to kill P815 cells *in vitro* was reduced in bg/bg mice when compared with +/bg mice (Saxena *et al.*, 1982). NK cells from mice carrying the *beige* mutation appear to be able to recognise and bind target cells, but lack the ability to mediate killing (Roder, 1979). Mice of this genotype are similar in many respects to humans with Chediak-Higashi Syndrome and like their human counterparts have giant lysosomal granules, pigment dilution and are susceptible to infection.

The biological response of mice to lipopolysaccharide or endotoxin is determined by the *Lps* gene (Sultzer, 1968). Mice carrying the *Lps*^{n} allele are sensitive to lipopolysaccharide, whereas certain mutant mice, C3H/HeJ and C57BL/10ScCr, for example, carry the *Lps*^{d} allele and are insensitive to lipopolysaccharide. Mice carrying the *Lps*^{d} allele are susceptible to *Salmonella typhimurium* infection and fail to control the early development of this organism in the spleen. However, further research has demonstrated that this mutation has wider implications and that macrophages from these mice are refractory to activation by numerous bacterial products unrelated as well as related to LPS (Chedid *et al.*, 1976). This suggests that macrophages from these mice have a dysfunction that generally results in the inability to become activated, a phenomenon demonstrated clearly in C3H/HeJ mice which have been shown not to

respond to IFN-γ activation *in vitro* and have limited tumouricidal activity (Meltzer & Nacy, 1985).

Murine NK cells have been shown to kill extracellular tachyzoites and *T. gondii* infected cells *in vitro* (Hauser & Tsai, 1986). Since the ability of NK cells from *beige* mice to kill target cells is impaired, it may be anticipated that mice carrying this mutation would be more susceptible to infection with *T. gondii* . The inability of macrophages from C3H/HeJ mice to become activated and more specifically to respond to IFN-γ (Meltzer & Nacy, 1985), an important mediator of protective immunity against *T. gondii* infection (Subauste & Remington, 1991; Chapter 1), may also have a dramatic bearing on the disease manifestations. The influence of these two mutations effecting two cell types that apparently play important roles in *T. gondii* infection (reviewed, Chapter 1) has therefore been examined.

5.3. Materials and Methods

Mice

C3H/He and C57BL/6J mice and their mutants C3H/HeJ and C57/BL/6-BGL were purchased from Harlan and Olac Ltd. (Oxon, England). Mice were used when 8-10 weeks old and groups comprised 6-7 mice as indicated.

Parasites

Mice were infected subcutaneously, intraperitoneally or orally as indicated with 20 cysts (RRA strain). Described in detail in Chapter 2.

Monitoring infection

Mortality was recorded daily and cyst numbers were were determined as described in Chapter 2.

5.4. Results

Comparison of mortality and cyst numbers following different routes of inoculation in C3H/He mice and their congenic mutant C3H/HeJ.

The influence of the *Lps* gene on mortality and brain cyst development was examined in C3H/He *(Lps^n)* and mutant C3H/HeJ *(Lps^d)* mice, infected subcutaneously or orally (Figure 5.1 and Table 5.1). Mortality was significantly higher in mice carrying the Lps^d allele (71.4%, 5/7 s.c.; 28.6%, 2/7 oral) compared with Lps^n mice (14.3%, 1/7 s.c.; 0%, 0/7 oral) for both routes of infection examined. Subcutaneous infection gave an overall greater mortality than oral infection in both Lps^n and Lps^d mice. Examination of brain homogenates of surviving mice, 66 days post infection revealed differences in cyst burdens between mouse strains and routes of infection. Orally infected C3H/He Lps^n mice had significantly more ($p<0.05$) cysts (12114 ± 2810, n=7) in their brains compared with C3H/HeJ Lps^d (4733 ± 1357, n=5). Subcutaneous infection gave similar results although the difference in cyst numbers between C3H/He, Lps^n mice (6440 ± 1675, n=6) and C3H/HeJ Lps^d mice (3500 ± 1500, n=2) was not significantly different ($p<0.375$). This may reflect the low number of surviving mice.

Comparison of mortality and cyst numbers following different routes of inoculation in C57BL/6J mice mutant C57BL/6-BGL.

C57BL/6 mice and the mutant C57BL/6-BGL were infected with *T. gondii* orally, intraperitoneally or subcutaneously and examined for their ability to survive and limit the formation of brain cysts (Figure 5.2 and Table 5.1). Mortality levels in C57BL/6J and C57BL/6-BGL mice infected subcutaneously were similar over the first 38 days post infection (14.3%, 1/7). However, by day 66 mortality had increased to 85.7% in C57BL/6-BGL mice while remaining at 14.3% in C57BL/6J mice. In contrast, intraperitoneal infection induced 33.3% (2/6) mortality in C57BL/6J mice compared with zero (0/6) in C57BL/6-BGL mice. Oral infection induced low mortality in both strains with C57BL/6-BGL (28.6%, 2/7) mice having a slightly higher rate than

C57BL/6J (14.3%, 1/7). Examination of the brain homogenates of surviving mice, 66 days post infection revealed differences in cyst burdens between mouse strains and routes of infection. The brains of the two C57BL/6-BGL mice that were infected subcutaneously and died due to toxoplasmosis on this day were also examined and included in the analysis. Mice infected by the subcutaneous route harboured significantly ($p<0.05$) more cysts in their brains (C57BL/6J, 5188$\pm$2176, n=6; C57BL/6-BGL , 22117$\pm$2998, n=3) than those infected intraperitoneally (C57BL/6J, 2347$\pm$669, n=5; C57BL/6-BGL, 7000$\pm$1071, n=5) or orally (C57BL/6J, 6220$\pm$1576, n=6; C57BL/6-BGL, 9640$\pm$2753, n=5). C57BL/6J mice infected subcutaneously or intraperitoneally had significantly less cysts than C57BL/6-BGL mice ($p<0.005$ and $p<0.05$, respectively). There was no significant difference in the number of cyst harvested from the brains of C57BL/6J and C57BL/6-BGL mice infected orally.

Table 5.1 Cumalative mortality and mean brain cyst burden in C57BL/6J, C57BL/6-BGL, C3H/He and C3H/HeJ mice 66 days post infection with 20 cysts *T. gondii* Beverley strain by different routes as indicated.

Mouse strain	Route of infection	Total cysts/brain ± S.E. at day 30	% Mortality at day 66
C57BL/6J	s.c.	5188±2176	14.3
	i.p.	2347±669	33.3
	oral	6220±1576	14.3
C57BL/6-BGL	s.c.	22117±2998	85.7
	i.p.	7000±1071	0
	oral	9640±2753	28.6
C3H/He	s.c.	6440±1675	14.3
	oral	12114±2810	0
C3H/HeJ	s.c.	3500±1500	71.4
	oral	4733±1357	28.6

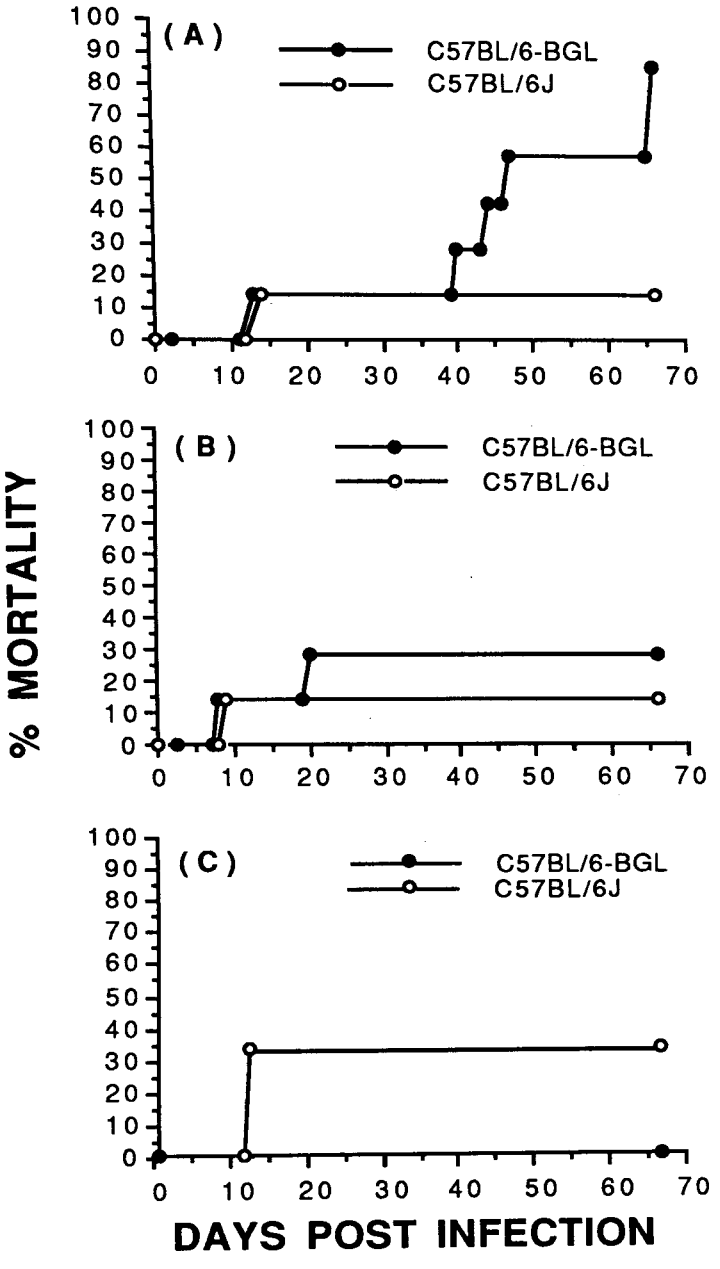

Figure 5.1. Percent mortality over 66 days in C57BL/6J and C57BL/6-BGL mice following (A) s.c., (B) i.p, or (C) oral infection with 20 cysts of the Beverley strain of *T. gondii*.

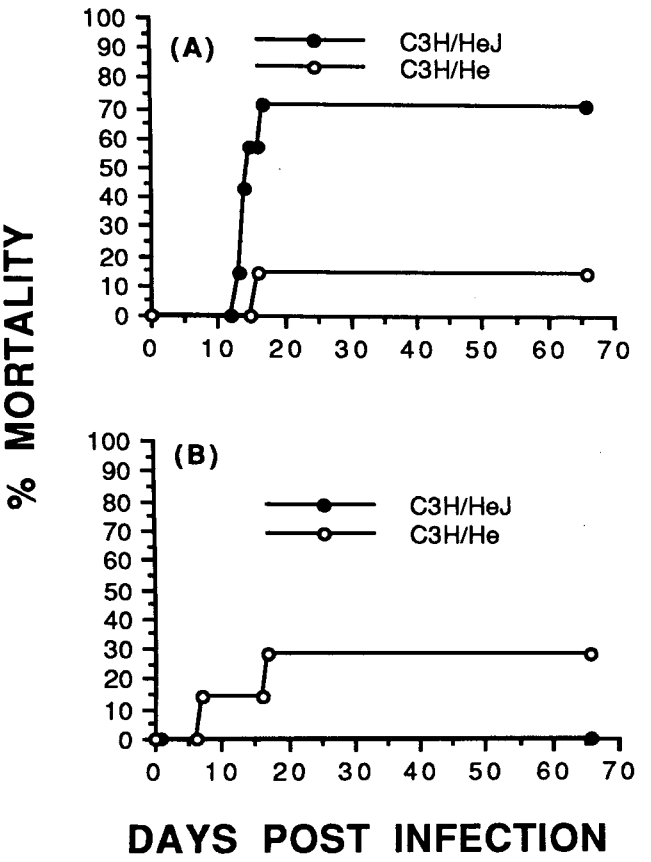

Figure 5.2. Percent mortality over 66 days in C3H/He and C3H/HeJ mice following (A) s.c. or (B) oral infection with 20 cysts of the Beverley strain of *T. gondii*.

5.5. Discussion

This study demonstrates that the ability of mice to resist infection with *T. gondii,* as measured by survival and limitation of brain cyst development, is influenced by both the *beige* and the *Lps* genes. Mice carrying the *beige* mutation are more susceptible to fatal *T. gondii* infection as well as harbouring larger brain cyst burdens than their congenic counterparts. Interestingly, C57BL/6J mice infected subcutaneously were more susceptible to brain cyst development than mice infected orally or intraperitoneally. This phenomenon was also observed in the parental strain although, somewhat lower in magnitude. The most dramatic difference in mortality between mice carrying the *beige* mutation and the parental strain of mice was when infection was *via* the subcutaneous route.

The apparent susceptibility of mice carrying the *beige* mutation which develop large cyst burdens and have high rates of mortality is not surprising. NK cells have been shown to be capable of killing extracellular parasites *in vitro* (Hauser and Tsai, 1986) and antigenic fractions from *T. gondii* have been shown to enhance human (Sharma *et al.,* 1984b) and mouse (Diez *et al.,* 1991) NK cell activity. Human and murine NK cells have been demonstrated to produce IFN-γ in response to *T. gondii* antigen, a cytokine that has been demonstrated to have many beneficial anti-*T. gondii* effects (reviewed, Subauste & Remington, 1991; Chapter 1). However, the *beige* mutation does not appear to have any effect on the ability of NK cells to produce IFN-γ (Kawase *et al.,* 1983), suggesting that the inability of mice carrying this mutation to develop higher levels of resistance is due to other functional defects; most likely impaired ability to lyse target cells.

The *beige* mutation, once thought to be solely responsible for a selective impairment of NK cells to lyse their target cells, is now known to cause defects in the $CD8^+$ T lymphocytes (Saxena *et al.,* 1982). Since T cells and their products play an important part in the development of protective immunity against *T. gondii* infection, the increased susceptibility to infection in mice carrying the *beige* mutation can not

unequivocally be attributed to NK cell dysfunction.

C3H/HeJ mice *(Lps^d)* were found to be more susceptible to acute infection than C3H/He mice *(Lps^n)* and as such, suffered greater levels of mortality in the initial phase of infection. However, mice carrying the *Lps^d* allele harboured fewer cysts at necropsy than those carrying the *Lps^n* allele.

It has been demonstrated that the inability of *Lps^d* mice to respond to LPS is a consequence of a more general dysfunction that prevents non-specific macrophage activation to a variety of bacterial products related and unrelated to LPS (Chedid *et al.*, 1976). *In vitro* experiments have demonstrated that macrophages from *Lps^d* mice infected with BCG have reduced cytotoxicity against *Rickettsia tsutsugamushi, Leishmania major, Schistosoma mansoni* and tumour cells compared with *Lps^d* mice (Meltzer & Nacy, 1985). Interestingly, macrophages from these mutant mice do not become activated in the presence of IFN-γ (Meltzer & Nacy, 1985).

Infection of mice with *T. gondii* has been shown to induce both TNF-α (Chapter 4), and IFN-γ (Shirahata & Shimizu, 1986) production, while *in vitro* stimulation of naive spleen cells with soluble antigen fractions from *T. gondii* tachyzoites induces IFN-γ production (Borges & Johnson, 1975; Chapter 10). Furthermore, IFN-γ (Anderson *et al.*, 1976) has been implicated as playing the major role in the activation of macrophages perhaps acting in synergy with TNF-α (Sibley *et al.*, 1991) to limit *T. gondii* infection by the induction of reactive nitrogen intermediates (Langermans *et al.*, 1992). IFN-γ (reviewed, Subauste & Remington, 1991; Chapter 1) and endogenous TNF-α have also been been demonstrated to play important roles in preventing mortality at the onset of infection (Johnson *et al.*, 1992; Chapter 4). Thus, given the important role of activated macrophages in the control of *T. gondii* infection (Remington *et al.*, 1972) and that macrophages from C3H/HeJ mice appear refractory to activation, it is not surprising that we find increased mortality in *Lps^d* mice.

Lps^{d} mice had fewer cysts in the brains at necroscopy, a surprising observation considering their apparent susceptibility to acute infection. However, recent evidence has suggested that certain as yet unidentified mediators released by mice as a consequence of LPS stimulation may cause leakiness in the blood brain barrier. In a recent study, Lustig *et al.*, (1992) demonstrated that administering LPS to C3H/He *(Lps*^{n}*)* mice allowed two viruses normally lacking neuroinvasive capacity, to invade the brain causing encephalitis. In contrast, LPS treatment of C3H/HeJ *(Lps*^{d}*)* mice did not allow neuroinvasion of either virus. Transfusion of serum from C3H/He mice to C3H/HeJ mice was sufficient to transfer susceptibility to neuroinvasion. The effects of this serum could be ablated by heat treatment, but not by the addition of neutralising anti-TNF-α antibodies. Similarly, it is likely that macrophages from C3H/HeJ mice do not become activated in response to *T. gondii* infection and consequently do not produce the inflammatory mediators that cause 'leakiness' of the Blood Brain Barrier associated with neuroinvasion. Reducing the number of tachyzoites that cross the blood brain barrier in the early stages of infection would ultimately result in fewer cysts developing in the brains of C3H/HeJ mice.

Although TNF-α, a cytokine shown capable of disrupting the integrity of the Blood Brain Barrier in other diseases (Sharief *et al.*, 1992), was ruled out as the sole mediator allowing neuroinvasion in the above study, many other cytokines have been demostrated to facilate lymphocyte migration into tissues. A recent report has demonstrated MIP-1β, an inflammatory product released by macrophages, increases expression of VCAM-1 adhesion molecules on vascular endothelial cells (Tanaka *et al.*, 1993), an important step in allowing lymphocyte migration into tissues (Springer, 1990).

CHAPTER 6

CD8+ T cells are the major lymphocyte subpopulation involved in the protective immune response to *Toxoplasma gondii* in the mouse.

6.1. Abstract

The ability of the major T cell subsets to adoptively transfer resistance to *Toxoplasma gondii* infection was studied. Spleen cells harvested from mice with a 3 month (chronic) *T. gondii* infection as well as spleen cells from uninfected mice were enriched for T cells by nylon wool purification. Adoptive transfer of these cells from both groups of donor mice led to a significant increase in the survival of syngeneic recipient mice infected intraperitoneally with 20 *T. gondii* cysts. Increased survival was mediated particularly by CD4 depleted but, also to a lesser extent CD8 depleted subpopulations. These results were confirmed in T cell reconstituted athymic nude mice. Unfractionated T cells from chronically infected donors produced a significant inhibition of cyst formation in the brains of recipient mice measured ten weeks after infection compared with control mice. The inhibition of cyst formation was ablated by pretreating T cells with anti-CD8 antibody and complement, but not anti-CD4 antibody and complement. Mice receiving cells from infected donors produced an early increase in their IgG1 and IgG2a antibody titres compared with mice given cells from uninfected animals. The depletion of either CD8+ or CD4+ immune cells appeared to have little effect on the antibody responses in recipient mice and there was no correlation between antibody levels and immunity. The results indicate that CD8+ T lymphocytes from convalescent *T. gondii* infected BALB/c mice are the principal mediators of resistance to *T. gondii* although CD4+ T cells appear to be involved during the acute phase of infection.

6.2. Introduction

The critical role of T lymphocytes for the development of protective immunity to *T. gondii* was demonstrated by the inability of athymic, nude mice to control infection (Lindberg & Frenkel, 1977). This study also found that the adoptive transfer of T cells could confer a degree of protection. Depletion of T cell subpopulations followed by cell transfer has suggested CD8+ T cells to be the principal mediators of resistance against acute (fatal) *T. gondii* infections (Suzuki & Remington, 1988). However, during chronic infections the elimination of CD4+ T cells has been shown in separate studies, using apparently identical experimental procedures to be capable of abolishing previously acquired immunity allowing disease reactivation (Vollmer *et al.*, 1987) and to reduce inflammation in the brains of the infected mice (Israelski, *et al.*, 1989). The following study was therefore undertaken to determine by adoptive T cell transfer the relative contribution to disease control of CD4+ and CD8+ T lymphocyte subsets as measured by control of cyst formation and survival.

6.3. Materials and Methods

Mice

Inbred BALB/c mice were bred and maintained in this laboratory under conventional conditions. Male mice were used for experiments when ten to twelve weeks old. Athymic nude (nu/nu) BALB/c mice were obtained from OLAC (1976) Ltd.

Infections

The brains from mice infected 17-21 months previously, with RRA (Beverley) strain of *T. gondii*, were used as the source of infective organisms. Mice were inoculated with 20 cysts intraperitoneally unless otherwise stated.

Preparation of spleen cells

Adoptive lymphocyte transfers were accomplished with cells from naive mice or animals recovered from a *T. gondii* infection. These mice were infected subcutaneously with 10 cysts suspended in 0.2ml saline and penicillin-streptomycin (10 units/ml,10mg/ml). Three months after infection with *T. gondii* the spleens of the mice were removed aseptically. Spleens from normal and infected mice were dissociated in RPMI medium. These cells were enriched for T lymphocytes by nylon wool purification (Julius *et al.*, 1973). Cell suspensions, adjusted to 10^7 cells per ml in RPMI with 0.3% BSA were incubated for 40 min on ice with either anti-CD4 or anti-CD8 monoclonal antibodies (Sera-lab, UK). Following washing, the cells were incubated for a further 40 min at 37°C with rabbit complement. Cell suspensions were washed and resuspended in RPMI for transfer. In each experiment cell viability was checked by trypan blue dye exclusion. Recipients were inoculated via the tail vein with 10^7 cells 24h before *T. gondii* challenge.

Fluorescence staining

Analysis of the frequency of lymphoid cell populations was performed with a fluorescence activated cell sorter (FACS, Becton Dickson or EPICS Profile, Coulter Electronics) using conventional techniques for the preparation and staining of cells

(Micklem *et al* ., 1980). Lymphocytes were incubated with monoclonal antibodies against the surface markers; CD8, CD4 or Thy1.2 (Sera-lab, UK). Dead cells were excluded on the basis of low-angle light scatter and similarly monocytes and granulocytes were gated out on their 90° angle light scatter. The results from FACS analysis were used to check the efficiency of the various enrichment and depletion processes undertaken during the course of the investigation. During a typical experiment, nylon wool purification yielded 77.8% Thy1.2+ cells of which 34.6% were CD8+ and 65.4% were CD4+.

Cell depletions by Flow Cytometry and reconstitution of nude BALB/c mice

Congenitally athymic nude (nu/nu) 9 week old male BALB/c mice were reconstituted with spleen cells from their euthymic (nu/+) female litter mates. Spleen cells were adjusted to 10^7 per ml in RPMI with 0.3% BSA and incubated for 40 min on ice with either anti-CD4 or anti-CD8 monoclonal antibodies (Sera-lab UK). Following washing, cells were incubated with TAGO Goat anti-Rat IgG-FITC conjugate for 40 min on ice. Fluorescent cells were removed from both populations using Flow Cytometry. Further analysis indicated that depletion was 95% successful. Recipient mice were inoculated with $5x10^5$ cells from the depleted population into a tail vein. As nude mice are severely immunocompromised they were infected with 10 rather than 20 cysts to allow increased time for the manifestations of T cell involvement.

Anti-T. gondii antibody measurement by ELISA

At intervals throughout the duration of the study, mice were bled into heparinised capillary tubes and plasma samples obtained by centrifugation. Absorbance readings are expressed as experimental results divided by uninfected (control) values.

Statistics

Values shown are the mean for each group of mice, ± one standard error. Statistical evaluations were made by Student's t-test and differences at the 5% level were considered significant.

6.4. Results

Adoptive transfer of resistance against acute T. gondii infection

The mortality rates of mice from the various experimental and control groups following infection with *T. gondii* are summarised in Table 6.1. Mice receiving medium alone were most susceptible, with 60% of the group dead by day 25. None of the mice given unfractionated T cell populations from either previously infected or, more surprisingly, from non-infected donor mice, died during the course of the experiment. Similarly all recipient mice survived from groups receiving CD4 depleted spleen cell populations from either infected or non-infected donor animals. Depletion of CD8 T lymphocytes however from the inoculated cell populations did increase the mortality rates of recipient mice, although not to the same degree as those mice receiving medium alone. These results were consistent throughout a number of subsequent experiments.

Increased resistance as indicated by increased survival time could be transferred to athymic nude (nu/nu) BALB/c mice with lymphocyte populations from their euthymic (nu/+) litter mates. A degree of resistance was transferred by both the CD4 depleted and CD8 depleted subpopulations as well as unfractionated cells (Figure 6.1a). Increasing the number of lymphocytes transferred greatly prolonged survival in the recipients of unfractionated T cells and the CD4 depleted populations (Figure 6.1b), although it may be significant that mice in the second experiment were infected orally and not intraperitoneally.

Adoptive transfer of resistance against cyst formation

Ten weeks after *T. gondii* infection the brains of surviving mice were removed and the number of cysts in each brain determined (Table 6.1). Cyst numbers were the highest in the mice given medium alone although the numbers were not significantly different from those mice receiving unfractionated or CD8 depleted T cells from non-infected mice. A marked inhibition of cyst formation was, however, noted in the

recipients of CD4 depleted lymphocytes from uninfected donors compared with mice receiving no cells or unfractionated cells from uninfected donors ($p<0.01$). The recipients of unfractionated T cells from chronically infected mice were however found to have significantly fewer cysts ($p<0.01$) than the recipients of T cells from uninfected donors. The resistance to cyst formation was as great in the recipients of CD4 depleted "immune" cell populations as it was in the recipients of unfractionated cells from infected animals and these two experimental groups consistently and significantly demonstrated the greatest resistance to brain cyst formation. Protection, however, as measured by cyst counts was not apparent in the recipients of CD8 depleted T cell populations.

Antibody response to T. gondii following adoptive cell transfer

Specific antibody titres in *T. gondii* infected mice are summarised in Figures 6.2 & 6.3. Those mice given cells from a chronically infected donor showed a significant ($p<0.01$) rise in their IgG2a titre, by as early as day 14 (Figure 6.2). This was not seen in the mice receiving cells from uninfected animals. A delayed increase in IgG2a antibody concentrations was seen in this group and by day 42 their IgG2a titre had increased to a level similar to that of the mice receiving primed cells. After this time antibody concentrations remained similar in all infected groups throughout the period of the experiment. The results also indicate that increased anti-*T. gondii* IgG2a levels were independent of the particular primed T cell population transferred. These results were similar for all classes and sub-classes of Ig tested including IgG1 (Figure 6.3.).

Groups of mice given spleen cells from *T. gondii* convalescent mice, but not subsequently infected with *T. gondii* were monitored for antibody production throughout the experiments. No anti-*T. gondii* response was ever observed and therefore no parasites had been transferred with the spleen cell populations.

Table 6.1. Effect of adoptive transfer of T cell subpopulations on survival and cyst formation in the brains of BALB/c mice.*

Spleen cell population	BALB/c donor	Survival(%)	No. of cysts/brain§
Unfractionated T cells	Non-infected	100	328.0±46.9
	Infected	100	105.2±23.7
CD4 depleted	Non-infected	100	200.0±24.6
	Infected	100	98.7±26.8
CD8 depleted	Non-infected	80	350.0±56.4
	Infected	90	230.0±56.4
No cells		40	396.0±106.7

*Mice were inoculated i.p. with 20 cysts RRA strain 24h after adoptive cell transfer of 10^7 cells.

§Mean ± one SE at the 10th week after infection.

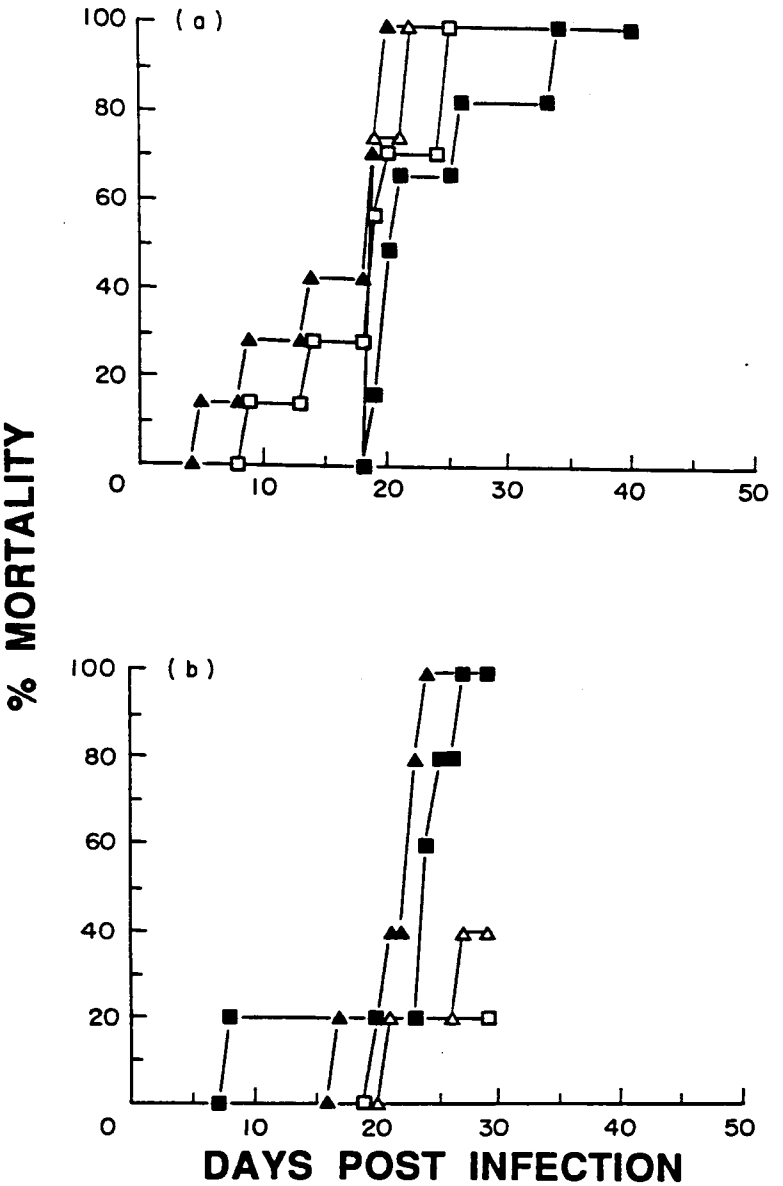

Figure 6.1a. The mortality of nude (nu/nu) BALB/c mice infected intraperitoneally with 10 *T. gondii* tissue cysts. Mice were reconstituted i.v. with either 5 x10^5 unfractionated spleen T cells (□), CD8- T cells (■), or CD4- T cells (△) from their hairy litter mates (nu/+). Control mice were inoculated i.v. with RPMI alone (▲).

Figure 6.1b. The mortality of nude (nu/nu) BALB/c mice infected orally with 10 *T. gondii* tissue cysts. Mice were reconstituted i.v. with either 10^7 unfractionated spleen T cells (□), CD8- T cells (■), or CD4- T cells (△) from their hairy litter mates (nu/+). Control mice were inoculated i.v. with RPMI alone (▲).

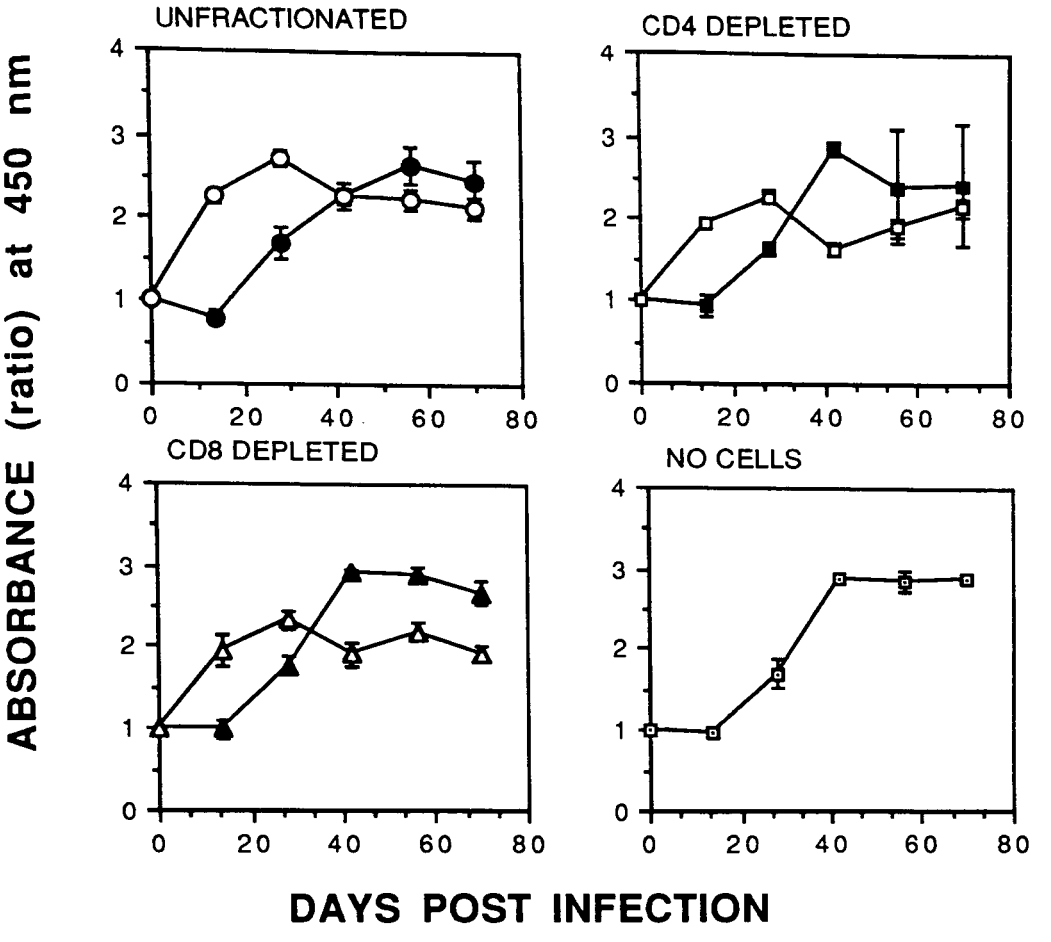

Figure 6.2. The effect of adoptive transfer of splenic T cells on the anti-*T. gondii* IgG2a antibody responses of BALB/c mice infected with 20 tissue cysts of *T. gondii*. Recipient mice were inoculated i.v. with 10^7 cells from either non-infected (●, ▲, ■) or chronically infected mice (○, △, □). The donor T cell populations consisted of either unfractionated populations (●, ○), $CD8^-$ (△, ▲) or $CD4^-$ populations (□, ■). Control mice were given RPMI medium only (⊡). The donor T lymphocyte population is indicated over each graph.

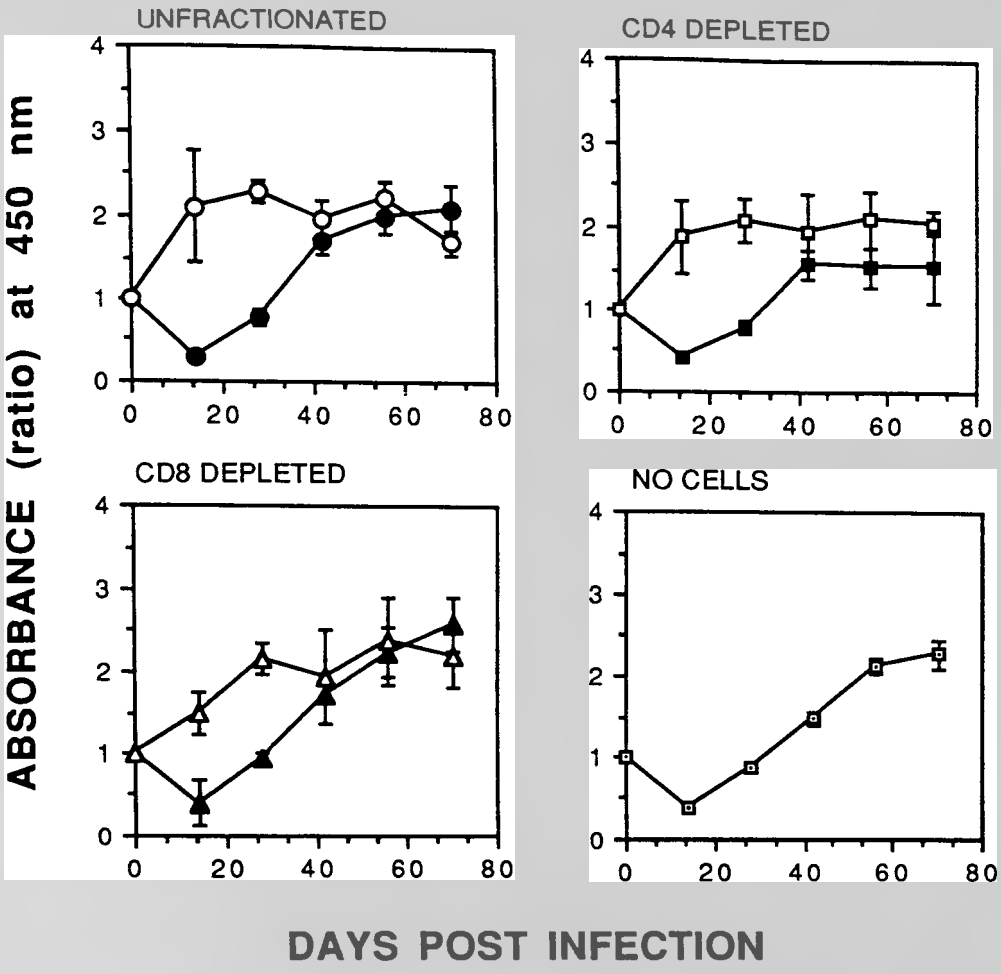

Figure 6.3. The effect of adoptive transfer of splenic T cells on the anti-*T. gondii* IgG1 antibody responses of BALB/c mice infected with 20 tissue cysts of *T. gondii*. Recipient mice were inoculated i.v. with 10^7 cells from either non-infected (●, ▲, ■) or chronically infected mice (○, △, □). The donor T cell populations consisted of either unfractionated populations (● , ○), $CD8^-$ (▲ , △) or $CD4^-$ populations (■ , □). Control mice were given RPMI medium only (⊡). The donor T lymphocyte population is indicated over each graph.

6.5. Discussion

This investigation has shown that a degree of immunity develops during chronic toxoplasmosis which is transferable to naive syngeneic recipients with T cell populations. More specifically the removal of CD8+ T cells reduced the protective ability of these cells as measured by both decreased host survival following infection with *T. gondii* cysts and in chronic infections by brain cyst formation. Although the adoptive transfer of CD8+ T lymphocytes and to a lesser extent CD4+ T lymphocytes increased the survival of *T. gondii* infected mice compared with animals not receiving T cells, this protection was independent of whether the donor animals had or had not been previously infected. Inhibition of brain cyst formation, however, could only be transferred to naive syngeneic recipients with CD8+ T cell populations and this ability was particularly associated with primed T cell populations from previously infected donors.

The ability of unprimed T lymphocytes from uninfected donors to transfer protection as measured by host survival and to a lesser extent cyst formation, is a surprising phenomenon. Increased resistance against *T. gondii* infection adoptively transferred by these T cell populations may simply reflect the increased number of potentially responsive cells present following cell transfer to recipients. As the level of challenge infection used by us in these experiments corresponds to the LD50 in BALB/c mice, any slight alteration in T cell numbers or balance could have profound effects on the outcome of the disease. However, the importance of primed *versus* unprimed cells in immunity, especially during different phases of infection, has previously been demonstrated by Bateman *et al.*, (1987) with Moloney Sarcoma Virus (MSV). They found that while unprimed CD4+ T cells were required for the primary rejection of MSV, only primed CD8+ T cells were able to transfer resistance to a secondary challenge.

Other workers have also found CD8+ T lymphocytes to play the most significant

protective role during acute *T. gondii* infection (Suzuki and Remington, 1988). Our results indicate that cells of this phenotype are almost entirely responsible for limiting cyst formation during infection. In the early stages of infection the presence of CD8+ T cells, which recognise class I rather than class II, H-2 molecules (Swain 1983), may be acting primarily as cytotoxic cells, limiting parasite dissemination either by direct recognition of infected cells or by the release of lymphokines. Indeed, *in vitro* studies have demonstrated that CD8+ T cells, from immunised mice (Khan *et al.*, 1988b; Hakim *et al.*, 1991) can kill extra-cellular tachyzoites (Khan *et al.*, 1988b) and*T. gondii* infected or antigen pulsed cells in a class I restricted fashion (Hakim *et al.*, 1991). Suzuki and Remington (1990) recently demonstrated that the ability of adoptively transferred immune CD8+ T cells to prevent mortality can be ablated by simultaneously administering a neutralising monoclonal antibody to IFN-γ. Nonetheless, the production of IFN-γ by CD8+ T cells, as well as other cells such as TH1 cells or NK cells, may mediate protection indirectly by activating macrophages to kill *T. gondii in vitro* (Remington *et al.*, 1972; Anderson & Remington 1974) by oxygen dependent (reviewed, Hughs, 1988) and L-arginine dependent mechanisms (Adams *et al.*, 1990). Treatment of mice with recombinant IFN-γ has been shown to confer significant resistance against acute *T. gondii* infection *in vivo* by preventing parasite proliferation (Suzuki *et al.*, 1988) and reducing mortality (McCabe *et al.*, 1984). While in the chronically infected host, it may prevent cyst rupture and toxoplasmic encephalitis (Suzuki, *et al.*, 1989b).

In agreement with the functional studies demonstrating the necessity of CD8+ T cells for the generation and maintenance of anti-*T. gondii* protective immunity, genetic studies have mapped resistance to toxoplasmosis, as measured by reduced cyst numbers (Chapter 3; Jones & Erb, 1985; McLeod *et al.*, 1989b; Brown & Mcleod, 1991) and increased survival (Williams *et al.*, 1978) to the D region within the H-2 complex.

Despite the evidence presented above demonstrating a major protective role for CD8+ T

lymphocytes during *T. gondii* infections, there is much evidence that CD4+ T cells are also important in the induction of protective immunity. Since this study, Gazzinelli *et al.*, (1991 & 1992b) have demonstrated the important synergistic relationship of CD4+ and CD8+ T cells in mediating protective immunity. These workers demonstrated that both CD4+ and CD8+ T cells isolated from mice immunised with the a temperature sensitive mutant (ts-4) could adoptively transfer immunity to *T. gondii* infection, reducing mortality in recipients. *In vitro*, stimulation with crude *T. gondii* antigen, of both CD4+ and CD8+ T cells isolated from mice immunised with the a temperature sensitive mutant (ts-4) produced IFN-γ, although only CD4+ T cells produced IL-2. Thus, immunity to toxoplasmosis could be attributed to CD4+ T cells of the TH1 subclass and IFN-γ producing CD8+ T cells. Interestingly, the depletion of CD4+ T cells alone in chronically infected mice was not in itself sufficient to permit disease recrudescence, although simultaneous depletion of CD8+ T cells or the administration of an neutralising antibody to IFN-γ resulted in encephalitis and increased cyst numbers (Gazzinelli, *et al.*, 1992b). This study, while demonstrating the necessity for IFN-γ in the prevention of disease reactivation, also demonstrated that CD8+ T cells can produce sufficient quantities of this cytokine to effect protective immunity. Nevertheless, CD4+ T cells may have an auxiliary role in the production of IFN-γ and would appear to be the sole producer of IL-2 (Gazzinelli, *et al.*, 1992b), and consequently may be instrumental in the induction of cytolytic CD8+ T cells (Maraskovsky *et al.*, 1989; Bass *et al.*, 1992).

However, the presence of CD8+ T cells may under certain circumstances be detrimental to the host. A significant elevation in CD8+ T cell numbers occurs in patients with prolonged symptoms due to acute infection (Luft *et al* ., 1984 and 1987, Sklenar *et al* ., 1986). This contrasted with asymptomatic patients where the number of CD8+ T cells was normal and the induction of suppressor activity minimal. It is possible that the presence of CD8+ T cells may simply reflect acute symptomatic

disease and in fact be beneficial in controlling the disease. In support of this theory, a rise in the number of CD8+ T cells is observed in the spleen of acutely infected BALB/c and BALB/K mice which accompanies and precedes a decline in brain cyst numbers in both mouse strains (Chapter 3). An alternative explanation is that CD8+ T cells may act as suppressor cells and cause prolonged symptoms. Recent evidence suggests that CD8+ T cells can be divided into at least two functional subsets based on the lymphokines they produce. One of these populations, like TH1 cells is characterised through the secretion of IFN-γ, but not IL-4 and the other like TH2 cells secretes IL-4 (reviewed, Bloom *et al.*, 1992). Under these circumstances one subset of CD8+ T cells through the secretion of IFN-γ and the consequent anti-*T. gondii* effects would be disease controlling. In contrast, the other IL-4 producing CD8+ T cell subset could through the release of cytokines be responsible for suppression of cell mediated immunity and consequently induce disease exacerbation.

In our experiments a role for antibody could not be demonstrated. However, it may be significant that as well as anti-*T. gondii* specific IgG1, anti-*T. gondii* specific IgG2a was detected in all groups of mice. Indeed, IgG2a antibody production was augmented by adoptively transferred immune T cells irrespective of whether they were unfractionated, depleted of CD4+ or CD8+ T cells. The IgG2a subclass of immunoglobulin is associated with the presence of IFN-γ and TH1, CD4+ T cells (Snapper and Paul, 1987). It is likely that IFN-γ producing CD8+ T cells would also augment IgG2a production and would be an explanation for the observed increase in this IgG subclass in mice receiving CD8+ T cells from immune donors.

In *Listeria monocytogenes* infections both CD4+ and CD8+ T cells are implicated in protection. Although the major bactericidal role has been attributed to the CD8+ T cell population, CD4+ cells are important in inducing an influx of inflammatory cells to the site of infection (Czuprynski and Brown 1986). Our results reinforce the fact that

there is complex interplay between different facets of the immune response, but indicate a significant protective role for $CD8^+$ T cells during both acute and chronic *T. gondii* infection.

CHAPTER 7

Detection of cytokine mRNA in the brains of mice with toxoplasmic encephalitis

7.1. Abstract

C57BL/10 ScSn mice infected with *T. gondii* developed a meningoencephalitis, characterised by areas of tissue destruction and cellular infiltration including foci of neutrophils. Large numbers of cyst stages were found throughout the brain but were not always associated with inflammation. The use of immunocytochemistry to detect glial fibrillary acidic protein, an astrocyte specific marker, showed a widespread astrocyte activation. This was particularly prominent in areas of intense inflammation but cysts were negative for glial fibrillary protein, indicating that astrocytes were not the host cells for the bradyzoites. The use of the polymerase chain reaction to assist in the amplification of total brain RNA allowed the characterisation of the cytokines being produced locally within the brains of infected animals. β-actin transcripts were detected in all of the infected and uninfected mice. In only one of the seven uninfected control mice were other transcripts found. Transcripts for TNF-α, IL-1α and IL-1β, IL-6, macrophage inflammatory protein -1 (MIP-1) and interferon-γ as well as the CD4 marker, were detected in all of the infected mice. However, transcripts for IL-2 and IL-4 were not detected. Several of the cytokines present are capable of initiating meningeal inflammation and may play a role in the immunopathogenesis of toxoplasmic encephalitis

7.2. Introduction

Infection of humans with *T. gondii* may be associated with severe neurological symptoms frequently involving the brain and the optic nerve (McCabe *et al.*, 1987; Cook 1990). Neurological manifestations are most common in the immunosuppressed, especially those infected with HIV. In the USA, toxoplasmic encephalitis is now the single largest cause of cerebral mass lesion (Luft & Remington, 1988), where approximately 50% of HIV patients sero-positive for *T. gondii* develop this frequently fatal complication (Zangerle *et al.*, 1991). Recrudescence of the latent cyst stages (bradyzoites), found throughout the the body, but most prominently in the brain, is responsible for the resultant encephalitis. Cyst rupture and the transformation of bradyzoites to invasive tachyzoites gives rise to active infection leading to the characteristic localised tissue necrosis and inflammation (Frenkel & Escajadillo, 1987; Tadros & Laarman, 1982). It appears that cyst rupture with subsequent parasite invasion and destruction of neighbouring cells leads to the attraction into the brain of large numbers of inflammatory cells with the resultant immunopathology (Suzuki *et al.*, 1989a; Ferguson *et al.*, 1991).

The weight of evidence indicates that protective immunity against toxoplasmosis is cell mediated and although CD8+ T cells have been shown to be the major lymphocyte population involved in this protection , CD4+ T cells, macrophages (reviewed Chapter 1 & 4) and NK cells (Hauser & Tsai, 1986) are also important. The ability of these cell populations to influence the course of infection is at least partially dependent on the cytokines they produce, some of which have been shown to be beneficial while others are detrimental. The cytokines IFN-γ and IL-2 have been demonstrated to mediate resistance to toxoplasmosis whereas the role of TNF-α appears to be ambivalent as it has been shown to contribute to host protection as well under certain circumstances shortening time to death (reviewed, Chapter 1; Chapter 4).

Recent work has also indicated the importance of cytokines in mediating inflammatory changes within the CNS. Administration of TNF-α, IL-1α, as well as a macrophage

inflammatory protein (MIP-1) have all been shown to initiate meningeal inflammation (Ramilo *et al.*, 1990; Saukonen *et al.*, 1990; Waage *et al.*, 1989). Whilst macrophages are one of the main sources of these cytokines, astrocytes share many of the same characteristics including the ability to produce TNF-α, IL-1α and IL-6 (Fontana *et al.*, 1984; Robbins *et al.*, 1987; Frei *et al.*, 1989; Lieberman *et al.*, 1989).

The C57/BL10 ScSn mouse is extremely susceptible to toxoplasmosis as demonstrated by high mortality rates and the development of large brain cyst burdens accompanied with encephalitis (reviewed, Chapter 1 and 3). The following work was undertaken to determine which cytokines are produced within the CNS of animals with toxoplasmic encephalitis and to correlate these with the histopathological events observed.

7.3. Materials and Methods

Mice and infections

C57BL/10ScSn mice were used when 6-8 weeks old. These mice have been shown to be highly susceptible to infection with *T. gondii* as demonstrated by low survival rates and the development of large numbers of tissue cysts in the brain (Chapter 3; McLeod *et al.*, 1987). Mice were infected with 10 cysts (RRA strain) as described in Chapter 2.

Tissue processing

Mice were perfused with PBS (pH 7.4) through the right ventricle to remove peripheral blood from the CNS. The brain was divided into its two hemispheres and one half fixed in neutral buffered formalin. After fixation the tissue was processed, paraffin embedded and sagittal sections taken. One section was stained with haematoxylin and eosin to allow the histopathological changes to be assessed. Blank sections were used for immunocytochemistry to identify activated astrocytes with a rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) as the primary antibody and an avidin biotin peroxidase system (DAKO) to visualise activity with diaminobenzamide as substrate (Hunter *et al.*, 1991). The other half of the brain was used to isolate total RNA using a guanidinum thiocyanate method (Chomczynski & Sacchi, 1987).

Reverse transcription and polymerase chain reaction

Five ug of total RNA was lyophilised and resuspended in 9ul of annealing buffer (250mM KCl, 10mM Tris HCl pH 8.3, 1mM EDTA) with 2ul (0.5ug) of the 3' primer, heated at 80°C for 3 min, 69°C for 20 min and allowed to cool to 40°C over 20 min. The mix was then quenched on ice and 15ul of cDNA/dNTP buffer (24mM Tris HCl, pH 8.3, 16mM $MgCl_2$, 8mM dithiothrietol, 0.4mM dNTPs) with 200 units of Moloney murine leukemia virus reverse transcriptase (BRL) added and incubated at 43°C for 45 min. The resulting cDNA was then amplified by adding 0.5ug of the 5' primer, 20ul of dNTPs (2.5mM) and 45ul of Taq polymerase buffer (100mM Tris HCl

pH8.8, 30mM $(NH_4)_2SO_4$, 2mM $MgCl_2$, 10mM β-mercaptoethanol) with 2 units of Taq polymerase (Replinase, Du Pont, UK). The mix was then taken through 35 cycles at 94°C for 1 min, 60°C for 2 min and 74°C for 3 min. PCR products were electrophoresed through a 2% agarose gel (Sigma, Poole, Dorset, UK) and visualised with ethidium bromide stain and UV illumination. Positive controls were obtained from a variety of different mouse RNA samples.

7.4. Results

All infected animals developed toxoplasmosis as evident from their loss of condition and weight (25% by day 35) and increasing mortality within the group (75% by day 35). These mice had high anti-*T. gondii* antibody titres as determined by ELISA (results not shown) and an infected control group sacrificed at this time had a mean of 25078 $\pm$ 9934 (n=6) cysts per brain.

Histology

Histologically there was a well developed encephalitis and an associated meningitis in all the infected mice examined with microglial inflammatory nodules also present. Figure 1 shows typical reactions seen in the CNS with areas of perivascular cuffing and encephalitis evident (Figure 7.1a). Foci of neutrophils were present in many of the brains examined, normally in areas where there were no signs of other inflammatory cells (Figure 7.1b). Tissue cysts were distributed throughout the brain, usually with few if any associated inflammatory cells (Figure 7.1a). Activated astrocytes, as detected by intensity of GFAP staining, were widespread in the brain and were particularly prominent in areas of inflammation (Figure 7.1b). In none of the mice examined were any of the cysts GFAP positive nor were activated astrocytes particularly associated with the cyst stages.

Detection of cytokine mRNA by PCR assisted amplification

The use PCR assisted amplification of total brain RNA detected the presence of transcripts for β-actin, IFN-γ, IL-6 and MIP-1 as well as IL-1α and β, CD4 and TNF-α (Figure 7.2) in all of the infected mice. However, transcripts for the cytokines IL-2 and IL-4 were not demonstrated, In only one of the seven B10 uninfected control mice tested were transcripts other than for β-actin found. These were for CD4 and IL-1α. This mouse had no signs of any inflammatory processes nor astrocyte activation. There were no histological signs of inflammation in the CNS in any of the control mice.

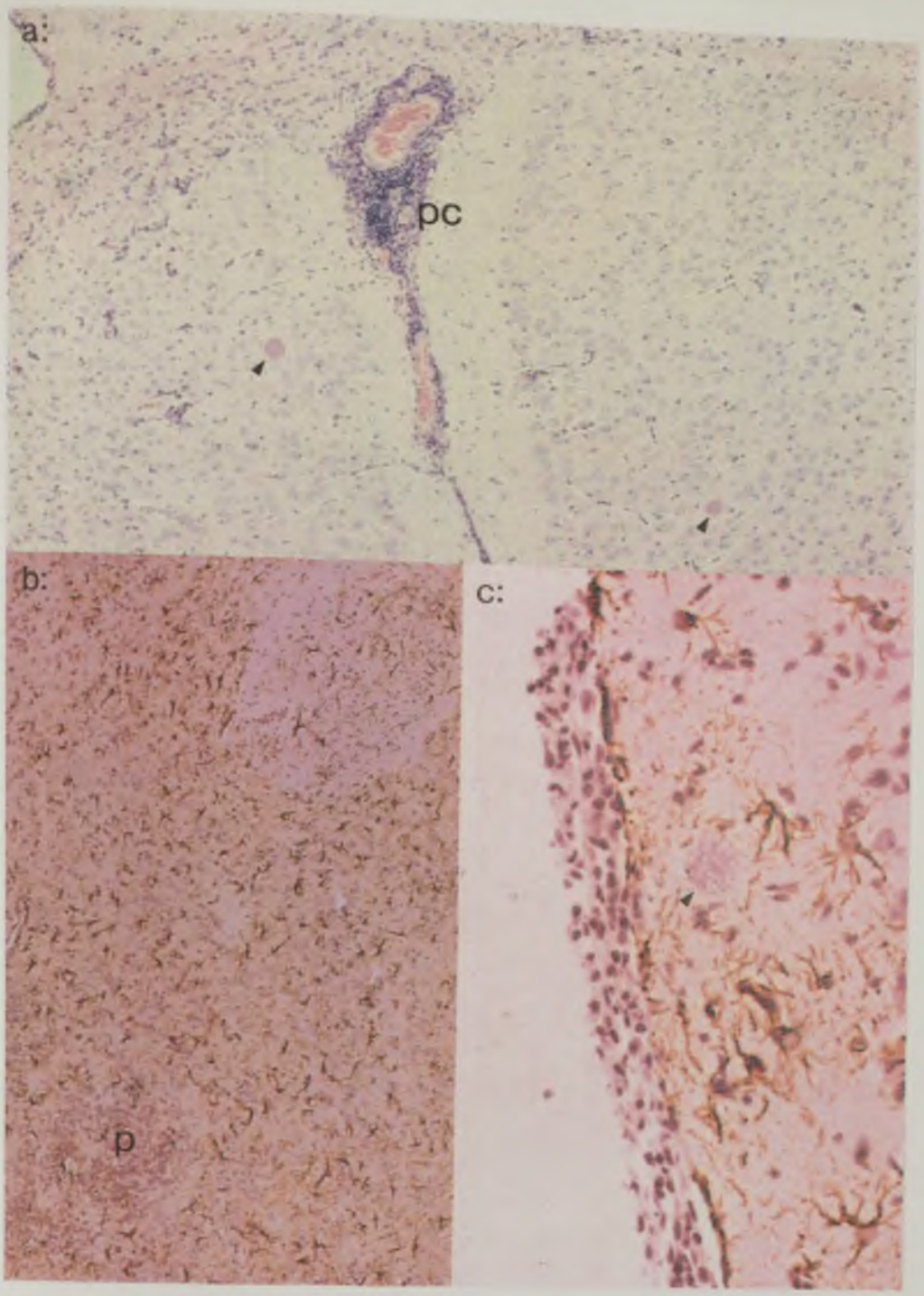

Figure 7.1. Brain sections of mice with chronic toxoplasmic encephalitis.
a: Large numbers of inflammatory cells are present with perivascular cuffing (pc) and an encephalitis. Cells infected with *T. gondii* cysts (◂) are evident throughout the brains of infected mice, often not associated with areas of inflammation (magnification x 50). **b:** Astrocyte activation, as detected by immunocytochemistry for GFAP, is wide spread. Foci of polymorhs (p) are frequently present in many areas (magnification x 100). **c:** Astrocyte activation, as detected by immunocytochemistry for GFAP, is wide spread. Cysts (◂) are not GFAP positive (magnification x 200).

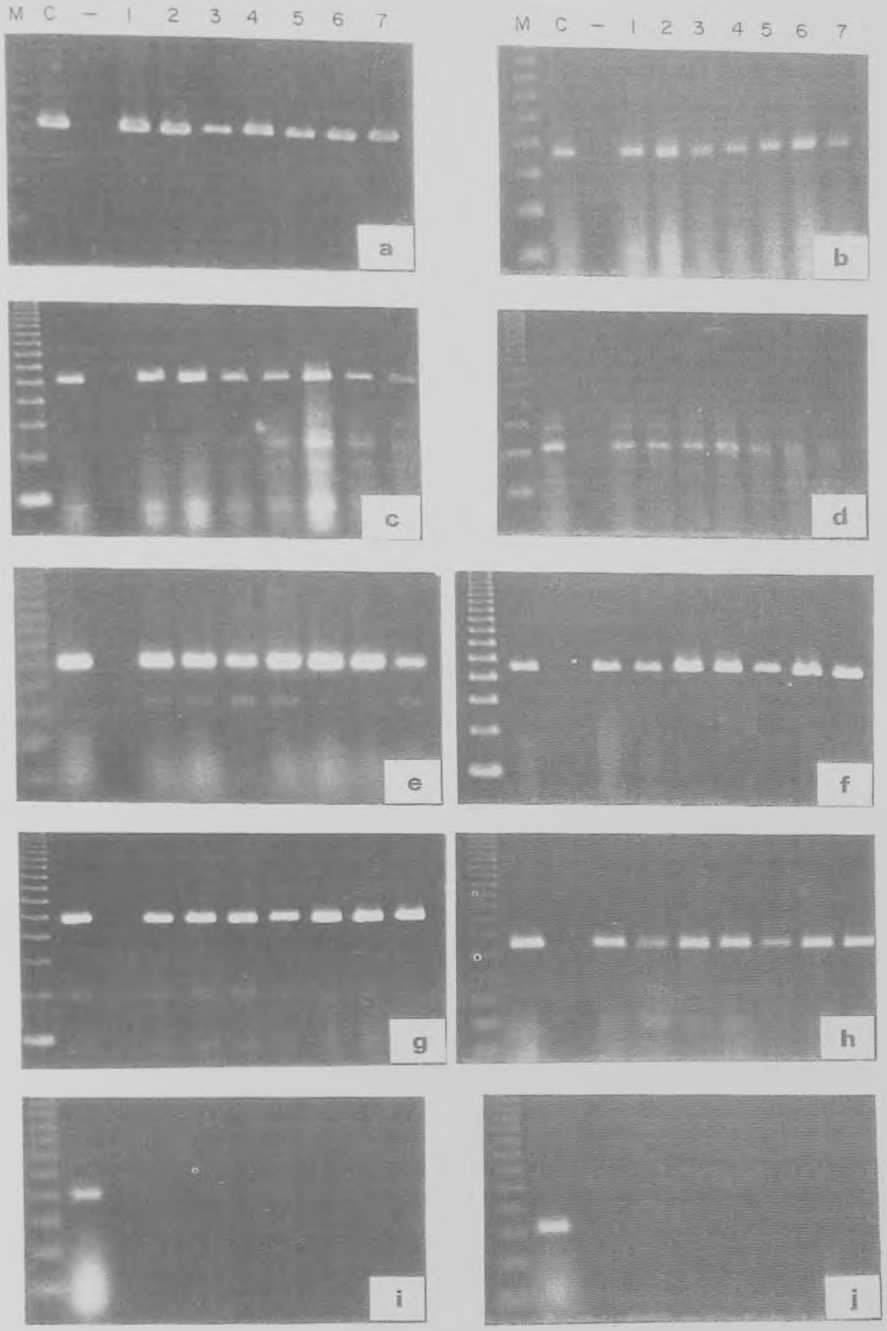

Figure 7.2. The use of PCR assisted amplification to detect cytokine transcripts. The left lanes (M) contain a ladder of 123bp fragments. A positive control (C) and a negative control (-) are also included. **Figure 7.2a**: b-actin (549 bp); **b**: IFN-γ (460bp); **c**: IL-6 (638 bp); **d**: MIP-1 (267 bp); **e**: IL-1α (625bp); **f**: IL-1β (563bp); **g**: CD4 (615bp); **h**: TNF-α (692 bp); **i**: IL-2 (502bp); **j**: IL-4 (399bp).

7.5. Discussion

The pathological reactions described in the results were indicative of several different stages of an ongoing inflammatory response and in agreement with previous reports (Ferguson *et al.*, 1991). As there were large numbers of mononuclear cells present in the inflammatory infiltrates, it was no surprise to detect transcripts for MIP-1, IL-1α & IL-1β, IL-6 and TNF-α, all of which can be produced by activated macrophages. MIP-1 has been shown to be involved in the chemotaxis of neutrophils as well as a variety of inflammatory processes, including those in the CNS (Wolpe *et al.*, 1988; Saukonnen *et al.*, 1990). Macrophages are not the only cells capable of producing these cytokines, activated microglial cells and astrocytes can produce IL-1α, IL-1β, IL-6 and TNF-α (Fontana *et al.*, 1984; Robbins *et al.*, 1987; Frei *et al.* 1989, Lieberman *et al.*, 1989). Indeed, the degree of astrocyte activation observed suggests that these cells play a significant role in toxoplasmic encephalitis.

Astrocyte proliferation, or gliosis, is often a consequence of damage to the CNS and is regarded as a part of the healing process within the CNS. The gliosis observed here may be a response to the damage caused by the inflammation and so may be an indirect rather than a direct result of an active *T. gondii* infection in the CNS. Alternatively, parasite induced production of cytokines by glial cells may be responsible for mediating the immunopathology associated with the disease. Whilst astrocytes are known to be one of the many host cells for tachyzoites, our findings indicate that astrocytes were not host cells for the cyst stage. However, whilst Ferguson & Hutchison (1987), reported that, in the brain, this stage was found exclusively in the neuronal cells of infected mice, Jones *et al.*, (1986) demonstrated that astrocytes could act as host cells for bradyzoites *in vitro* .

The role of TNF-α in toxoplasmosis remains controversial, as administration of recombinant TNF-α has been shown to shorten the time to death of acutely infected mice (Black *et al.*, 1989) whilst other workers (Chang *et al.*, 1990) have reported that recombinant TNF-α protects mice from infection as does IL-1. Nonetheless, both

TNF-α and IL-1 have been shown to mediate meningeal inflammation (Ramilo *et al.*, 1989; Saukonnen *et al.*, 1990). IL-6 has not been linked directly to inflammation in the CNS, it may be involved in inducing proliferation of astrocytes (Benveniste *et al.*, 1989) as well as the production of local antibody (Benveniste *et al.*, 1989). Indeed intrathecal antibodies have been found in toxoplasmosis (Potasman *et al.*, 1990). Plasma cells are often seen as a cellular infiltrate associated with perivascular cuffing (Figure 7.3.)

The detection of IFN-γ mRNA transcripts in mice with progressive toxoplasmic encephalitis was surprising. Current evidence would indicate that IFN-γ would prevent cyst rupture (Jones *et al.*, 1986) and the use of neutralising antibodies by other workers exacerbated the encephalitis associated with the disease (Suzuki *et al.*, 1989). IFN-γ has several functions that may be associated with pathogenisis in the CNS; it is capable of increasing class I and II expression on astrocytes (Fontanna *et al.*, 1987) as well as the expression of adhesion molecules on endothelial (Mantovini & Dejanae, 1989) and glial cells (Satoh *et al.*, 1991). The cytokine transcripts detected may play a role in pathogenesis as well as controlling toxoplasmosis. Indeed, microglial cells activated by IFN-γ or endotoxin have been shown to be capable of killing cocultured neuronal cells *via* L-arginine dependent mechanisms (Chao *et al.*, 1992a) and IFN-γ has been demonstrated to mediate lethality in mice infected with *Plasmodium vinckei* (Kremsner *et al.*, 1992) or challenged with endotoxin (Doherty *et al.*, 1992).

Although identification of the CD4 marker might suggest the presence of helper T cells in the CNS, the absence of transcripts for IL-2 and IL-4, either or both of which are produced by all T helper cells (Mosmann & Moore, 1991), would indicate that this cell type is not in fact present. As activated microglial cells have been shown to be $CD4^+$ (Perry & Gordon, 1987, Chen *et al.*, 1989), the presence of this marker, in the absence of helper T cells is not unreasonable or unlikely. mRNA for IFN-γ in the CNS is most likely derived from $CD8^+$ T cells, although NK cells cannot be ruled out as a source of this cytokine (Hauser & Tsai, 1986). Numerous studies have indicated

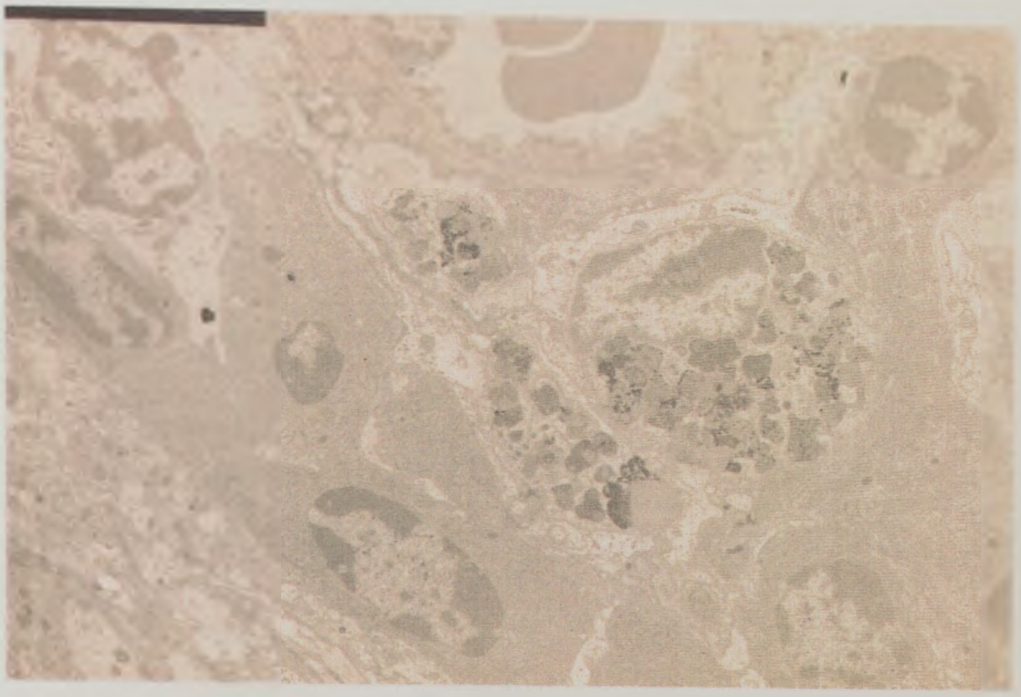

Figure 7.3. Transmission Electron Micrograph of the brain of a chronically infected mouse. Several plasma cells are evident within the brain tissue, having transversed the vascular endothelium. This E.M. was kindly supplied by David Ferguson, University of Oxford.

CD8+ T cells are the major T cell subset involved in the protective immune response against *T. gondii* (Brown & McLeod, 1990; Suzuki and Remington, 1988; Parker *et al.*, 1991; Chapter 6), and these appear only to function effectively if operating synergistically with CD4+ T cells (Suzuki & Remington 1988; Gazzinelli *et al.*, 1991). Thus, mice depleted of CD4+ T cells before infection with *T. gondii* developed exacerbated disease (Araujo, 1991), while those depleted of CD4+ T cells during chronic infection lost previously acquired immunity (Vollmer *et al.*, 1987). The apparent absence of CD4+ T cells from the CNS might in fact explain their extreme susceptibility as demonstrated by high cyst numbers, severe toxoplasmic encephalitis and high mortality. In the following chapter these observations are studied in more detail using a more sensitive assay.

CHAPTER 8

Kinetics of cytokine mRNA production in the brains of mice with progressive toxoplasmic encephalitis

8.1. Abstract

C57BL10 ScSn (B10) mice infected orally with *Toxoplasma gondii* were killed on days 5, 10, 15, 20 and 30 post infection and their brains excised. These were either used to count total tissue cyst numbers or divided for RNA purification and histopathological studies. The first signs of inflammation were detected on day 10 post infection, before the appearance of cysts in the brain, and correlated with the presence of activated astrocytes. These mice had a mild meningitis with areas of encephalitis. Small numbers of cyst stages were first observed in the brain on day 15 and by day 20 the cyst numbers had increased dramatically but were not always associated with inflammation. After this time point, total cyst numbers did not increase significantly though there developed a marked variation in tissue cyst size with larger cysts becoming more numerous. The use of the polymerase chain reaction to assist in the amplification of brain RNA allowed the characterisation of the kinetics of cytokine production within the brains of these animals. Only IL-1α was found to be expressed constitutively in control mice. Transcripts for other cytokines associated with activated monocytes, microglial cells and astrocytes (TNF-α and IL-6) were present on day 10, (IL-6) and day 15 (TNF-α) post infection. Thereafter these cytokines were present in all infected animals. Of the T cell-associated cytokines, IL-4, a characteristic product of the TH2 cell subset, was detected on days 10 and 15, while GM-CSF which can be produced not only by this cell type but also by TH1 cells and CD8+ T cells, was also present on day 15 but not thereafter. Transcripts for IFN-γ, present from day 15 post infection, were probably produced by CD8+ T cells, as IL-2 which would indicate TH1 cell involvement was only detected 30 days after infection. The continual presence of IFN-γ and TNF-α, cytokines with reported anti-toxoplasmic activity, in the CNS of B10 mice throughout the latter half of the experimental period did not diminish the severity of infection. These results indicate that the TH2 subset may allow a rapid rise in cyst numbers and so be important in determining susceptibility to toxoplasmic encephalitis.

8.2. Introduction

Many *in vivo* studies have used administration of exogenous recombinant cytokines or neutralising antibodies to influence the course of toxoplasmosis (reviewed, Chapter 1). Although such treatments often markedly alter disease processes in the central nervous system (CNS) effects are probably indirect because of the inability of the materials to cross the blood brain barrier. Thus, this form of experimentation sheds little knowledge on local inflammatory responses occurring in the brain naturally during *T. gondii* infection.

In Chapter 7 we characterised cytokine production within the CNS of animals with advanced toxoplasmic encephalitis and demonstrated the presence of transcripts for TNF-α, macrophage inflammatory protein-1 (MIP-1) and interleukin-1 (IL-1), cytokines which are able to initiate meningeal inflammation (Ramilo, *et al.*, 1990; Saukkonen *et al.*, 1990; Waage *et al.*, 1989). Transcripts for IFN-γ were also detected but not IL-2 or IL-4. However these animals were approaching death and the information obtained probably revealed more of the immunopathology associated with toxoplasmic encephalitis than the immunological processes responding to and influencing parasite infection. Consequently, the following work was undertaken to determine the kinetics of cytokine production within the CNS of animals as they progressed through acute infection to the development of chronic toxoplasmic encephalitis and to correlate these with the histopathological events observed.

8.3. Materials and Methods

Mice and infections

C57BL/10ScSn mice (6-8 weeks old) were infected orally with *T. gondii* (RRA strain) as described in Chapter 2. Groups of 5 mice were killed on days 5, 10, 15, 20, and 30 days post infection and assessed pathologically for CNS involvement. Total RNA was used to characterise the kinetics of cytokine production within the brains of these mice. A further three mice were killed at each time point plus day 25 and 35 and their total number of brain cysts calculated.

Histopathology

At time of killing, mice were perfused through the left ventricle with 50 ml of PBS (pH7.4) to remove peripheral blood, and the brain divided for histopathology and RNA purification. Brains processed for histopathology were fixed in neutral buffered formalin and 5um sagittal sections cut and stained with hematoxylin and eosin.

RNA purification and reverse transcription

Total RNA was purified from brain tissue using a modified guanidinium thiocyanate method (Chomczynski & Sacchi, 1987). Reverse transcription was carried out using approximately 5ug of total RNA assembled in a total volume of 20ul of 1xTaq polymerase buffer (Promega, Madison, W1) with 2.5mM of each dNTP (Pharmacia, Uppsala Sweden), 200 U of Moloney murine leukemia virus reverse transcriptase (BRL, Uxbridge, GB), 20 U RNase inhibitor (Promega) and 0.5 ug of the 3' primer, to be used PCR (see below). The reaction mix was left at room temperature for 10 min to allow annealing of the primer to its target sequence and then incubated at 43°C for 45min for the reverse transcription reaction.

Polymerase chain reaction

Oligonucleotide primers designed to amplify cytokine cDNA by PCR and produce bands of different sizes depending on the cytokine being analysed (Brenner, *et al.*, 1989) were either purchased from Clonetech (Cambridge, GB) or synthesised on an

applied Biosystems (Foster City, CA) oligonucleotide synthesiser. These were used for the characterisation of the cytokines being produced within the CNS of infected animals. For PCR the reverse transcription reaction was made up to 100ul with 1xTaq polymerase (Promega) and subjected to 30 cycles of amplification at 94°C for 1 min, 60°C for 2 min and 72°C for 3 min. Analysis of the PCR reactions was carried out using agarose gel electrophoresis with ethidium bromide staining and was visualised by ultraviolet illumination.

Slot blot analysis

Internal probes were used to further characterise the degree of cytokine message present. Sixty microlitres of the PCR reaction was alkali denatured with 61ul of 1.7M NaOH and left for 10 min before the addition of 121.3ul of 0.2xSSC and the sample applied to a filter (Genescreen plus. DuPont, Herts, GB), presoaked in 0.4M Tris (pH7.7), using a slot blot manifold (Bio-Rad, Watford, GB). After 30min, a vacuum was applied and and then each slot washed twice with 150ul 0.4M NaOH and twice with 150ul 2xSSC. The filter was air dried, baked for 2 hr at 80°C before prehybridisation in 5xSSPE containing 0.6% SDS and 50ug/ml of salmon sperm DNA. The appropriate (γ-^{32}P) ATP labelled internal probe was then added and the filter probed overnight 5-8°C below the Tm 50 of the probes. Internal probe (40ng) was labelled with (γ-^{32}P) ATP using T4 kinase (Boehringer Mannheim, Mannheim, FRG) and separated from unincorporated isotope using Sephadex G-50 (Pharmacia) spin column. Filters were washed twice in 5xSSC, 0.1% SDS for 15min and 0.3xSSC, 0.1% SDS for 15min before being allowed to dry. Appropriate concentrations of SSC and SSPE solutions were prepared from 20xSSC (3M NaCl, 0.3M $C_6H_5Na_3O_7$; BDH Ltd., Dorset, U.K.) and 20xSSPE (3M NaCl, 0.23M $NaH_2PO4.H_20$, 25mM EDTA; BDH Ltd., Dorset, U.K.) Filters were exposed to Kodak XAR-5 film overnight at -70°C for 1-3 days.

8.4. Results

Histopathology

No evidence of any infection or inflammation was detected by either histological means or cytokine analysis on day 5 post infection. The first histological signs of inflammation were on day 10 post infection, before the appearance of cysts in the brain. These mice had a mild meningitis with areas of encephalitis and widespread reactive astrocytes which had intensified by day 20. Microglial nodules were infrequent at day 10 but were prominent by day 15 post infection (Figure 8.1). Small numbers of cyst stages were first seen in the brain on day 15 and by day 20 cyst numbers had increased dramatically in number (Figure 8.2). The cysts were not always associated with inflammation. From day 20 post infection onwards cyst numbers remained high but as time progressed they became more varied in size with many large cysts present.

Detection of cytokine mRNA transcripts by PCR and slot blot-analysis

The use of PCR to assist in the amplification of total brain RNA allowed the characterisation of the cytokines being produced locally within the brains of infected animals (Figure 8.3). Transcripts for γ-Actin were detected in all of the uninfected (results not shown) and infected mice.

IL-4 was the first T cell associated cytokine detected, in one animal on day 5 post infection and thereafter in all mice on days 10 and 15 post infection with two mice still positive on day 20. IL-6 also appeared early in the course of the experiment on day 10 post infection. By day 15 post infection transcripts for TNF-α were present as were the T cell associated cytokines GM-CSF and IFN-γ. IL-4, IL-1α and IL-6 were also detected at this time. In those animals killed later, *i.e.* days 20 and 30 post infection, no transcripts for IL-4 or GM-CSF could be detected, although IL-1α, TNF-α, IL-6 and IFN-γ were present at all these time points in all these animals. Transcripts for IL-2 was only detected in the CNS of those mice killed on day 30 post infection. In uninfected mice, there was no histological signs of any CNS inflammation and only

transcripts for IL-1α were detected at similar levels to those mice killed on day 5 post infection (results not shown).

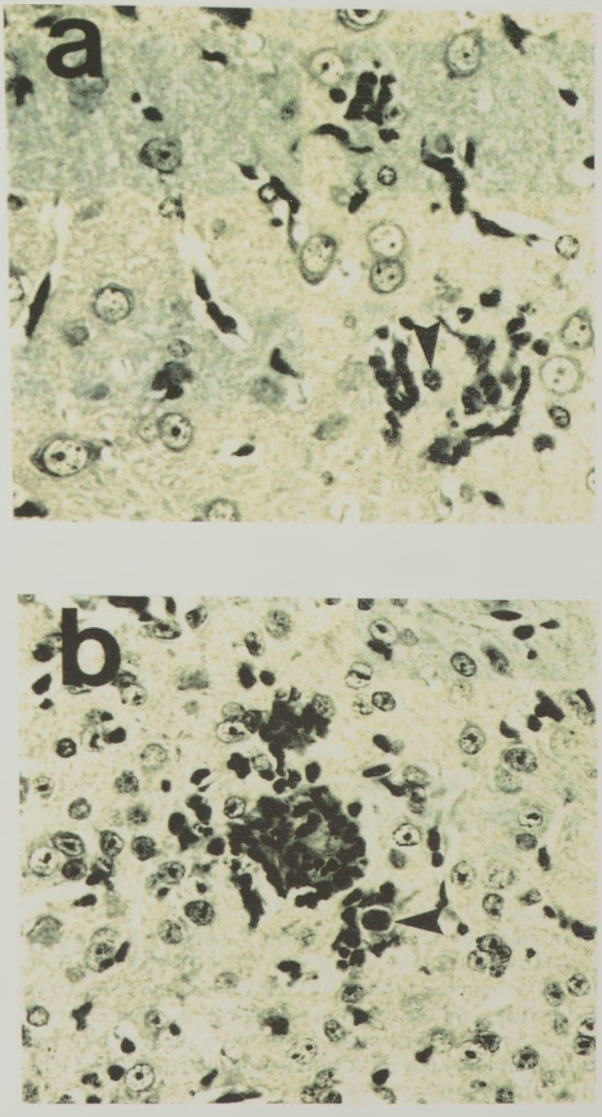

Figure 8.1. (a) Microglial response to parasites from mouse killed on day 15 post infection. The multiplying tachyzoites apparent (arrowed) with a few microglial cells evident. (b) Microglial nodule from a mouse killed on day 20 post infection. The intense microglial reaction in forming the nodule, presumably to multiplying parasites, is obvious with little reaction to the parasite cyst (arrowed) (magnification x 100).

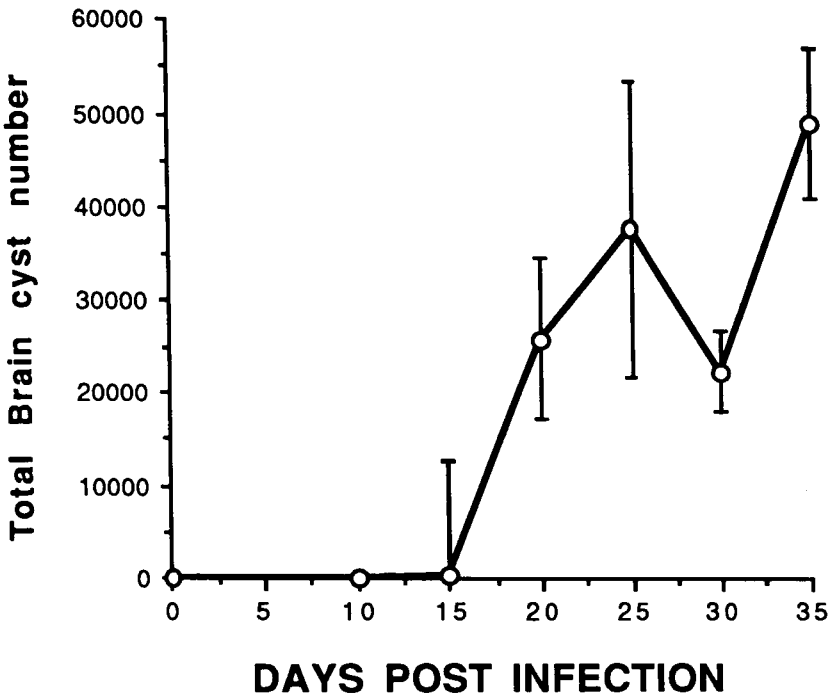

Figure 8.2. The total cyst counts in the brains of C57BL/10ScSn mice infected orally 5, 10, 15, 20, 25, 30, and 35 days beforehand with 10 tissue cysts.

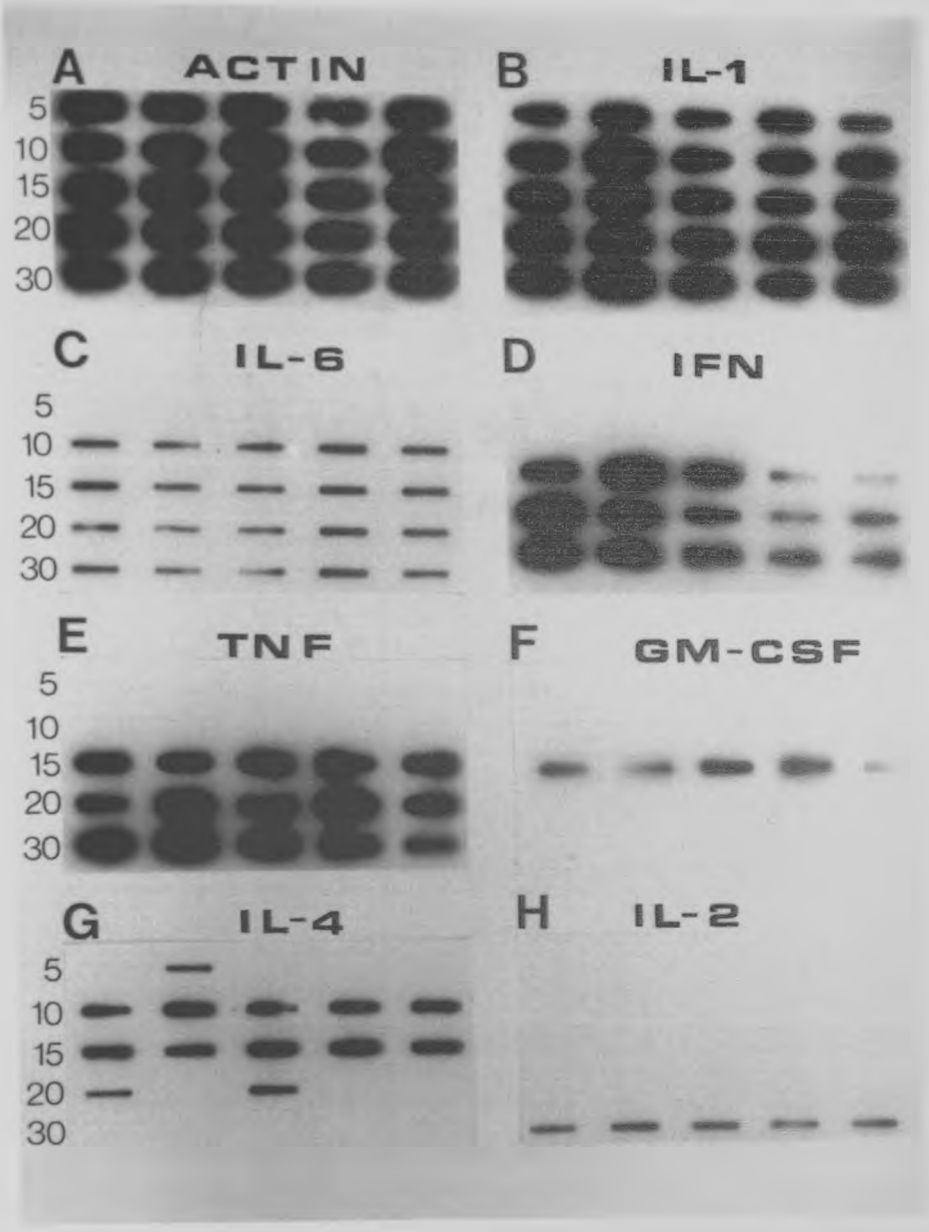

Figure 8.3. Slot-blot analysis of cytokine PCR mixes. Each mouse killed had PCR carried out for each of the above cytokines and the reaction was slot-blotted and probed as described in materials and methods, The same position on the slot-blot was used for each individual mouse. Cytokine profiles for days 5, 10, 15, 20, and 30 are shown.

8.5. Discussion

In *T. gondii* infected B10 mice, a variety of cytokines can be detected in the CNS as the infection progresses. These different cytokine profiles, demonstrated at different stages of infection may in part represent the evolution of T cell subsets in the pathological response associated with toxoplasmic encephalitis. Whereas some cytokines appeared transiently (IL-4, IL-2, GM-CSF), others were present throughout most of the infection (IL-1α, IL-6, TNF-α, IFN-γ). IL-4 is a product of the TH2 CD4+ subset which can also produce GM-CSF, while IL-2 and GM-CSF are particularly associated with the TH1 CD4+ cell subset (Mosmann, 1991). CD8+ T cells are also a possible source of these latter two cytokines. IFN-γ, on the other hand, may be produced by the CD4+ TH1 cell subset, CD8+ lymphocytes (Fong & Mosmann, 1990) or NK cells (Hauser & Tsai, 1986). Of the other cytokines detected, IL-6 and TNF-α appear early and persist throughout the infection. They are generally associated with activated macrophages and in the CNS, with microglial cells and astrocytes, (Fontana *et al.*, 1984; Robbins *et al.*, 1987; Frei *et al.*, 1989; Fontana *et al.*, 1987; Lieberman *et al.*, 1989). It is therefore, significant that astrocyte activation and microglial nodules are apparent at an early stage of infection.

Apart from IL-1α, which was expressed constitutively in all mice, IL-4 was the earliest cytokine detected in the brain and it occurred concurrently with the onset of inflammation, 10 days after infection. This cytokine is produced by the TH2 CD4+ T cell subset and is associated with antibody production and depression of classical cellular immunity (Mosmann & Moore, 1991). GM-CSF which can also be produced by this T cell subset and IFN-γ which is not (Mosmann & Moore, 1991), were the next T cell products detected at day 15 post infection. IFN-γ was probably produced by CD8+ lymphocytes rather than the CD4+ TH1 subset as IL-2 was not detected until 15 days after IFN-γ was first present. The apparent appearance of a CD4+ T cell population before that of CD8+ T cells was surprising as it is well established that CD8+ T cells bind preferentially to cerebral endothelium (Pryce*et al.*, 1991).

However, CD4+ T cell help may be needed for stimulation of CD8+ lymphocytes which would result in their products being detected latter in infection (Gazzinelli *et al.*, 1991).

The apparent presence of the TH2 and CD8+ T cell subsets does not result in any significant protection in these mice which are extremely susceptible to T. *gondii* infection and develop progressive fatal toxoplasmic encephalitis. Previous studies suggest that a specific CD8+ T cell response is primarily involved in protection against *T. gondii* (Chapter 6; Gazzinelli *et al.*, 1991; Parker *et al.*, 1991; Brown & McLeod, 1990; Subauste *et al.*, 1991; Khan *et al.*, 1991), although it appears that CD4+ T cells can influence infection (Chapter 6; Gazzinelli *et al.*, 1991; Parker *et al.*, 1991; Vollmer *et al.*, 1987; Israelski *et al.*, 1989; Araujo, 1991). In a vaccine study using the avirulent mutant (ts-4), CD4+ T cells of the TH1 subset have been shown to operate synergistically with CD8+ T cells in the early development of protective immunity (Gazzinelli *et al.*, 1991). Other studies indicated that depletion of CD4+ T cells using the monoclonal antibody GK 1.5 before infection exacerbated disease and increased eventual cyst number (Araujo, 1991), while similar treatment of chronically infected mice induced recrudescence of latent infection (Vollmer *et al.*, 1987). However, a further report contradicted the latter observation suggesting that CD4+ T cell depletion with this antibody reduced inflammation cyst numbers during chronic infections (Israelski *et al.*, 1989). These two observations, apparently irreconcilable at the time of publication may now be explained in light of a recent study that found that partial depletion of CD4+ T cells induced a predominately TH2 response (Field *et al.*, 1992). It is therefore possible that the differences in the above two studies could be explained if the efficacy of depletion also differed and these apparently contradictory results might reflect the different influences of each population.

The detection of IFN-γ mRNA transcripts in mice with progressive toxoplasmic encephalitis was surprising as this cytokine has been thought to be the major mediator

of resistance against *T. gondii* (Suzuki *et al.*, 1991). Current evidence would indicate that IFN-γ would prevent cyst rupture, but not induce cyst formation (Jones *et al.*, 1986) and the use of IFN-γ neutralising antibodies by other workers exacerbated the encephalitis associated with this disease (Suzuki *et al.*, 1991). However, IL-4, the first lymphocyte associated cytokine detected, has been demonstrated in other experimental systems to inhibit the effects of IFN-γ (Liew *et al.*, 1989). An IL-4-mediated inhibition of IFN-γ may explain the massive rise in cyst burden between days 15 and 20 post infection in these experiments. Alternatively the inability of IFN-γ to control infection in B10 mice could simply be a quantitative effect.

The detection of IL-2, a cytokine reported as promoting protective responses to T. *gondii* (Sharma *et al.*, 1985; Subauste & Remington, 1991), on day 30 of infection was also surprising as a previous study did not detect this cytokine on day 35 of infection (Chapter 7). The increased sensitivity of our assays may be responsible, but its presence does not prevent the onset of fatal infection. Although IL-2 is normally associated with TH1 cells it can also be produced by CD8+ cells (Fong & Mosmann, 1990) and has also been associated with virgin or memory CD4+ T cells (Mosmann & Moore, 1991). As GM-CSF, which is also a product of TH1 cells (Mosmann & Moore, 1991), is not detected on day 30, other T cell subsets would appear likely sources of IL-2.

The production and release of cytokines within the CNS may have several consequences. TNF-α and IL-1α can both initiate meningeal inflammation (Ramilo *et al.*, 1990; Saukkonen *et al.*, 1990; Waage *et al.*, 1989) and these results add further weight that they play an important role in CNS inflammatory processes. The TNF-α and IFN-γ transcripts detected can both induce MHC expression on a variety of glial cells including astrocytes (Benveniste *et al.*, 1990; Lavi *et al.*, 1988; Male *et al.*, 1987; Massa *et al.*, 1987). Whether the reactive astrocytes observed are involved in antigen presentation is unknown. However, astrocytes are able to prime CD8+ T cells but not CD4+ T cells although they can perpetuate the activation of CD4+ T cells (Sedgwick,

et al., 1991). TNF-α, IL-6 and IFN-γ can also act alone or synergistically to induce adhesion molecules on astrocytes, as well as endothelial cells (Satoh *et al.*, 1991; Frohman *et al.*, 1989; Mantovani & Dejanae, 1989; Pober *et al.*, 1983), an important step in allowing inflammatory cells to access the CNS. Several of these cytokines are also possible candidates for inducing the astrogliosis observed. TNF-α in particular may act as an autocrine regulator of astrocyte function (Bethea *et al.*, 1990). These cytokines may also have a paracrine effect if produced by inflammatory cells or microglial cells. Other consequences of cytokines being produced within the CNS include the direct toxic effects TNF-α which can cause lysis of oligodendrocytes and myelin damage (Selmaj *et al.*, 1988), while IL-6 induced nerve growth factor secretion by astrocytes (Frei *et al.*, 1989) may be involved in tissue repair processes. Alternatively, IL-6 in its capacity as a B cell-stimulating cytokine may be involved in intracerebral antibody synthesis, a phenomenon which has been shown to occur in toxoplasmosis (Potasman *et al.*, 1988).

These experiments detail the cytokine events in the brain of highly susceptible *T. gondii* infected B10 mice. The indication is that the early response in the CNS is of the TH2 subset. This fails to control parasite growth and so allows the development of the characteristic pathology associated with toxoplasmic encephalitis.

CHAPTER 9

Studies on a murine model of congenital toxoplasmosis: vertical disease transmission only occurs in BALB/c mice infected for the first time during pregnancy

9.1. Abstract

The incidence of congenital toxoplasmosis was determined by an ELISA in the litters of BALB/c mice which had been infected 8 weeks before mating, on day 12 of pregnancy, or on both these occasions. Of those mice given the infection for the first time on day 12 of pregnancy, 5 out of 6 gave birth to infected litters with approximately 50% of the individuals in each litter being infected. BALB/c mice which had been infected 8 weeks before mating did not give birth to infected litters even if they were re-infected on day 12 of pregnancy. Following infection BALB/c mice were found to harbour significantly fewer tissue cysts than the congenic H-2 derivative BALB/K strain. However, chronically infected BALB/K mice also failed to produce infected litters indicating that tissue cyst burden in the dam did not influence congenital infection at least on the BALB background. This study demonstrates that BALB/c dams chronically infected with *T. gondii* have immunity capable of protecting their foetuses from congenital infection, even if the dams are re-infected during pregnancy. Our results demonstrate that the BALB/c mouse can be used as a model of human or ovine congenital *T. gondii* infection suitable for testing putative vaccines.

9.2. Introduction

The development of a vaccine which protected the adult and consequently reduced the incidence of congenital toxoplasmosis would be highly desirable. Such a vaccine may prevent vertical disease transmission without necessarily inducing sterile immunity. For this reason the development of a suitable laboratory model of congenital toxoplasmosis is essential to test the efficacy of putative vaccines. Such a model system has been studied in this laboratory, using outbred mice and has proven useful in studying ophthalmological, behavioural and neurological sequelea (Hay *et al.*,. 1981, Hutchison *et al.*, 1982, Hay *et al.*,. 1985, McMenamin *et al.*, 1986 and Dutton *et al.*, 1986). However, the value of this model for immunological studies or vaccine design has never been assessed. Indeed to analyse, dissect and characterise the nature of protective immunity as well as guaranteeing reproducibility of results it is essential that the disease model should comprise the use of inbred mice. The suitability of a murine model of congenital infection could, however, be questioned due to the apparent differences in the disease transmission patterns between the mouse and humans or ovids. The literature would indicate that while vertical transmission through successive generations is the normal situation in mice (Beverley 1959) it is not in either humans (Cook 1990) or ovids (Beverley & Watson 1971) where only infection for the first time during pregnancy results in congenital infection. These differences might suggest that mice would make poor substitutes for studying immunoprophylaxis of human or ovine congenital disease. The following study was therefore undertaken to determine whether chronically infected *T. gondii* BALB/c mice produced healthy, disease free litters and whether a prior infection decreased the likelihood of congenital infection following a second infection during pregnancy. The results would indicate the usefulness of this model for vaccine studies.

9.3. Materials and methods

Mice

Inbred BALB/c, BALB/K and C57BL/10 mice were bred and maintained in this laboratory under conventional conditions. Mice were used when eight to ten weeks old unless otherwise stated.

Infections

Cysts of the RRA (Beverley) strain of *T. gondii* were harvested and counted as described in Chapter 2. Mice were infected orally with 5 cysts either 8 weeks prior to mating, or with 20 cysts on day 12 of pregnancy or on both these occasions with 5 and then 20 cysts respectively. Other mice were not inoculated and acted as *prima gravid* age-matched controls. The experimental protocol is summarised in Table 9.1. Infections were confirmed in all cases by the detection of anti-*T. gondii* IgG in serum samples taken 8 weeks after administration of infective brain homogenate.

Mating

Virgin female mice, uninfected or with an 8 week chronic infection were housed 3 to a cage with 1 male. Mice were inspected daily for the presence of vaginal plugs and the day of discovery designated day 0. As soon as possible after birth, litters were fostered to uninfected lactating dams, to avoid possible infection through lactation.

Detection of Congenital Infection

(i) Enzyme-Linked Immunosorbant Assay (ELISA) :

Serum samples were obtained by bleeding the offspring *via* their tail vein when they were 8 weeks old. Plasma was separated by centrifugation and used at 1 in 2000 dilution in the ELISA test as described in Chapter 2. Optical densities of >3x the negative control were taken as positive, although positives were generally >10x the control value.

(ii) Sub-inoculation into C57BL/10 mice

Offspring were sacrificed when they were 9 weeks old and their brains were placed in 1ml of 0.9% saline and passed 6 times through a number 21 gauge needle. C57BL/10 mice were injected intraperitoneally with 0.2 ml of brain suspension. Four weeks later the C57BL/10 mice were sacrificed and thirty microlitres of brain suspension examined microscopically for the presence of tissue cysts as described previously.

Table 9.1. Experimental Protocol

	Treatment				
	Week -8	Day 0	Day 12	Week 3	Week 11
Group 1	5 Cysts orally	Mate	____	Foster pups	Bleed pups
Group 2	____	Mate	20 Cysts orally	Foster pups	Bleed pups
Group 3	5 Cysts orally	Mate	20 Cysts orally	Foster pups	Bleed Pups
Group 4	____	Mate	____	Foster Pups	Bleed pups

9.4. Results

The incidence of congenital toxoplasmosis in the various experimental groups is summarised in Figure 9.1 and Table 9.2. An infected individual was one that had a significant specific *T. gondii* antibody response as determined by ELISA 8 weeks after birth (Figure 9.1). As clearly demonstrated there was no ambiguity in the specific antibody levels between infected and non-infected individuals. Disease positive individuals displayed an extremely high absorbance value compared with non-infected mice. Of 9 BALB/c mice infected with 5 cysts 8 weeks prior to mating, none of their offspring were found to be infected. Five of 6 litters from BALB/c mice infected for the first time on day 12 of pregnancy did have infected individuals. Of a total of 28 surviving pups from this group, 14 were infected. One pup in this group had bilateral cataracts 8 weeks after parturition. No congenital infections were detected however in the 39 pups born to the 9 BALB/c dams which had been infected with *T. gondii* 8 weeks prior to mating and reinfected on day 12 of pregnancy.

All of the mice with elevated anti-*T. gondii* specific IgG levels were found to have cysts in their brains at death and brain homogenates from these mice were also infective to naive recipients. Cysts were totally absent from the brains of antibody negative individuals and brain homogenates from antibody negative pups also failed to infect *T. gondii* susceptible C57BL/10 mice. This clearly demonstrates that the presence of *T. gondii* specific IgG is a reliable indicator of infection.

The mean number of pups in each litter during these experiments was significantly smaller than those we have come to expect from normal breeding stock BALB/c mice (Table 9.2). However, the number of offspring in litters born to mice with a *T. gondii* infection, either chronic or acute, was not significantly different from age-matched *prima gravid* control mice. Due to rejection after fostering, neonatal death was high (14.3%) in all litters born to dams infected with *T. gondii* at any time before or during pregnancy.

Chronically infected BALB/K mice have significantly more cysts in their brains (3120±766) than BALB/c mice (193±58) infected for a similar time period (8 weeks). Nevertheless litters of seven BALB/K mice (34 pups in total) infected 8 weeks before pregnancy with *T. gondii* were found to be completely free of infection as measured both by ELISA and inoculation of brain tissue into susceptible C57BL/10 mice. Post-mortem examination of the brain of the BALB/K mothers demonstrated heavy tissue cyst burdens. Approximately 50% of the offspring of BALB/K mice infected for the first time on day 12 of pregnancy were infected. The results with this strain are therefore similar to those we have obtained using BALB/c mice and vertical disease transmission is limited to infection during pregnancy.

In these experiments BALB/c mice were infected on day 12 after mating. In previous experiments when 10 BALB/c mice were infected orally with 20 tissue cysts 7 days after mating, 5 mothers died before giving birth, 3 did not litter and were found on post-mortem examination to have resorbed their embryos and 2 litters were aborted. Of 10 mice infected on day 12 after mating all littered, most pups survived and approximately 50% were infected as determined by ELISA, a result similar to that described above.

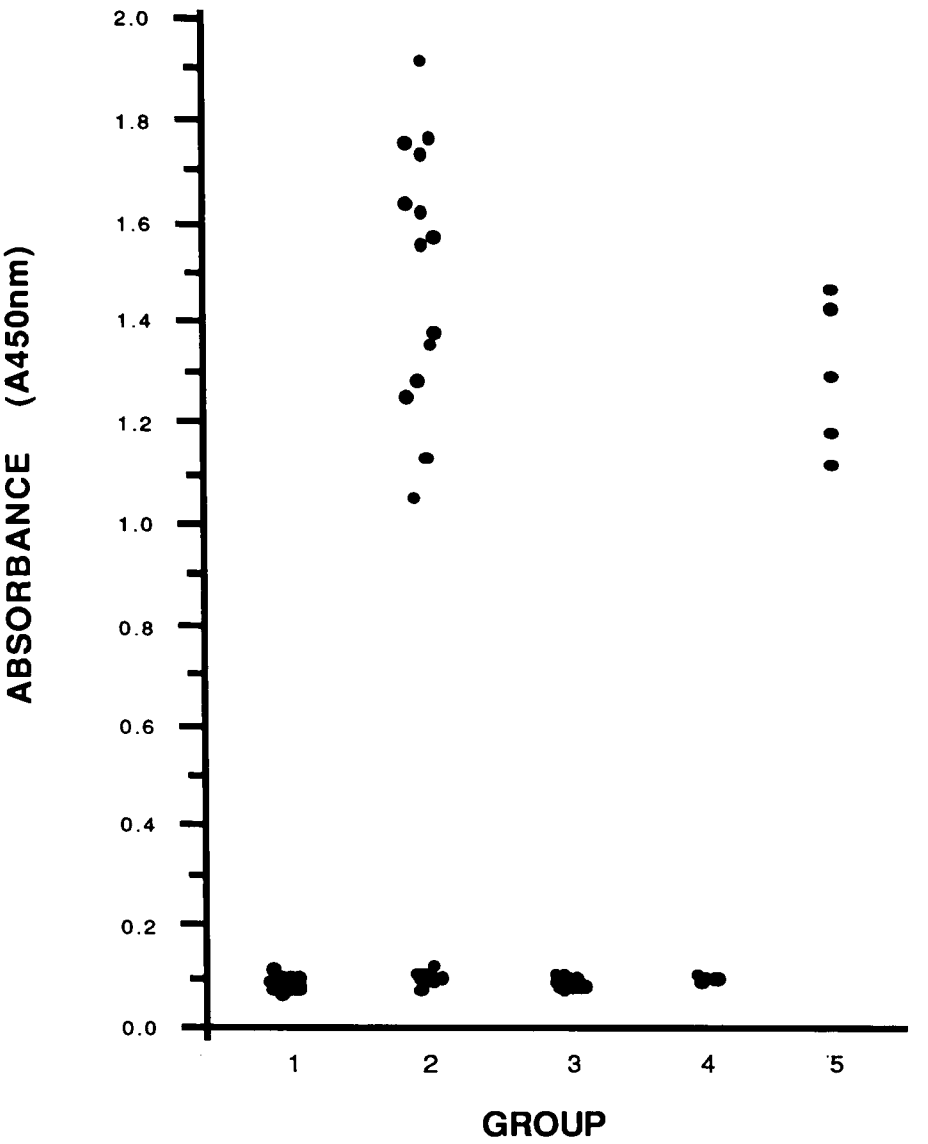

Figure 9.1. Absorbance at 450nm of plasma samples tested by ELISA, from the offspring of BALB/c dams infected, either 8 weeks prior to mating (1), on day 12 of pregnancy (2), or 8 weeks prior to mating and on day 12 of pregnancy (3). Normal uninfected adult BALB/c mice (4) and BALB/c mice infected as adults (5).

Table 9.2. The incidence of congenital toxoplasmosis infection and mean number of pups in litters of BALB/c mice.

	Time of infection of the dams	No. of infected litters/total litters	No. of infected pups/total pups born	Mean no. of pups/litter
Group 1	8 weeks before mating*	0/9	0/50	5.5±0.4
Group 2	Day 12 of pregnancy§	5/6	14/36	6.0±0.4
Group 3	8 weeks before mating* and on day 12 of pregnancy§	0/9	0/47	5.2±0.7
Group 4	Age matched uninfected controls	0/10	0/40	4.0±0.63
Group 5	Stock BALB/c uninfected	0/99	0/836	8.4±0.25

* 5 cysts inoculated orally

§20 cysts inoculated orally

9.5. Discussion

This study clearly demonstrates that chronically infected BALB/c mice do not allow vertical disease transmission and congenital infection in these mice only occurs if the mother is infected for the first time during pregnancy. Therefore, vertical disease transmission is limited to one generation in this mouse strain. This finding contrasts with numerous other studies on congenital toxoplasmosis using rats (Wildfuhr, 1954), mice (Beverly, 1959), rats and mice (Remington, Jacobs & Melton 1961) and mice and hamsters (De Roever Bonnet 1969), where vertical transmission has been demonstrated in chronically infected animals from generation to generation. These early studies used outbred animals, or in the case of Remington *et al.*, (1961) NIH mice. However, Dubey and Shen, (1991), recently demonstrated that chronically infected Sprague-Dawley rats do not transmit the disease vertically, although this study did not ascertain if these rats had sufficient immunity to prevent vertical disease transmission on reinfection during pregnancy. Our observations are based primarily on the BALB/c inbred mouse and its H-2 congenic, BALB/K, derivative.

Using the laboratory model originally described by Hay *et al.*, (1981) infection of outbred mice with *T. gondii* on day 12 of pregnancy is the most effective time at which to produce infected pups. We have confirmed these results using inbred BALB/c mice and our additional observations indicate that infection earlier in pregnancy results in resorption or abortion, while infection later markedly reduces the incidence of congenital infection. As previous reports indicated that *T. gondii* can be transmitted *via* lactation (Eichenwald, 1948), litters are routinely fostered onto lactating dams, while pups were not tested for *T. gondii* specific IgG until 8 weeks of age in order to insure that maternal antibody was not measured and false positives obtained. It has recently been demonstrated that the offspring of chronically infected mice show a delayed antibody response to *T. gondii* challenge infection which can last for as long as 8 weeks (Suzuki & Kobayashi, 1990). Maternal antibody can persist for a surprisingly long time (up to 8 wks) and suppresses the onset of a specific immune response in the pups during this period. Nevertheless, positive anti-*T. gondii*

ELISA at 8 weeks of age in pups in our experiments show 100% correlation with *T. gondii* infection as detected by sub-inoculation of brain tissue into naive mice and microscopical examination.

It is well documented that different mouse strains exhibit different levels of resistance to *T. gondii* infection (Williams, Grumet and Remington, 1978; Johnson, 1984; Jones and Erb, 1985; McLeod *et al.*, 1989b). BALB/c mice infected intraperitoneally with the Pe strain are relatively resistant and harbour few cysts in their brain, contrasting with C57BL/10 mice which harbour many (Jones and Erb, 1985). In this laboratory we have observed a similar pattern when infecting orally with the RRA strain; BALB/c mice have moderate mortality and harbour few cysts, whereas C57BL/10 mice have many cysts accompanied with severe wasting and eventual death (Chapter 3). Given the stark differences in the disease pattern of non-congenitally acquired toxoplasmosis between different inbred strains of mice, it would be surprising if there was not also a difference in the transmission of congenital disease. Intuitively it would be anticipated that those mice harbouring large numbers of cysts would be more likely to allow vertical disease transmission than those harbouring few cysts. However, no congenital transmission was detected in the litters of chronically infected BALB/K mice, although the mothers themselves were found to have extremely high cyst counts.

The number of offspring born in a litter to all *T. gondii* infected dams did not differ significantly from non-infected age matched *prima gravid* mice but, was significantly reduced compared with stock BALB/c mice from our breeding colony. Stock BALB/c mice are mated for the first time when 8-10 weeks old, whereas the experimental and control groups were mated when 16-18 weeks old. Fertility obviously drops dramatically in this mouse strain with age.

Previous studies have also indicated that a degree of immunity can be conferred in a mouse model of congenital *T. gondii* infection (McLeod *et al.*, 1988). In this case immunisation was achieved by inoculating a temperature sensitive mutant intra-intestinally into Swiss mice before pregnancy. The incidence of infection in litters

born to mice treated in this way was 64%, whereas 94% of litters born to non-immunised animals were infected. In contrast we find that BALB/c mice infected before pregnancy with the cyst forming RRA strain, are resistant to such a degree that none of their offspring become infected. The advantages of the disease model reported in this study are self-evident.

Finally, except in the case of immunodepressed mothers (Desmonts *et al.*, 1990) it has been the general consensus that congenital infection in humans or sheep only occurs if the mother acquires the infection for the first time during pregnancy. This indicates that a vaccine generating sufficient protective immunity could prevent congenital infection. Our results indicate that the BALB/c mouse can be used as a model of human or ovine congenital toxoplasmosis suitable for testing new vaccines and chemotherapeutic agents as well as identifying those elements of the immune system promoting disease resistance.

CHAPTER 10

Congenital toxoplasmosis in the BALB/c mouse: prevention of congenital infection and foetal death by vaccination.

Abstract

The vaccine potential of STAg (Soluble Tachyzoite Antigen) formulated in FCA or NISV was assessed by their ability to reduce cyst burdens in subsequently infected mice. Mice inoculated s.c. with STAg, STAg with NISV or STAg incorporated in NISV at 2 x 2 week intervals before infection, had significantly fewer cysts at necropsy compared with control mice receiving PBS. However, mice receiving STAg incorporated in NISV were found to have significantly fewer cysts in their brains at necropsy than all other vaccinated groups ($p<0.05$). No significant reduction in brain cyst burdens was found in mice receiving STAg emulsified in FCA compared with control mice receiving PBS, and indeed mice receiving PBS emulsified in FCA alone were found to harbour significantly more cysts in their brains at necropsy ($p<0.01$). The litters from dams vaccinated with STAg incorporated in NISV and infected on day 12 of pregnancy were completely protected from foetal death and had a reduced incidence of congenital infection (12%) compared with litters of control dams which suffered not only 50% foetal death, but 50% of the surviving offspring were infected. Mice vaccinated with STAg incorporated in NISV were found to have enhanced T cell proliferative responses over all the other groups tested while mice vaccinated with STAg emulsified in FCA had decreased T cell proliferative responses compared with mice vaccinated with STAg in PBS. IFN-γ concentrations measured in the supernatants of the T cell proliferation assays by ELISA were significantly higher in mice vaccinated with the STAg/NISV preparation compared with mice vaccinated with the FCA/STAg preparation ($p<0.025$). These results demonstrated that a degree of protection against adult acquired disease can be induced by immunisation with STAg and this protection can be enhanced by incorporating STAg into NISV. Immunisation in this fashion which is associated with high T cell proliferative responses and the enhanced production of IFN-γ, confers significant immunity against congenital disease and foetal death .

Introduction

Infection of humans with *T. gondii* for the first time during a pregnancy can result in foetal infection or abortion although individuals infected before pregnancy have sufficient immunity to prevent foetal death or infection (Cook, 1990). Similarly, in sheep, an experimental or natural infection before mating is effective in preventing foetal infection and abortion even if challenged during pregnancy (Beverley & Watson, 1971). A medical or veterinary vaccine against *T. gondii* would ideally give a similar degree of protection. In recent years a live attenuated vaccine has been licensed for use in veterinary animals and is capable of reducing the incidence of foetal death in vaccinated ewes (Buxton *et al.*, 1991). Unfortunately, this vaccine does not prevent congenital disease transmission and its use may ironically result in increased numbers of infected lambs and consequently a greater public health hazard. In addition, live attenuated vaccines such as this are unlikely to be licensed for medical use and remains far from ideal in a veterinary context. A safe defined subunit vaccine must therefore be sought.

Although, recent technical advances have facilitated the identification and purification of potentially immunoprotective components of pathogens, these components usually lack immunogenicity (Bomford, 1989). The inclusion of an adjuvant may circumvent this problem, but past experience illustrates that great care must be taken in the choice of adjuvant if the vaccine is to be successful. For example, studies of synthetic peptides derived from the primary sequence of the major surface protein of *Leishmania* (gp63) have demonstrated that an otherwise exacerbative peptide could be rendered protective when prepared in an adjuvant (Jardim *et al.*, 1990). Similarly, a major surface protein from *T. gondii* (p30) has been shown to be capable of causing disease exacerbation when formulated in FCA (Kasper *et al.*, 1985) but is protective when encapsulated in liposomes (Bulow & Boothroyd, 1991). As new vaccine technologies have progressed, the importance of selecting appropriate and developing new safe adjuvants has been highlighted.

Despite the vast array of adjuvant systems currently under development (liposomes, NISV(niosomes), ISCOMS and pluronics), aluminium hydroxide is the only adjuvant currently licensed for use in humans (Glenny *et al.*, 1926). Unfortunately, aluminium hydroxide has been demonstrated not to promote cell-mediated immunity (Bomford, 1980; Grun and Maurer, 1989). Consequently, the development of effective vaccines against intracellular pathogens such as *T. gondii*, requiring a cell mediated immune response (reviewed in Chapter 1) has been hampered by the inadequacies of available adjuvants.

Recently, Brewer & Alexander (1992) demonstrated that non-ionic surfactant vesicles (NISV) greatly enhance the immunogenicity of entrapped antigens. In addition, NISV have been shown to be at least as efficient as Freunds Complete Adjuvant (FCA) in stimulating the production of IgG2a specific antibody in the absence of any of the toxicity problems associated with FCA (Brewer & Alexander, 1992). The production of the IgG2a antibody subclass has been associated with the activation of the Th1 subset of T lymphocytes and the production of IFN-γ (Snapper & Paul, 1987). As the protective immune response against *T. gondii* has been shown to be dependent on IFN-γ production (reveiwed, Chapter 1: Subauste & Remington, 1991), this adjuvant system may prove ideal for an anti-*T. gondii* vaccine.

In addition, NISV are composed of chemically defined compounds which are stable in air atmospheres (Handjani-vila *et al.*, 1979; Kiwada, 1985) and unlike liposomes the compounds used in their formation are totally synthetic and do not resemble biological material.

In pursuit of a vaccine against toxoplasmosis different workers have studied many antigen fractions including the excretory/secretory antigens (Duquesne *et al.*, 1991), the membrane antigen p30 (Kasper *et al.*, 1985; Khan *et al.*, 1991; Bulow & Boothroyd, 1991) and the soluble antigens derived from the tachyzoite (Alexander *et al.*, 1993). In this study we examine the capability the soluble antigen fraction (STAg)

emulsified with FCA or entrapped in NISV to protect mice from both post-natally acquired *T. gondii* infection and congenital disease.

Materials and Methods

Mice

BALB/c and BALB/K mice were bred and maintained in this laboratory under conventional conditions and used when 8-12 weeks old.

NISV formulation and entrapment of antigen

Non-ionic surfactant vesicles were formed by the method of Brewer and Alexander (1992). A mixture of the non-ionic surfactant, 1-mono palmitoyl glycerol, Cholesterol and Dicetyl phosphate (Sigma, Poole, Dorset, UK.) was mixed and melted slowly. Vesicles were formed by the addition of PBS (pH 7.4), and immediately vortexing for 1 minute. STAg was incorporated into preformed vesicles by the dehydration rehydration method (Kirby & Gregoriadis, 1984). After dehydration and rehydration the vesicles were washed 3 times at 100 000 x g and the entrapped protein concentration estimated by the method of Bradford (1976), after vesicle lysis with propanol.

Vaccination protocol

Mice were inoculated subcutaneously with 50ug of antigen 2 weeks and 4 weeks before infection with *T. gondii*. Antigen was either in a free form, emulsified in FCA or entrapped in NISV. Control mice were inoculated with PBS.

Assessment of cyst numbers in the brains of mice

The number of cysts in the brains of mice was assessed as described in Chapter 2.

Detection of congenital infection

The incidence of congenital infection in the litters of BALB/c mice was assessed by ELISA as described in Chapter 2 and 9.

T cell proliferation Assays and determination of IFN-γ production

The spleens from BALB/c mice injected subcutaneously 1 week previously with 0.2ml of PBS or 50 ug STAg in PBS, entrapped within NISV or emulsified in FCA were removed aseptically and placed in RPMI 1640 supplemented with 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.05 mM β-mercaptoethanol (Gibco, Paisley, U.K.) . Cell suspensions were prepared by gently teasing the spleens apart with forceps after which the resulting cell suspensions were centrifuged at 200 x g for 5 min. Erythrocytes were removed by resuspending the pellet in 3ml Boyles Solution (0.17M Tris, 0.16M Ammonium chloride; BDH Ltd., Dorset, UK) and incubating at 37°C for 3 min. After 2 washes in RPMI medium, viable cells as detected by trypan blue exclusion were counted and cell suspensions adjusted to $5x10^6$/ml in RPMI 1640 supplemented as above and containing 10% Controlled Process Serum Replacement-2 (CPSR-2; Sigma, Dorset, U.K.). 100μl aliquots containing $5x10^5$ cells were added to the wells of 96 well flat bottomed tissue culture plates (Costar, Cambridge, M.A.) which contained 100μl per well of STAg at concentrations of 100, 33, 11, 3.7 and 0 μg/ml or Con A (5ug/ml) in triplicate. These plates containing the cells were incubated for 60 hrs at 37°C, 5%CO_2 after which 150ul of supernatant from each well was removed (and stored at -70°C for IFN-γ assay) and replaced with 150ug of complete medium containing the appropriate amount of STAg or Con A. At this time, 0.25uCi tritiated thymidine (specific activity 35Ci/mmol; ICN/Flow, High Wycombe, U.K.) was added to each well. After a further 12 hrs incubation at 37°C the cells were harvested onto filter paper (ICN/Flow, High Wycombe, UK.) using a cell harvester (Skatronas, Lier, Norway). Thymidine incorporation was measured by liquid scintillation on a β-counter (Pharmacia LKB Biotech, Milton keynes, U.K.) using 1ml of Optiscint (Pharmacia Biosystems, Milton Keynes, U.K.) added to the filter discs in vials (Hughes & Hughes, Somerset, U.K.) and counted for 5 each.

IFN-γ assay

IFN-γ production by stimulated and non-stimulated cells from the various groups of

immunised mice was measured by an ELISA (TechGen International, London, U.K.). The culture supernatants were assayed in duplicate diluted 1/15 in PBS containing sodium azide (pH 7.4) as described below. 150µl samples of supernatants and standards (0-7000 pg/ml IFN-γ) were added in duplicate to wells of a microtitre plate precoated with monoclonal anti-IFN-γ and incubated at 37°C for 60 minutes. After washing three times with PBS/Tween (pH7.4, 0.05% Tween 20), 100µl of detector complex prepared by incubating biotinylated anti-IFN-γ with streptavidin linked alkaline phosphatase, was added to each well. After incubation for 60 minutes at 37°C, the wells were washed as above and 100µl of paranitrophenyl-phosphatase prepared in diethanolamine (pH 10.1) added to each well. After a further 30 minute incubation at 37°C absorbances were measured at 405nm on a Titertek Multiskan plate reader (Flow Laboratories, Irvine, Ayrshire, U.K.).

IFN-γ concentrations in the diluted samples were determined from the standard curve (regression coefficient, r = 0.985).

Table 10.1. Experimental Protocol

	Treatment				
	Weeks -4 & -2	Day 0	Day 12	Week 3	Week 15
Group 1	STAg/NISV (s.c.)	Mate	20 Cysts orally	Foster pups	Bleed pups
Group 2	PBS (s.c.)	Mate	20 Cysts orally	Foster pups	Bleed pups

Results

The efficacy of STAg emulsified in FCA as a vaccine as measured by prevention of brain cyst formation.

Vaccinating mice with STAg emulsified in FCA did not result in lower cyst burdens in the brains of mice, 32 days post infection. However, mice given FCA without STAg had significantly ($p<0.01$) increased cyst burdens in their brains compared with control mice (Figure 10.1a).

The efficacy of STAg entrapped in NISV as a vaccine as measured by prevention of brain cyst formation and reduced weight loss

Mice receiving STAg, STAg entrapped in NISV or STAg mixed with NISV were found to harbour significantly less cysts at necropsy than control mice receiving PBS. However, vaccination with STAg entrapped within NISV produced significantly ($P<0.05$) reduced cyst numbers compared with all other groups (Figure 10.1b). Control mice receiving PBS only, lost over 10% of their body weight over the first 21 days although immunisation with STAg in PBS or STAg entrapped in NISV could reduce weight loss considerably over the same time period. Surprisingly, mice receiving NISV without STAg also had showed reduced weight loss compared with mice receiving PBS. Mice receiving STAg entrapped in NISV were the only mice to show an overall increase in body weight over the period of study (Figure 10.2)

The efficacy of vaccination with STAg entrapped in NISV in the prevention of congenital infection

The incidence of congenital infection in the litters of BALB/c mice vaccinated 2 and 4 weeks prior to mating with STAg entrapped in NISV and subsequently infected with 20 cysts RRA was assessed by ELISA and compared with non-vaccinated controls (Figure 10.3). Vaccination of mice resulted in greatly enhanced antibody production as measured by ELISA, in serum samples taken at necropsy 8 weeks after giving birth (Figure 10.4a). In addition to this vaccinated dams had significantly fewer cysts in their brains than non vaccinated control mice (Figure 10.4b.). The vaccinated dams,

(11 in total) gave birth to 67 mice all of which survived and only 9 were found to be infected. In comparison, the control dams (9 litters in total) gave birth to a total of 59 mice of which 30 were dead at or within 24 hours of birth. Of the 29 surviving mice in this group 15 were found to be infected by ELISA. (Figure 10.3 and Table 10.2).

Table 10.2. Summary of the fate of offspring born to vaccinated and control dams infected orally with 20 RRA strain *T. gondii* on day 12 of pregnancy.

	VACCINATED STAg/NISV	CONTROL
Litters born	11	9
Litters infected	6	8
Total number offspring	67	59
Total deaths	0	30
Total survivors	67	29
Total infected	9	15

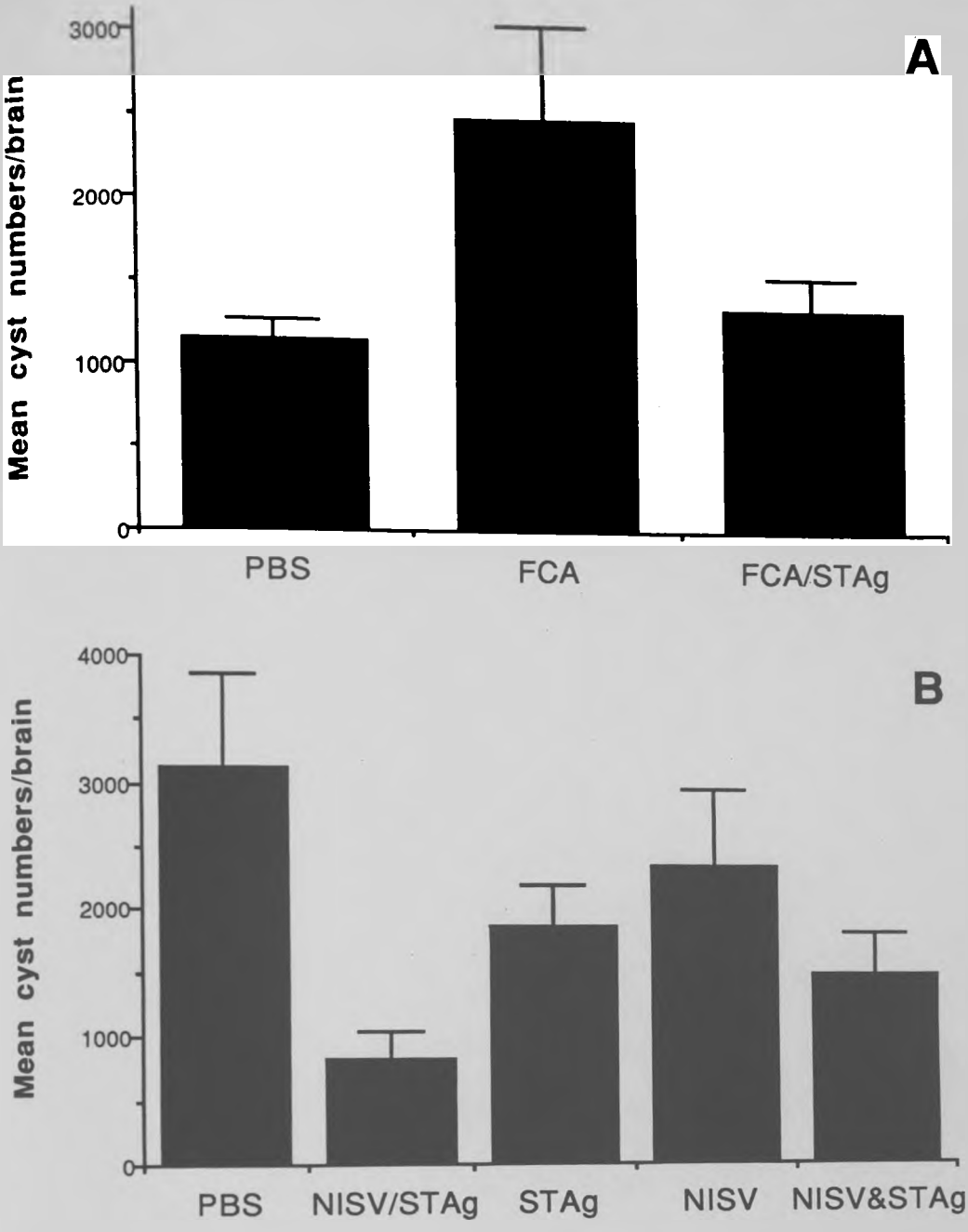

Figure 10.1. The mean number of cysts (± S.E.), 32 days post infection in the brains of BALB/K mice infected orally with 20 RRA cysts. Mice were inoculated subcutaneously with PBS, FCA or 50ug STAg in FCA (Figure 1a) or PBS, empty NISV, STAg in PBS (50ug), empty NISV mixed with STAg (NISV&STAg; 50ug), or STAg entrapped in NISV (STAg/NISV; 50ug) (Figure1b), 2 weeks and 4 weeks before infection.

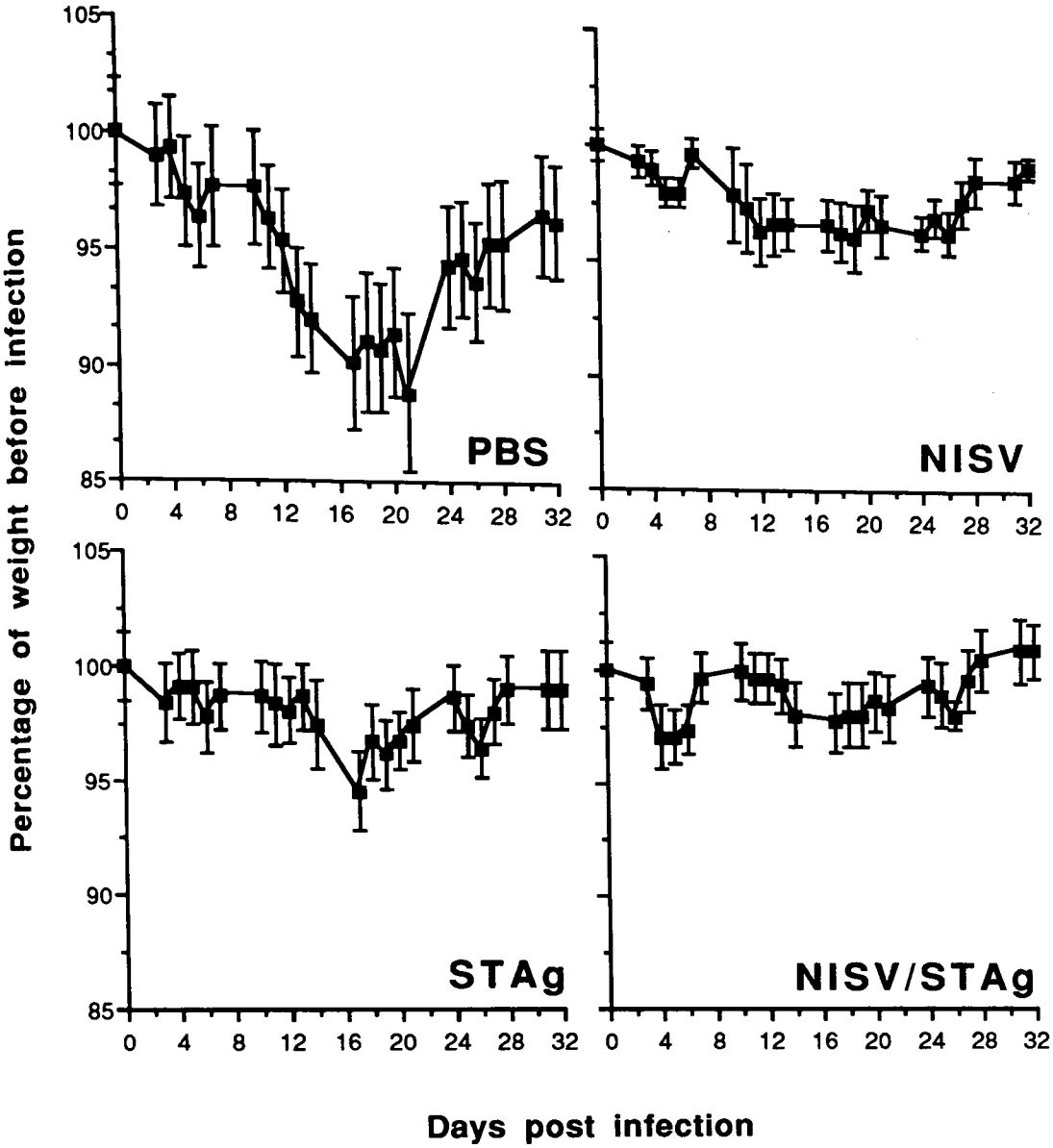

Figure 10.2. Effect of vaccination on reduction in mean weight due to infection with *T. gondii*.. Mice were inoculated with PBS, NISV or 50ug STAg in PBS or entrapped within NISV 2 weeks and 4 weeks before infection. Mean weights are expressed as percentages of the initial weight before infection (± S.E.).

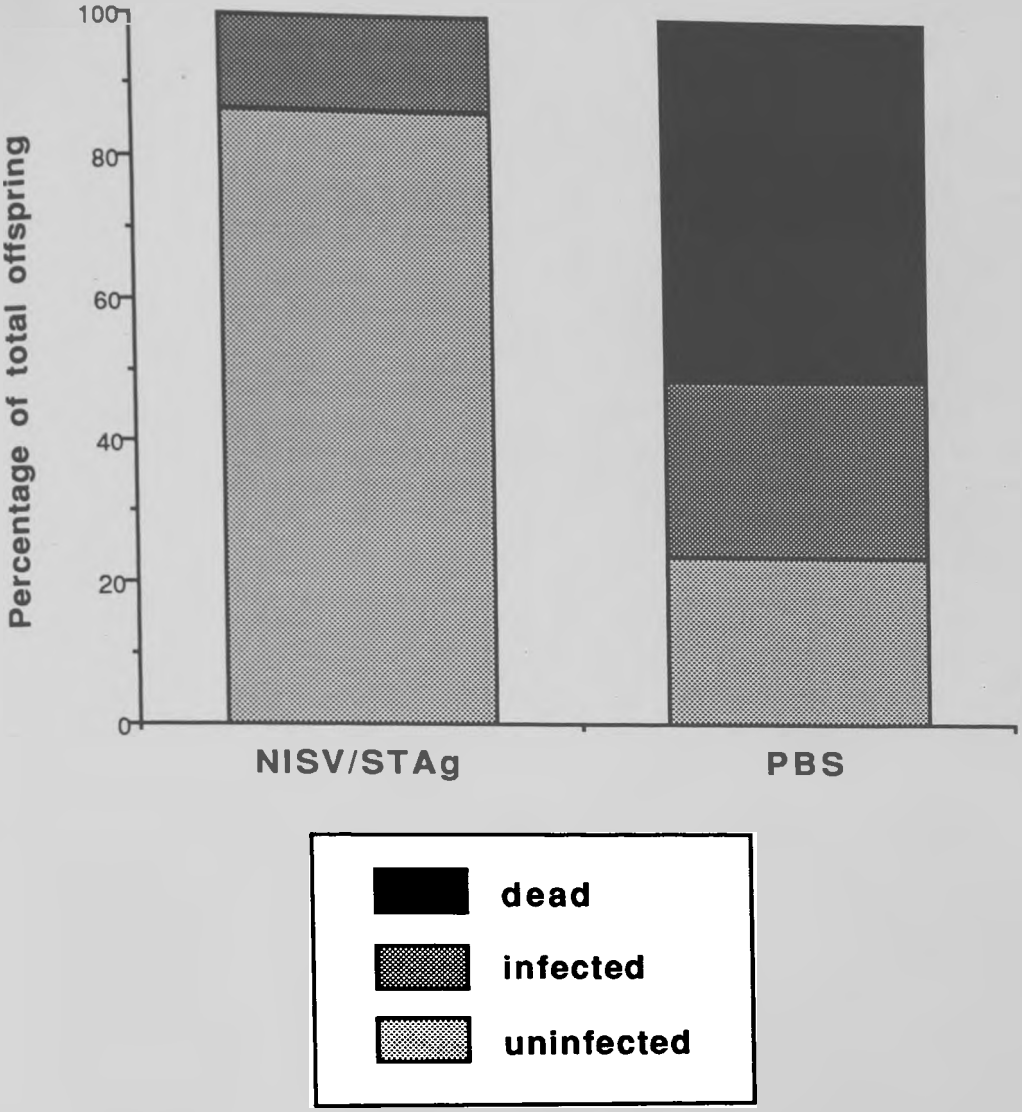

Figure 10.3. The effects of treatment with STAg encapsulated with NISV on the fates of the offspring of the BALB/c dams described in Figure 10.4. The percentage of surviving pups infected with *T. gondii* was determined eight weeks after birth by ELISA.

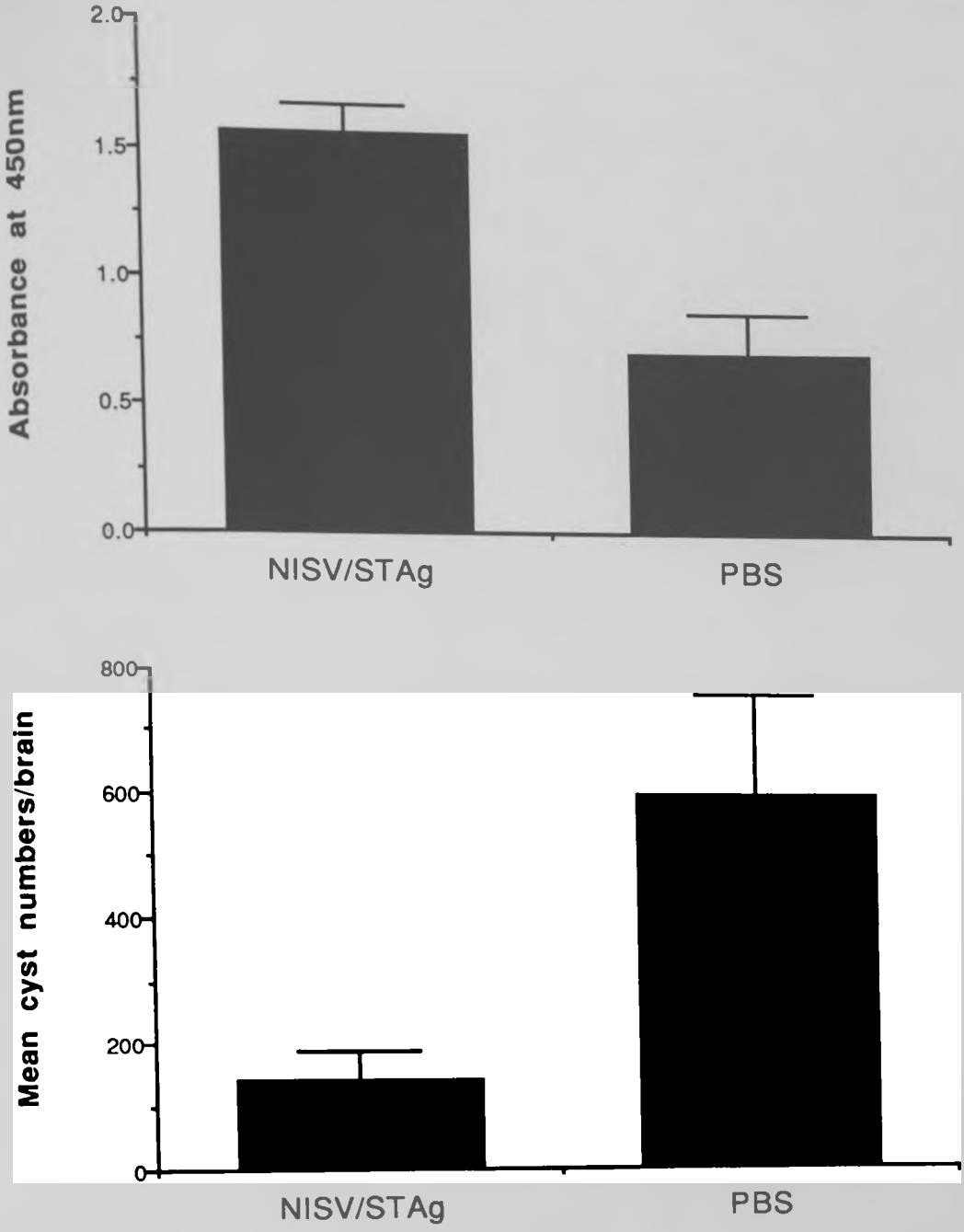

Figure 10.4. The mean antibody titres ± S.E. (Figure 4a) and the mean cyst numbers ± S.E. (Figure 4b) in the brains of BALB/c dams eight weeks post infection with 20 cysts orally. The mice were inoculated subcutaneously with either PBS or STAg entrapped within NISV two and four weeks before infection on day 11 or 12 of pregnancy.

T cell proliferation assays and IFN-γ assays

Spleen cells from mice vaccinated with STAg in PBS, STAg entrapped in NISV or STAg emulsified in FCA were tested for proliferative responses in an *in vitro* assay. (Figure 10.5). Spleen cells from all immunised groups of mice proliferated in response to *in vitro* stimulation with STAg. Immunisation with STAg entrapped in NISV induced higher mean proliferative responses (S.I.=8.11$\pm$0.79) than immunisation with STAg emulsified in FCA (S.I.=5.61$\pm$0.83), ($p<0.05$) or STAg in PBS (S.I. =6.50$\pm$1.11), ($p<0.075$). In fact, spleen cells from mice immunised with STAg emulsified in FCA had reduced proliferative responses compared with spleen cells from mice given STAg in PBS ($p<0.05$). Similarly, when the IFN-γ concentrations in the supernatant of these cultures were assayed, spleen cells derived from mice immunised with STAg entrapped in NISV produced more IFN-γ compared with those immunised with STAg in PBS ($p<0.075$) and those immunised with STAg emulsified in FCA ($p<0.025$).

Incubation of spleen cells from naive donors with 100ug/ml STAg *in vitro,* did not induce significant proliferation over cells cultured in the absence of STAg (Figure 10.6). However, analysis of the culture supernatants demonstrated significant IFN-γ production by spleen cells from naive donors incubated with STAg (Figure 10.6).

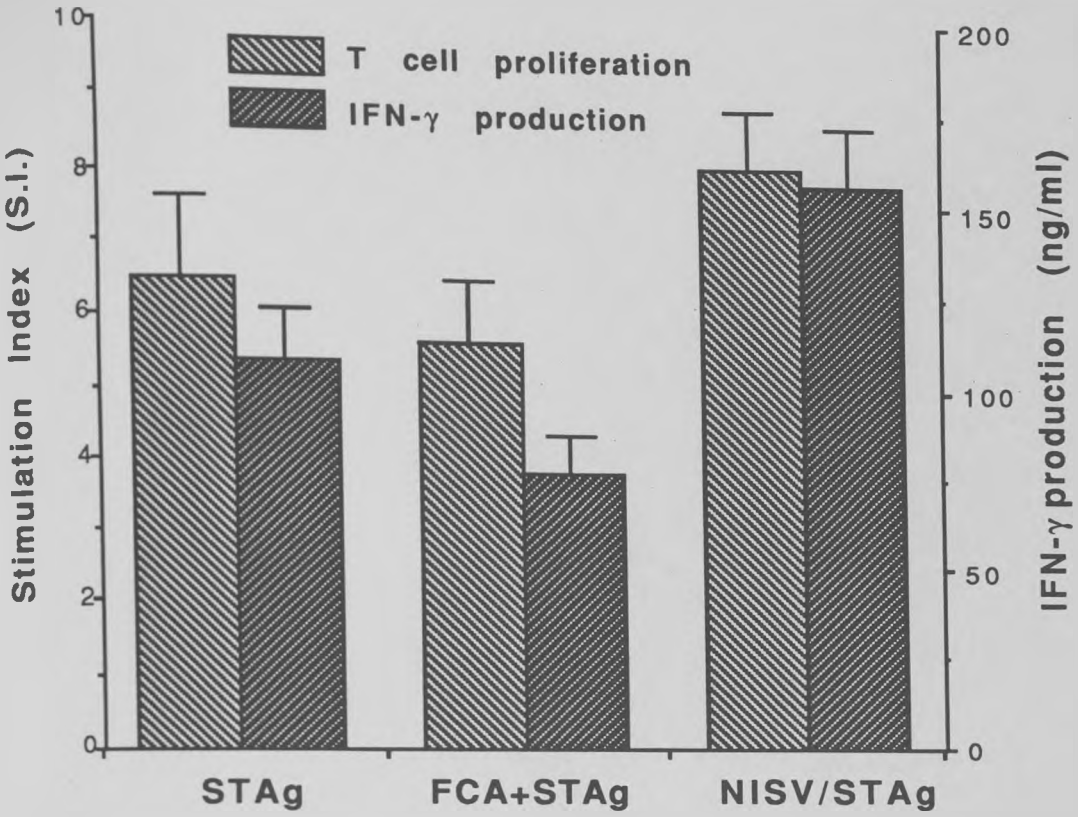

Figure 10.5. The STAg specific proliferation of BALB/c spleen cells as determined by thymidine incorporation and the levels of IFN-γ detected in the supernatants of the spleen cell cultures by ELISA. Mice were inoculated with 50ug of STAg either in PBS, emulsified in FCA (FCA+STAg) or entrapped in NISV (NISV/STAg). Spleen cells were stimulated *in vitro* with 100ug STAg one week after vaccination.

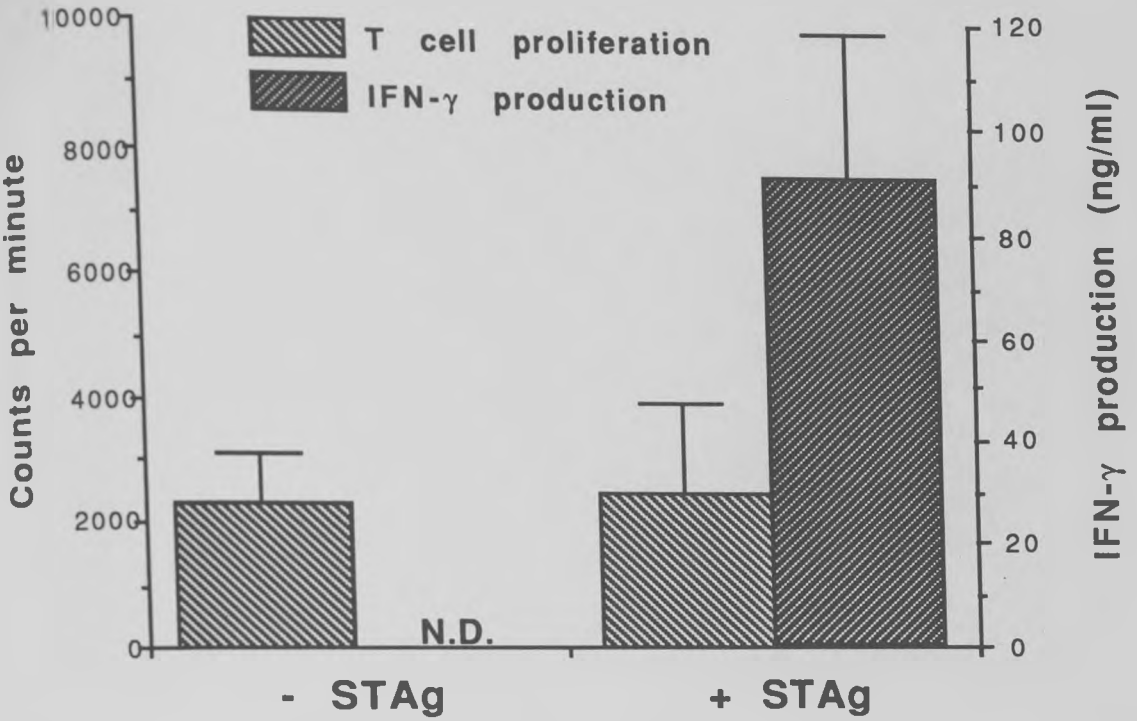

Figure 10.6. Effects of *in vitro* incubation with STAg on proliferation of and IFN-γ production by spleen cells from nonimmunised BALB/c mice. (N.D.= not detectable).

Discussion

This study demonstrates that vaccinating mice with STAg can induce a degree of immunity capable of reducing the eventual cyst number in the adult. This protective effect associated with STAg was enhanced by entrapment in NISV and not merely by co-administration, but inhibited by emulsifying in FCA. The requirement for the STAg to be entrapped in the NISV was not surprising as the adjuvant effects of NISV and liposomes, another vesicular adjuvant, have previously been demonstrated to be dependent on the antigen being entrapped within the vesicles (Brewer & Alexander, 1992; Shek & Sabiston, 1981). Vaccinating mice with STAg entrapped in NISV 2 and 4 weeks before mating could reduce foetal death and the incidence of congenital infection in the litters of mice subsequently infected on day 12 of pregnancy. Mice vaccinated with this preparation showed enhanced anti-*T. gondii* specific T cell proliferation responses over mice vaccinated with STAg alone. In contrast mice vaccinated with STAg emulsified in FCA had diminished anti-*T. gondii* specific T cell proliferation responses. Similarly, vaccination of mice with STAg entrapped within NISV could increase the ability of donor spleen cells to produce IFN-γ in response to *in vitro* stimulation with STAg. The role of IFN-γ in toxoplasmosis has been extensively reviewed (Subauste & Remington 1991; Chapter 1) and is reported to be important in the prevention of acute fatal disease (McCabe, 1984) as well as the prevention of toxoplasmic encephalitis (Suzuki & Remington, 1990). Therefore, it is not surprising that vaccination of mice with STAg entrapped in NISV is associated with protection against disease. NISV as an adjuvant has been reported to favour the production of IgG2a, the immunoglobulin subclass associated with the production of IFN-γ, the activation of the TH1 CD4+ T cell subset and the development of cytotoxic $CD8^+$ T cells (Snapper & Paul, 1987; Snapper & Mond, 1993). This study demonstrates the ability of NISV to enhance the production of IFN-γ and although the cell population responsible for its production is unknown, possible sources include NK cells, the TH1 CD4+ T cell subset or CD8+ T cells, all of which are capable of producing this cytokine and all of which have been implicated in the development of an effective anti-*T. gondii* immune response (reviewed, Chapter 1).

The finding that FCA can promote disease exacerbation in murine toxoplasmosis is not a new concept. Kasper *et al.*, (1985) found that using FCA in a vaccination preparation with the major membrane antigen P30, could exacerbate disease, causing increased cyst numbers. In contrast, Bulow and Boothroyd (1991) reported that reconstitution of the same antigen into liposomes, a vesicular adjuvant system similar to NISV, gave enhanced protection. One possible explanation as to why different adjuvants should exert such a dramatic difference over the eventual quality and nature of the immune response is that different adjuvants may preferentially target distinct populations of antigen presenting cells (APC). Macrophages are believed to favour the stimulation of the TH1 subset of $CD4^+$ T cells as opposed to the TH2 subset (Gajewski *et al.*, 1991) and consequently vesicular adjuvants such as liposomes or NISV which are avidly internalised by macrophages (Fidler *et al.*, 1980; Poste *et al.*, 1980) but not B cells (Dal Monte & Szoka, 1989) would ultimately favour stimulation of TH1 cells. Macrophages have also been shown to be the major cell type involved in the presentation of foreign antigen *via* class I MHC (Debrick *et al.*, 1991). Even within the one population of APCs, the type of adjuvant can have an important bearing on the type of immune response generated. For example, antigens delivered to the cytoplasm of the APC are generally processed via the endogenous pathway, resulting in class I MHC restriction, whereas those processed in endosomes are, i.e. the exogenous pathway are usually presented *via* class II MHC (reviewed, Braciale & Braciale, 1991). Numerous studies have demonstrated that membranous vesicles analogous to NISV can allow introduction of exogenous antigen into the class I MHC processing pathway and subsequent $CD8^+$ activation (Zhou *et al.*, 1992; Harding *et al.*, 1991; Hale *et al.*, 1980; Reddy *et al.*, 1992; Lopes & Chain, 1992).

In recent years there have been numerous reports of recombinant, purified or crude protein preparations able to induce a degree of immunity against the adult acquired disease. However, only a few have dealt with protective immunity against congenital disease transmission. McLeod *et al.* (1988) reported that intra-intestinal vaccination with a live temperature sensitive mutant (ts-4) could reduce the incidence of congenital

transmission from 94% in control groups to 64% in vaccinated groups. Vaccinating sheep with a similar live incomplete strain before pregnancy has been demonstrated to prevent foetal death upon challenge during pregnancy although the incidence of congenital infection is still relatively high (66%). Despite the inability of this strain to form cysts in vaccinated sheep, it is still capable of persisting for up to six weeks. It is unlikely that such a vaccine would ever be licensed for medical use. If it was licensed it could have the effect of decreasing *T. gondii* induced abortion while increasing the number of infected children. Similarly this vaccine may increase the public health hazard by increasing the number of infected lambs reaching the market. In this study we describe a vaccine that can prevent foetal death and markedly reduce the incidence of congenital transmission in a murine model.

CHAPTER 11

General Discussion

This thesis has dealt in detail with many aspects of toxoplasmosis including the genetic control and the immunological mechanisms associated with resistance and susceptibility to disease. In particular, I have been interested in examining cyst development in the brain and its associated immunopathology. Finally, I have been involved in developing a murine model of congenital toxoplasmosis

Genetic susceptibility to disease in these studies as well as others has been demonstrated to be quantitatively linked to the immune response. Thus, a quantifiable difference in spleen cell lymphocyte populations is discernible in BALB/c and BALB/K congenic mice, the kinetics of which relates to the stage of the infection (Chapter 3). Furthermore, ongoing work (results not shown), has shown that spleen cells from naive BALB/K mice produce around 8 times more IFN-γ than cells from BALB/c mice in response to *in vitro* stimulation with STAg. If BALB/K mice respond to an initial *T. gondii* infection in a similar manner, the effects of the IFN-γ could have two effects. Firstly, the IFN-γ produced could protect the mice from early mortality as does the administration of rIFN-γ (McCabe *et al.*, 1984). Secondly, the IFN-γ could encourage encystment as *in vitro* studies have recently suggested (Bohne *et al.*, 1993). Indeed, analysis of results reported in Chapter 3 confirm that BALB/K mice do exhibit greater resistance to acute mortality and develop larger cyst burdens than BALB/c. These differences in lymphocyte subset expansion and IFN-γ production may jointly account for the dramatic disparity in the number of cyst stages harboured in the brains of these two strains of mice and the difference in their rate of appearance and clearance.

In close agreement with other studies we demonstrate that mice that are H-2D/L$^{b \text{ or } k}$ are susceptible to large cyst numbers and encephalitis. The recent demonstration of polymorphism in the promoter region of the TNF genes has given one possible explanation as to why these mice suffer severe encephalitis and other mice of different haplotypes (H-2D^{d} for example) do not. Despite the circumstantial evidence, the effect of this RFLP and higher detectable levels of TNF-α transcripts in the brains of these infected animals (Freund *et al.*, 1992) high TNF-α production can not be irrevocably

linked to high cyst burdens. Indeed there is much evidence, both direct and indirect that the class I genes in the H-2D/L region, involved in antigen presentation are important in determining cyst number (reviewed Chapter 1). The lack of an appropriate congenic mouse strain is prohibitive in determining the relative contributions of each of these regions. However, the ability of parasites to exploit a cytokine for its own benefit has been demonstrated in other diseases. TNF-α for example has been demonstrated to augment egg laying by*Schistosoma mansoni* and *Mycobacterium avium* can apparently use IL-6 as a growth factor (reviewed, Colley and Nix, 1992). It is not impossible that TNF or an associatedn cytokine has a *T. gondii* cyst growth promoting effect. Interestingly, by an immunostaining technique, Chang *et al.,* (1992) were able to detect TNF-α inside cysts in the brains of infected mice suggesting that cysts may take up this cytokine. Furthermore, IFN-γ has been demonstrated to be required for the maintainance of cysts *in vitro* (Jones *et al.,* 1985) and more recently has been implicated in the induction of tachyzoite to bradyzoite transformation *in vitro* (Bohne *et al.,* 1993). TNF or other inflammatory cytokines could also increase the number of cysts in the brain indirectly by allowing more tachyzoites to gain access to the brain. By increasing the expression of adhesion molecules on the vascular endothelium or increasing the permeability of the vascular endothelium, TNF-α would actively help or passively allow more parasitised cells or extracellular parasites to gain entry to the brain. In support of this suggestion, we have detected greater amounts of TNF-α in the serum of acutely infected B10 mice which develop high cyst burdens compared with BALB/c mice which develop low cyst burdens (Chapter 4).

In Chapter 7 the inflammatory response in the brains of mice with progressive toxoplasmic encephalitis is examined and transcripts for many different cytokines several of which are associated with inflammation are detected. In agreement with Freund *et al.,* (1992) transcripts were detected for TNF-α and in addition this study detects transcripts for IL-1α, IL-1β, IL-6, MIP-1 and IFN-γ. Chapter 8 details the kinetics of cytokine production in these mice over the first 30 days of infection and it is hypothesised that the cytokines detected are suggestive of the presence of the TH2

$CD4^+$ T cell subset, which allows the disease to progress unchecked. Subsequent work still in progress has found that after this time point (day 35) the number of tissue cysts in the brains of the surviving mice is significantly less (see appendix 1). Therefore it would appear that like BALB/c and BALB/K mice (Chapter 3), after an initial period of cyst growth in the brain of the B10 mouse, the immune system of these animals is able to reduce cyst numbers. Analysis of mRNA transcripts by PCR in these mice surprisingly demonstrated that resolution of cyst number was accompanied by the expression of IL-10 and the disappearance of IFN-γ. Interestingly, a similar cytokine profile accompanied with a resolution of cyst numbers can be detected in the brains of BALB/K (see appendix 2) mice although the time scale differs dramatically from that of the B10. This appears to contradict the literature in many ways as IFN-γ is widely accepted as beneficial to the host and IL-10 would be expected to have deleterious effects by antagonising the effects of TH1 and $CD8^+$ T cells allowing the expansion of TH2 cells (Fiorentino *et al.,* 1989; Mosmann *et al.,* 1991). However, other workers studying the progression of experimental autoimmune encephalomyelitis (EAE) in the CNS of animals, using a PCR system, similar to that described in this thesis, find that resolution of this inflammatory disease was also associated with the appearance of IL-10 and the disappearance of IFN-γ (Kennedy *et al.,* 1992). IL-10 once called 'cytokine synthesis inhibitory factor', may act to inhibit the production of other cytokines and in this way reduce inflammation. Furthermore, the disappearance of IFN-γ may result in cyst rupture allowing the immune cells already in the brain to clear parasites from this site.

In chapter 3, looking at BALB/c and BALB/K mice it was observed that this period of resolution of cyst burdens in BALB/c and BALB/K mice was preceded and accompanied by a relative increase in splenic $CD8^+$ T cells. The increase in $CD8^+$ T cells may be important as this population has been shown to be capable of killing infected cells *in vitro* , although NK cells are another population that have also been shown to be important effecter cells in killing both extracellular tachyzoites and infected cells. Indeed, in agreement with numerous other studies, results from this

thesis demonstrate the important protective role of CD8+ T cells in toxoplasmosis (Chapter 6). CD8+ rather than CD4+ T cells have been shown to preferentially bind to brain endothelium (Pryce *et al.*, 1991) and the adhesion of CD8+ T cells to vascular endothelium in this manner, has recently been attributed to increased expression of adhesion molecules such as VCAM-1 (Vascular cell adhesion molecule-1) on the surface of endothelium, in response to MIP-1β (Tanaka *et al.*, 1993). It is therefore significant that transcripts for MIP-1β were detected in the brains of animals with encephalitis (Chapter 7 & 8). It is widely acknowledged that adhesion is the first step in the process of diapedesis that allows cells to cross endothelium to gain entry to inflamed sites.

In Chapter 9 the development of a murine model of congenital toxoplasmosis is described, demonstrating that BALB/c mice with chronic infections do not permit vertical disease transmission even if they are re-infected during pregnancy. The similarity in this respect of the host parasite relationship to that observed in humans and sheep should make this a valuable model in which to test vaccines, chemotherapeutic regimes or to study the immunoprophylaxis of vertical disease transmission. This model was employed in Chapter 10 in order to test the efficacy of an anti-*T. gondii* vaccine comprising the soluble fraction of the tachyzoite encapsulated in NISV. This formulation induced a high degree of protection in both the adult, and in the prevention of congenital disease. In contrast administration of FCA had the effect of increasing brain cyst burdens in adult mice while administration of STAg emulsified in FCA had no significant effect on brain cyst burdens. Preliminary work has indicated that a 120kd antigen from STAg, seperated by polyacrylamide electrophoresis, can reduce brain cyst numbers in vaccinated mice.

In recent years, progress in immunology has facilitated a greater understanding of toxoplasmosis as a disease of the immunocompetent and the immunocomprimised host. The mechanisms of protective immunity and immunopathology are better understood and in the near future, may enable the rational development of a safe, effective and well defined vaccine suitable for human and veterinary use .

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APPENDICES

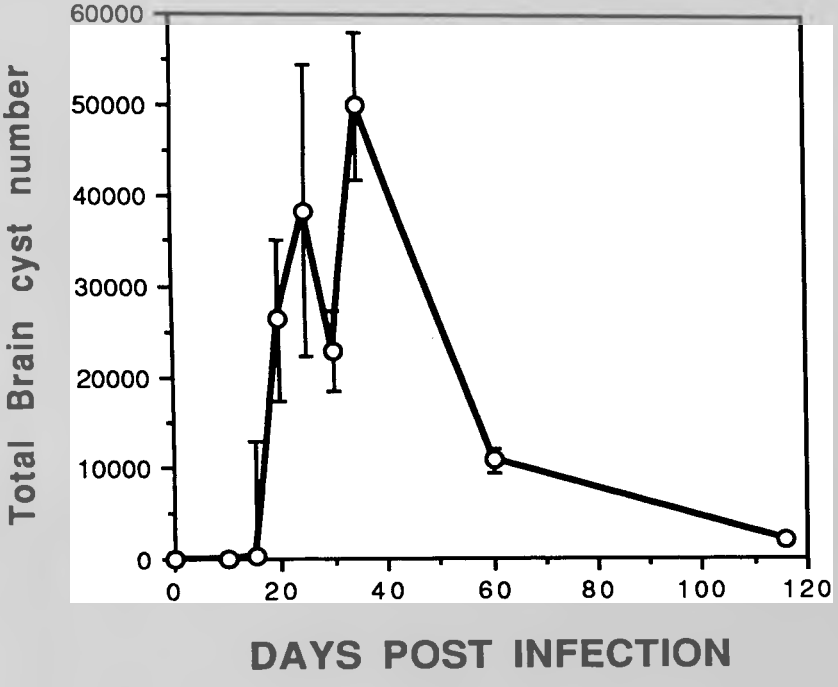

Figure A.1. The total cyst counts in the brains of C57BL/10ScSn mice infected orally 5, 10, 15, 20, 25, 30, 35, 60 and 116 days beforehand with 10 tissue cysts.

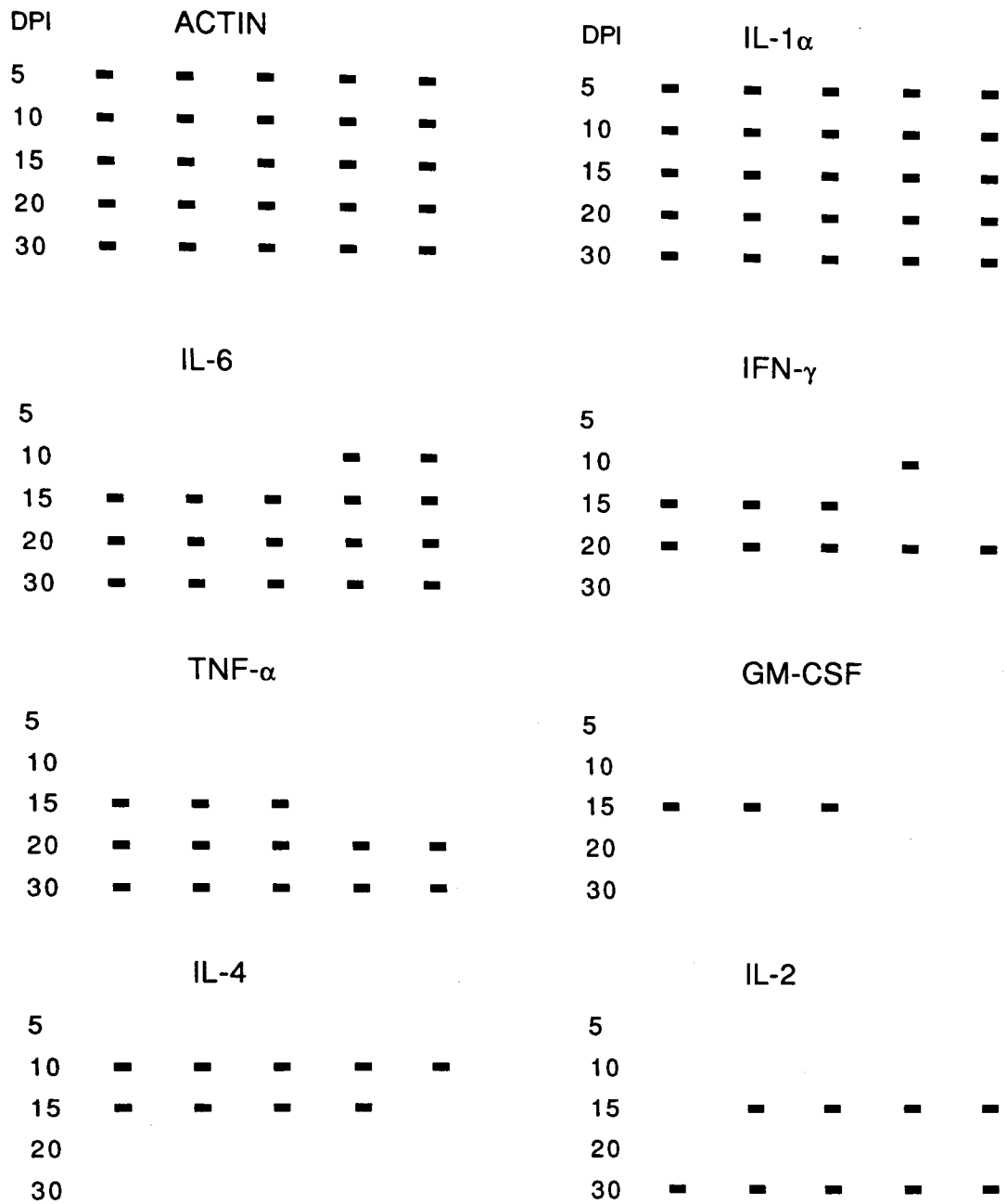

Figure A.2. Analysis of PCR cytokine production in BALB/K mice 5, 10, 15, 20 and 30 days following oral infection with 10 cysts *T. gondii* (Beverley strain). Each mouse was killed on alotted days and mRNA extracted and and subjected to PCR as described in Chapter 8. The same position was used for each individual mouse.