

## Kinetics of Changes in Abundance of Transcripts Encoding Cytokines, Fas, and Fas Ligand in Susceptible and Resistant Mice Infected with *Toxoplasma gondii*

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**Abstract:** The kinetics of changes in the abundance of mRNAs encoding various cytokines (interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-4 (IL-4), and (IL-6)) and Fas, Fas ligand were measured by reverse transcription-PCR at various sites in both susceptible C57BL/6 and resistant BALB/c mice infected with the Fukaya *Toxoplasma gondii* (*T. gondii*). The abundance of transcripts encoding IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 increased markedly from two weeks after infection, reaching a plateau at four weeks, in the brains of C57BL/6 mice ( $p < 0.05$ ). In spleen, the abundance of IFN- $\gamma$  and TNF- $\alpha$  mRNAs was increased two weeks after infection and then decreased in both mouse strains. Neutralization of endogenous IFN- $\gamma$  markedly increased parasite number in the brain of C57BL/6 mice, whereas injection of exogenous IFN- $\gamma$  had no effect. This observation indicates that endogenous IFN- $\gamma$  is essential but not sufficient for inhibition of *T. gondii* growth *in vivo*. No changes in the abundance of IL-4 mRNA were observed in the brain or spleen of either mouse strains. The abundance of Fas mRNA was slightly higher ( $p < 0.05$ ) in the resistant BALB/c mice than in the susceptible C57BL/6 mice and the abundance of Fas ligand mRNA was slightly higher ( $p < 0.05$ ) in the susceptible C57BL/6 mice than in the resistant BALB/c mice. This observation suggests that Fas may contribute to a protective role in resistant BALB/c mice and that increased Fas ligand expression in brain of susceptible C57BL/6 mice may result from inflammation of toxoplasmic encephalitis.

*Key words:* IFN- $\gamma$ , TNF- $\alpha$ , IL-4, IL-6, Fas, Fas ligand

### INTRODUCTION

*Toxoplasma gondii* (*T. gondii*) is an intracellular parasite that causes toxoplasmic encephalitis, a life threatening infection in immunocompromised individuals (Luft, et al., 1984; Luft and Remington, 1988). Inbred C57BL/6 (H-2<sup>b</sup>) mice show a higher mortality rate and a larger number of cysts in the brain than inbred BALB/c (H-2<sup>d</sup>) mice after infection with Fukaya strain. Genes of the major histocompatibility complex (MHC), especially of the H-2 region, as well as non-MHC linked genes, regulate the susceptibility to toxoplasmic en-

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cephalitis and influence cyst formation (Brown and McLeod, 1990; Deckert-Schluter, et al., 1994; McLeod, et al., 1993). To explore the mechanisms of immune responses of host to *Toxoplasma* infection in susceptible C57BL/6 and resistant BALB/c mice, we evaluated roles of cytokines (interleukin-4 (IL-4), IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interferon- $\gamma$  (IFN- $\gamma$ )) implicated in the host immune response (Gazzinelli, et al., 1993a; Hunter, et al., 1993; Hunter, et al., 1992; Schluter, et al., 1993; Suzuki and Joh, 1994) in toxoplasmic encephalitis by measuring mRNAs of these cytokines. The roles of Fas and Fas ligand, which mediate cytotoxicity by apoptosis, were also evaluated.

## MATERIALS AND METHODS

### *Mice.*

Inbred BALB/c and C57BL/6 mice (Charles River, Yokohama, Japan) were housed in the Laboratory Animal Center for Biomedical Research at Nagasaki University School of Medicine.

### *T. gondii and infection.*

An avirulent Fukaya strain of *T. gondii* was obtained from Y. Suzuki (Jikei Medical University, Tokyo, Japan). Cysts of this strain were prepared from C57BL/6 mice six weeks after oral infection with 20 cysts. C57BL/6 and BALB/c mice infected orally with 20 cysts of *T. gondii* were then sacrificed by asphyxiation with ethyl ether, one, two, four, or six weeks after infection. Spleen and brain were collected for the determination of the abundance of Fas, Fas ligand, IL-4, IL-6, IFN- $\gamma$  and TNF- $\alpha$  mRNAs.

### *Reverse transcription (RT)-PCR for Fas, Fas ligand and cytokine mRNAs.*

Total RNA was isolated with TRIzol reagent (Life Technologies, Gaithersburg, MD), and one  $\mu\text{g}$  was subjected to cDNA synthesis in a total volume of 20  $\mu\text{l}$  with an RNA PCR kit (TaKaRa, Shiga, Japan). The reaction mixture that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM of each deoxynucleoside triphosphate, 20 U of RNase inhibitor, 5 U of avian myeloblastosis virus reverse transcriptase XL, and 2.5 mM random nine-nucleotide oligomers, was incubated at 30°C for 10 min, 50°C for 30 min, 100°C for 5 min, and 4°C for 10 min. The reaction mixture was then diluted to 100  $\mu\text{l}$  with double-distilled H<sub>2</sub>O, and 5  $\mu\text{l}$  of the diluted cDNA were subjected to PCR with primers specific for IL-4, IL-6, TNF- $\alpha$ , IFN- $\gamma$ , Fas or Fas ligand. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA was also amplified as a standard to ensure that cDNA concentrations in different reaction mixtures were approximately equal. The band intensities of PCR products after electrophoresis were measured with an IPLab Gel densitometer (Signal Analytical Corp. Vienna, VA). Data were expressed as an S/G ratio ((optical density of sample (S))/optical density of GAPDH (G)) $\times$ 100. Contamination of total RNA with genomic DNA was examined by attempting to amplify cytokine genes from total RNA by conventional PCR; no cytokine genomic DNA was detected in one  $\mu\text{g}$  of total RNA.

*Neutralization of endogenous IFN- $\gamma$  or treatment with exogenous IFN- $\gamma$ .*

BALB/c or C57BL/6 mice were injected intraperitoneally with one mg of a monoclonal antibody (mAb) (R4-6A2) to mouse IFN- $\gamma$  five and 12 days after infection with 20 cysts of the Fukaya strain of *T. gondii*. Other animals were injected with 5000 U of mouse IFN- $\gamma$  (supernatant of cultured IFN- $\gamma$  producing cells (X63 BCMG Neo (PUC) mIFN- $\gamma$ )) (Rolink, et al., 1991) on day five and 12 after infection. Control mice were injected intraperitoneally with phosphate-buffered saline. After six weeks of infection, *T. gondii* numbers were determined by quantitative competitive PCR (QC-PCR) (Luo, et al., 1995).

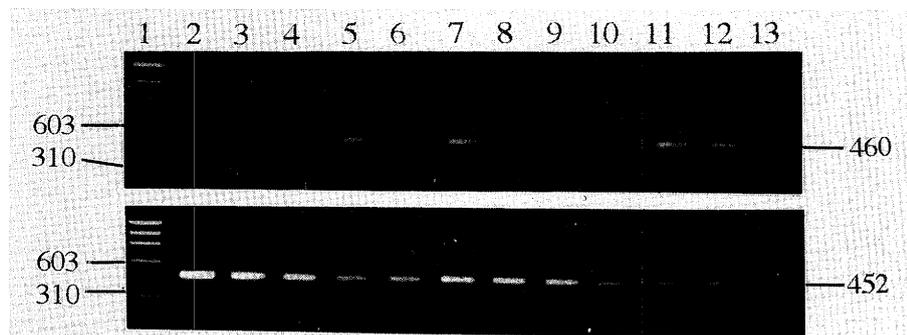
*Statistical analysis.*

Statistical evaluations of differences between means of experimental groups were determined by paired student's *t*-test.

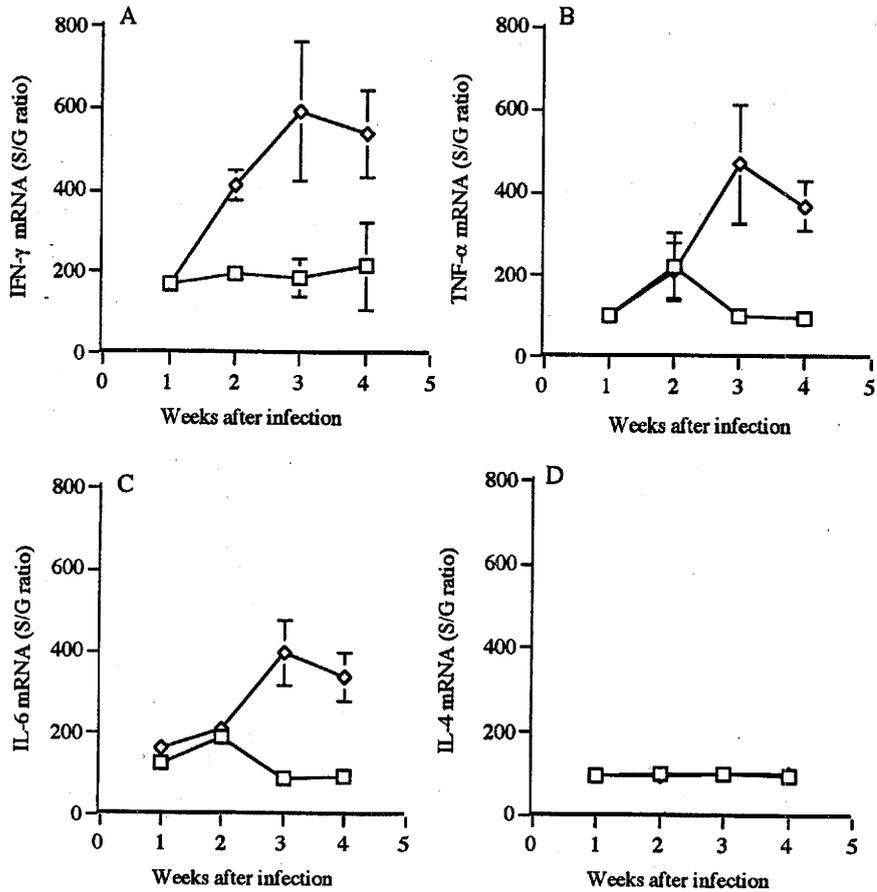
## RESULTS

*Cytokine mRNA abundance in the brain.*

The abundance of transcripts encoding IFN- $\gamma$ , TNF- $\alpha$ , IL-4, and IL-6 in the brains of BALB/c and C57BL/6 mice was measured by RT-PCR one, two, four, and six weeks after infection. IFN- $\gamma$  mRNA was detected in small amounts in both C57BL/6 and BALB/c mice one week after infection, and subsequently increased in abundance only in C57BL/6 mice ( $p < 0.05$ ) (Fig. 1, Fig. 2A). The abundance of TNF- $\alpha$  mRNA was low at one week and moderately increased at two weeks after infection in both strains of mice, and thereafter increased further in C57BL/6 mice ( $p < 0.05$ ) and decreased in BALB/c mice (Fig. 2B). The abundance of IL-6 mRNA remained low in the brains of BALB/c and C57BL/6 mice for two weeks after infection, and thereafter increased in C57BL/6 mice ( $p < 0.05$ ), but not in BALB/c mice (Fig. 2C). No significant difference of IL-4 mRNA abundance was found during infection in both mouse strains (Fig. 2D).



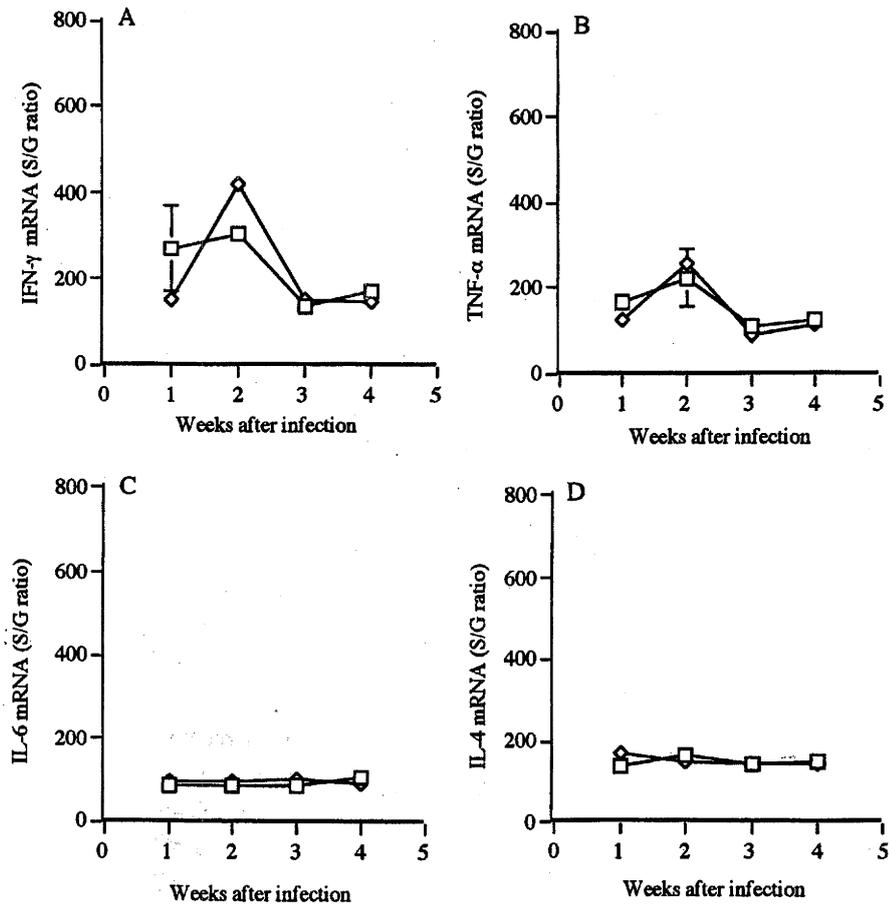
**Fig. 1.** Abundance of IFN- $\gamma$  mRNA in brains of BALB/c and C57BL/6 mice at two and four weeks after infection with *T. gondii*. Lane 1, molecular size marker (*Hae* III digest of  $\phi$ X174); Lanes 2 to 4, BALB/c mice two weeks after infection; lanes 5 to 7, C57BL/6 mice two weeks after infection; lanes 8 to 10, BALB/c four weeks after infection; lanes 11 to 13, C57BL/6 mice four weeks after infection. Upper panel shows the IFN- $\gamma$  RT-PCR products (460 bp). Lower panel shows the GAPDH RT-PCR products (452 bp).



**Fig. 2.** Time courses of changes in the abundance of mRNAs encoding IFN- $\gamma$  (A), TNF- $\alpha$  (B), IL-6 (C), and IL-4 (D) in the brains of BALB/c (□) and C57BL/6 (◇) mice after infection with *T. gondii*. Data are means  $\pm$  SD of three animals and are expressed as the ratio of cytokine mRNA signal to GAPDH mRNA signal  $\times$  100 (S/G ratio).

#### *Cytokine mRNA abundance in spleen.*

Because of the blood brain barrier, the immune response to pathogens in the brain may differ from that in other organs. We therefore examined cytokine mRNA abundance in the spleen of mice infected with *T. gondii*. The abundance of IFN- $\gamma$  (Fig. 3A) and TNF- $\alpha$  (Fig. 3B) mRNAs was low one week after infection, increased slightly at two weeks, and thereafter decreased in both BALB/c and C57BL/6 mice. Transcripts encoding IL-6 (Fig. 3C) or IL-4 (Fig. 3D) did not change significantly during infection of either mouse strain with *T. gondii*.



**Fig. 3.** Kinetics of changes in the abundance of mRNAs encoding IFN- $\gamma$  (A), TNF- $\alpha$  (B), IL-6 (C), and IL-4 (D) in the spleens of BALB/c (□) and C57BL/6 (◇) mice after infection with *T. gondii*. Data are means  $\pm$  SD of three animals.

*Effects of neutralization of endogenous IFN- $\gamma$  or injection of exogenous IFN- $\gamma$ .*

The role of IFN- $\gamma$  in the immune response to *T. gondii* infection was further investigated by injecting mice intraperitoneally with a mAb to IFN- $\gamma$  or IFN- $\gamma$  itself five and 12 days after infection. Injection of the mAb to IFN- $\gamma$  markedly increased the number of *T. gondii* in the brains of C57BL/6 mice ( $p < 0.05$ ), but not in those of BALB/c mice, at six weeks after infection (Fig. 4). In contrast, injection of IFN- $\gamma$  did not affect *T. gondii* numbers in the brains of either strain of mice.

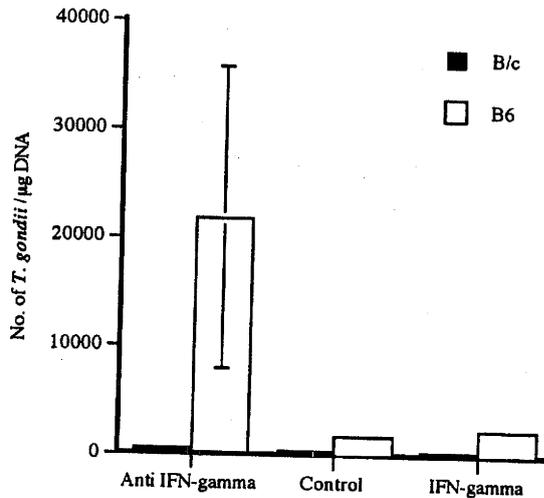
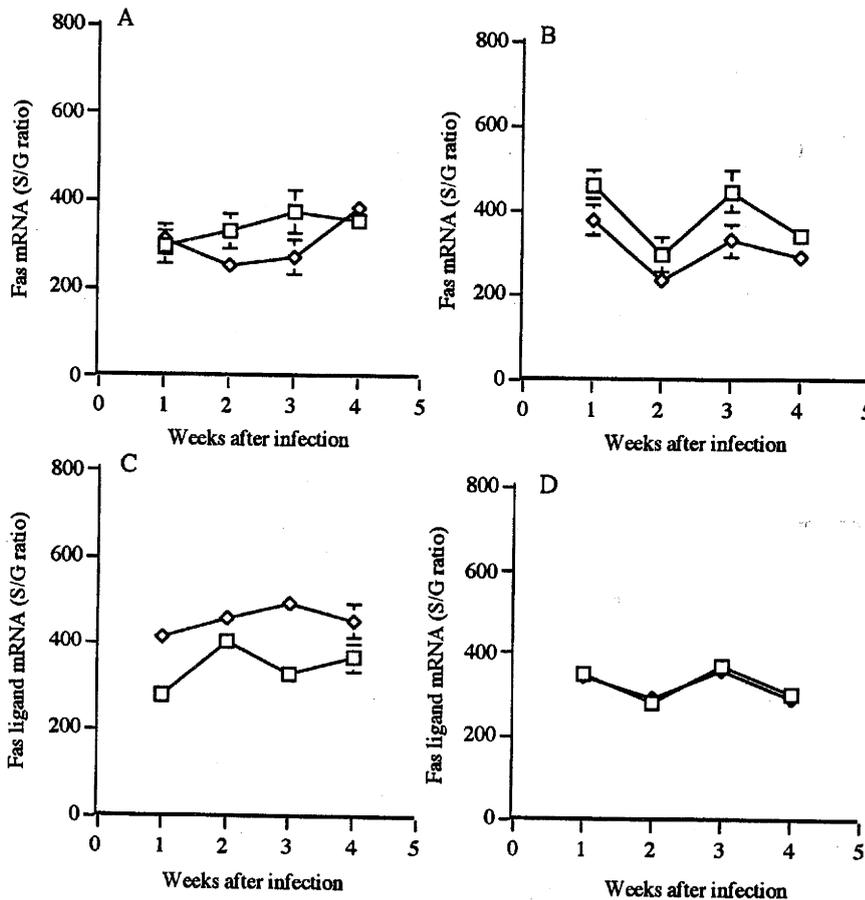


Fig. 4. Numbers of *T. gondii* in the brains of C57BL/6 (open bars) and BALB/c (closed bars) mice treated with a mAb to IFN- $\gamma$ , IFN- $\gamma$  itself, or phosphate-buffered saline (control) five and 12 days after infection. Data are means  $\pm$  SD of three animals.

#### *Abundance of Fas and Fas ligand mRNAs in brain and spleen.*

A decrease in the number of lymphocytes in the spleen in response to *T. gondii* infection was previously shown to be markedly greater in susceptible C57BL/6 mice than in resistant A/J mice (McLeod, et al., 1989). To investigate the mechanism of apoptotic cell death during the immune response to *T. gondii* infection, we measured the abundance of Fas and Fas ligand mRNAs in the brain and spleen of C57BL/6 and BALB/c mice. The abundance of Fas mRNA showed a small increase with time in the brain of BALB/c mice, whereas it tended to be lower and more variable in the brain of C57BL/6 mice ( $p < 0.05$ ) (Fig. 5A). The amount of Fas mRNA in spleen was also greater in BALB/c mice than in C57BL/6 mice ( $p < 0.05$ ) (Fig. 5B). The abundance of Fas ligand mRNA in brain was slightly higher in C57BL/6 mice than in BALB/c mice throughout the period observed ( $p < 0.05$ ) (Fig. 5C). Low and variable amounts of Fas ligand mRNA were apparent in the spleen of both strains of mice (Fig. 5D).



**Fig. 5.** Kinetics of changes in the abundance of mRNAs encoding Fas (A and B) and Fas ligand (C and D) in the brain (A and C) and spleen (B and D) of BALB/c (□) and C57BL/6 (◇) mice after infection with *T. gondii*. Data are means  $\pm$  SD of three animals.

## DISCUSSION

We have now demonstrated a markedly higher abundance of IFN- $\gamma$  mRNA in the brain in C57BL/6 mice than in BALB/c mice. These transcripts may be produced by CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes, because larger numbers of these cells are present in the parenchyma and perivascular and meningeal infiltrates of susceptible mice (Brown, et al., 1995), or they may be produced by natural killer cells (Schluter, et al., 1993). Neutralization of endogenous IFN- $\gamma$  markedly increased *T. gondii* numbers in the brains of C57BL/6 mice, although injection of exogenous murine IFN- $\gamma$  had no effect on *T. gondii* number in either mouse strain. These observations indicate that endogenous IFN- $\gamma$  is essential but not sufficient for inhibition of *T. gondii* growth *in vivo*. Furthermore, other group (Shirahata, et al., 1986)

detected differences in serum IFN- $\gamma$  concentration after infection with the relatively avirulent S-273 strain of *T. gondii* between resistant BALB/c and susceptible C57BL/6 mice at early stage. Detailed mechanisms of IFN- $\gamma$  mediated genetic control of susceptibility to *T. gondii* infection remains to be elucidated. Although the presence of TNF- $\alpha$  in serum and the brain of mice infected with *T. gondii* has been described by several groups (Brown, et al., 1995; Deckert-Schluter, et al., 1994; Freund, et al., 1992; Gazzinelli, et al., 1993a; Hunter, et al., 1993; Hunter, et al., 1992; Roberts, et al., 1995; Schluter, et al., 1993), the role of this cytokine in the pathogenesis of toxoplasmic encephalitis remains to be elucidated. The presence of TNF- $\alpha$  mRNA in the brains of susceptible C57BL/6 mice, but not of resistant BALB/c mice, was demonstrated after 30 days of chronic infection with the Me49 strain of *T. gondii* (Brown, et al., 1995; Freund, et al., 1992). We detected large amounts of TNF- $\alpha$  mRNA in the brains of susceptible C57BL/6 mice from two weeks after infection. This may reflect differences in the ability of these mice to produce TNF- $\alpha$  in response to inflammatory stimuli or differences in these stimuli.

The concentration of IL-6 was shown to be increased in serum and cerebrospinal fluid of SCID and CB-17 mice infected with the DX strain of *T. gondii* (Schluter, et al., 1993). Increased amounts of IL-6 mRNA were previously described in the brains of C57BL/10 Sc SN mice infected with the Beverley strain of *T. gondii* (Hunter, et al., 1992), and in the brains of SCID (Hunter, et al., 1993) and C57BL/6 (Gazzinelli, et al., 1993a) mice infected with the Me49 strain of *T. gondii*. This cytokine markedly enhances the intracellular replication of *T. gondii* after the invasion of macrophages prepared from Swiss-Webster mice, and it impairs the IFN- $\gamma$ -mediated toxoplasmicidal activity of macrophages when both cytokines are either administered together/or sequentially/ before infection (Beaman, et al., 1994). The survival of SCID mice infected with *T. gondii* was prolonged by treatment with antibodies to IL-6 (Hunter, et al., 1993). In central nervous system, microglia are believed to be derived from bone marrow monocytes that populate the central nervous system during fetal development. These glial cells appear to be functionally equivalent to macrophages in other tissues. Thus, our results suggest that the increased abundance of IL-6 mRNA in the brain of susceptible mice may inhibit microglial cell-mediated killing of *T. gondii*, even in the presence of high concentrations of IFN- $\gamma$  and TNF- $\alpha$ .

Th1 and Th2 subsets of CD4<sup>+</sup> T cells play differing roles in the immune response to parasitic infections. Th2 CD4<sup>+</sup> T cells produce cytokines (IL-4 and IL-10) that inhibit the ability of IFN- $\gamma$  to activate macrophages to limit the growth of *Trypanosoma cruzi* and *T. gondii* (Sher, et al., 1992). We could not detect any changes in IL-4 mRNA abundance in the brain or spleen of either strain of mouse, consistent with the observation of Brown *et al* (Brown, et al., 1995). Other groups detected an increased abundance of IL-10 mRNA in the brains of SCID mice and susceptible mice (C3H/HeJ, B10. RKDB, BALB/c-H-2<sup>dm2</sup>, C57BL/6) infected with *T. gondii* (Brown, et al., 1995; Hunter, et al., 1993). Neutralization of IL-10 increased the survival time of infected SCID mice (Hunter, et al., 1993). On the other hand, neutralization of IL-12 decreased the survival time of the mice (Gazzinelli, et al., 1993b). Furthermore exogenous IL-12 was demonstrated protective effect in SCID mice

infected with *T. gondii*. The combination of IL-12 and TNF- $\alpha$  induced the production of IFN- $\gamma$  from whole spleen cells of infected mice to a greater extent than that observed with spleen cells from uninfected mice (Hunter, et al., 1994).

The productions of IFN- $\gamma$ , TNF- $\alpha$ , IL-6 were comparable to the number of *T. gondii* in brain. We did not find any significant difference in *T. gondii* load in brain and other organs at day one, two, three, and seven after infection between BALB/c and C57BL/6 mice when *T. gondii* number was examined by using quantitative competitive PCR method. This may reflect that cytokine production by brain is host response to *T. gondii* infection.

The immune responses of brain may be different from those of other organs because of the blood brain barrier. We examined the cytokine productions in spleen after infection of *T. gondii*. No significant difference could be found between two mouse strains. This suggests that cytokine production in spleen did not correlate with cyst formation in brain.

We previously showed that cytotoxic CD4<sup>+</sup> T cells effectively kill *T. gondii*-infected human melanoma cells (Yang, et al., 1995). Cytotoxic Th1 CD4<sup>+</sup> T cells may induce cell death by triggering a Fas-dependent apoptotic pathway (Stalder, et al., 1994). In present study, the abundance of Fas mRNA was slightly higher in resistant BALB/c mice than in susceptible C57BL/6 mice and the abundance of Fas ligand mRNA was slightly higher in susceptible C57BL/6 mice than in resistant BALB/c mice. This observation suggests that Fas may contribute to a protective role in resistant BALB/c mice and that increased Fas ligand expression in brain of susceptible C57BL/6 may result from or in inflammation of toxoplasmic encephalitis.

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