Jpn. J. Trop. Med. Hyg., Vol. 25, No. 2, 1997, pp. 59-67

CYTOKINE PRODUCTION ASSAYED BY RT-PCR IN PREGNANT MICE INFECTED BY *TOXOPLASMA GONDII* AS A MODEL OF CONGENITAL TOXOPLASMOSIS

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Received January 31, 1997/Accepted April 9, 1997

Abstract: To explore the mechanisms of immune responses of host to *Toxoplasma gondii* (*T. gondii*) infection in pregnant mice, we evaluated roles of cytokines [interferon gamma (IFN-γ), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and interleukin 4 (IL-4)] by measuring mRNAs of these cytokines in placentas, lungs and spleens. The pathogenic effects of time and duration of the Fukaya infection on cytokine mRNA levels in pregnant mice were analyzed. The abundance of mRNAs encoding these cytokines was measured by reverse transcriptase (RT)-PCR at early and late stages of pregnancy in various organs of both susceptible C57BL/6 and resistant BALB/c pregnant mice infected with *T. gondii*. IFN-γ and TNF-α but not IL-6 or IL-4, were predominant in the immune responses of placentas, lungs and spleens of BALB/c and C57BL/6 mice during *T. gondii* infection. Levels of IFN-γ and TNF-α mRNA in placentas of early stage pregnant BALB/c mice (infected at one-week pregnancy and examined on day 4 after infection; 1W4D) were higher than those in corresponding C57BL/6 pregnant mice, which might correlate with the fact that higher parasite numbers in placentas and lungs of C57BL/6 mice (infected at one-week pregnancy and examined on day 11 after the Fukaya infection; 1W11D) were observed than those in placentas and lungs of corresponding BALB/c mice, but not correlate with the result of parasite numbers (*T. gondii* No./mg tissue) in spleens of C57BL/6 (0) and BALB/c (120±56) pregnant mice. In the late stage of pregnancy, levels of IFN-γ and TNF-α did not show definite correlations with *T. gondii* loads in placentas, lungs and spleens. These results indicate that endogenous IFN-γ and TNF-α of early stage pregnancy may be essential for inhibition of *T. gondii* growth in some organs (placentas and lungs), but not in spleens, and the mechanisms of genetic influence involved in the susceptibility and resistance to acute *T. gondii* infection may include several immune responses acting together.

Key words: *Toxoplasma gondii*, cytokine mRNA, congenital toxoplasmosis

INTRODUCTION

Human congenital toxoplasmosis is caused by maternal transplacental transmission of *T. gondii* parasites to the fetus, mainly by the acute initial maternal infection during pregnancy (Yokota, 1995). Any route of *T. gondii* infection leading to a maternal parasitemia during pregnancy may result in toxoplasmosis of the placenta and transmission of the protozoa to the offspring before birth (Cowen and Wolf, 1950). In human congenital infection of *T. gondii*, severity of disease appears to be strongly correlated with trimester of maternal acquisition (Luft and Remington, 1982). The depressed immune response during pregnancy would be expected to have a bearing on the severity of *T. gondii* infection and hence provides a greater opportunity for transplacental spread to occur (McLeod *et al*., 1989).

Pathological changes are much more common and more severe in the placenta than in the fetus, and placental damage is probaly the primary cause of fetal death (Loke, 1982). These facts clearly demonstrate that immune responsiveness of the placenta is critically important for resistance to the parasite entering the fetus and study of the pathology of the placenta is of great practical importance.

There have been several reports on maintenance of

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pregnancy, and recording the placental expression and/ or synthesizes of both harmful and protective cytokines, including interleukin 3 (IL-3), IL-4, IL-6, IL-10, IFN-γ, TNF, colony stimulating factor-1 (CSF-1), granulocyte macrophage-CSF (GM-CSF), and transforming growth factor β (TGF-β) (Chen *et al.*, 1993; Clark *et al.*, 1995; De *et al.*, 1992; Hunter *et al.*, 1993; Yelavarthi *et al.*, 1991). These two patterns of harmful and protective cytokines overlap substantially with the Thl and Th2 cytokine patterns that were originally identified among a panel of mouse Th clones (Mosmann *et al.*, 1986), and mutually inhibit the differentiation and/or activation of the other (Fiorentino *et al.*, 1989). Thus, the inflammatory/anti-inflammatory balance of cytokines in the placenta can alternatively be viewed as a Thl/Th2 balance. The protective Th2 cytokine pattern (together with TGF-β) may be functionally dominant over potentially harmful Thl cytokines during normal, successful pregnancy (Krishnan *et al.*, 1996). Among Th2 cytokines, IL-4 has the ability to drive precursor T cells into a subpopulation of T helper effectors known as Th2 cells, which are important regulations of humoral immunity, eosinophilia, and mastocytosis. In some infectious disease models, administration of anti-IL-4 at the time of infection will divert the ensuing response away from Th2 cells toward the Thl subpopulation of T helper effectors (Sadick *et al.*, 1990). We reported that the Thl-like human $CD4^+$ cytotoxic T lymphocytes specific for *T. gondii*-infected melanoma cells were existent in peripheral blood lymphocytes of patients with toxoplasmosis and were secreting IFN-γ and IL-6 (Yang *et al.*, 1995). Both IFN-γ and IL-6 enhanced HLA-DR molecules of *T. gondii*-infected melanoma cells, and these cytokines may play a role in pathogenicity of toxoplasmosis. IL-6 enhances intracellular replication of *T. gondii* and reverses IFN-γ-mediated activation of murine peritoneal Mϕ, and that certain of the interactions between these two cytokines may be at the level of TNF-α triggering (Beaman *et al.*, 1994). Conversely, an acute *T. gondii* infection during pregnancy may disrupt the maternal-fetal immunological balance in favor of antiparasitic proinflammatory abortogenic cytokines such as IFN-γ and TNF-α (Roberts *et al.*, 1995). Since IFN-γ and TNF-α may be necessary for resistance to *T. gondii* infection, it is perhaps more realistic to view the situation in pregnancy as an altered ballance between the two types of cytokines, which can shift in either direction for a variety of reasons (Clark *et al.*, 1995).

Our present work studied the immune responses of the placenta to *T. gondii* infection as compared to other organs of resistant (H-2^d) or susceptible (H-2^b) strains of pregnant mice. The cytokine (IFN-γ, TNF-α, IL-6 and IL-4) mRNA levels in placentas, lungs and spleens were analyzed by RT-PCR.

Materials and Methods

Mice and matings. Inbred female and male BALB/c (H-2^d) and C57BL/6 (H-2^b) mice (Charles River, Yokohama, Japan) were housed at the Laboratory Animal Center for Biomedical Research in Nagasaki University School of Medicine under conventional conditions. Eight- to ten-wk-old mice were used for mating. One male and two females were housed per cage overnight for mating and the presence of the vaginal plugs in the females was checked on the following morning. The day on which the plug was observed was considered to be day 0 of pregnancy. Pregnant females thus identified were removed from the mating cages and housed randomly with other female mice.

***T. gondii* strains and infections of mice.** A virulent strain RH and an avirulent cyst-forming strain Fukaya (Asai and Suzuki, 1990; Watanabe *et al.*, 1993) of *T. gondii* were used for infection experiments and prepared as previously described (Luo *et al.*, 1995).

For the experiment with the RH strain, tachyzoites were adjusted to 10^7 viable organisms/0.25 ml in phosphate-buffered saline (PBS) for inoculation. One- or two-week pregnant mice were infected intravenously via their retro-ocular venular plexus with 0.25 ml RH suspension. All pregnant mice died on day 6 after infection, therfore the infected mice were sacrificed on day 4 after infection. The organ samples, placentas, lungs and spleens, were collected to analyze the abundances of certain cytokines.

For the experiment with the Fukaya strain, the brains from mice infected 2 months earlier with the Fukaya strain of *T. gondii* were removed and homogenized in 5 ml of PBS with a mortar and pestle. Then 50 μl of brain suspension was placed on a glass microscope slide and mounted with a cover-slip and the number of cysts determined microscopically by scanning the entire preparation under 100× magnification. One- or two-week pregnant mice were infected by gavage with 0.5 ml of a brain tissue homogenate in PBS suspension, containing 20 cysts. The mice which were infected during the first week of pregnancy were sacrificed on day 4 after infection. Mice which were infected at two weeks of pregnancy were sacrificed and examined on day 4 after infection, just before delivery. Each experimental group consisted of three animals. The number of

Table 1 Summary of experimental groups

Mouse strain	*T. gondii* strain	Pregnant stage when infected	Analysis time post infection	Abbreviation
BALB/c, C57BL/6	RH, Fukaya	one week	day 4	1W4D
			day 11	1W11D
		two-week	day 4	2W4D

examined placentas were 9 to 20 in each group. Every experiment was performed two or three times and the results were shown to be essentially identical. The mice used in this present study were sacrificed by anaesthetic overdose.

Experimental groups. Experimental groups of pregnant BALB/c and C57BL/6 mice infected with the RH or the Fukaya strain are summarized in Table 1. One- or two-week pregnant mice were infected with *T. gondii* and the infectivities in these mice were examined on day 4 or 11 after infection. These experimental groups are abbreviated as 1W4D, 1W11D and 2W4D respectively. We tried to establish an experimental group in which the mice were infected on day 0 of pregnancy however, these mice did not become pregnant.

Detection of cytokine mRNA by reverse transcriptase (RT)-PCR. Total cellular RNA was extracted with Trizol (Gibco BRL, Grand Island, NY, U.S.A.) from placentas, lungs and spleens of *T. gondii*-infected or non-infected pregnant mice according to the instructions of the manufacture. After extraction, the RNA concentration was determined spectrophotometrically using GeneQuant (Pharmacia LKB Biochrom Ltd, Science Park, Cambridge CB4 4FJ, England). One μg of total RNA was reversed transcribed in a final volume of 20 μl using the RNA PCR kit (R019A, Takara, Shiga, Japan) according to the manufacturer's instructions. The quantity of RNA used for RT and RT-PCR was decided as manufacturer's recommendations. The reaction mixture contained 10 mM Tris•HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM of each deoxynucleotide triphosphate (dATP, dUTP, dCTP and dTTP), 20 U of RNase inhibitor, 5 U of avian myeloblastosis virus reverse transcriptase XL, and 2.5 mM random nine-nucleotide oligomers and was incubated at 30°C for 10 min, 50°C for 30 min, 99°C for 5 min, and 4°C for 5 min. The RT product mixture (cDNA) was then diluted to 100 μl with double-distilled water, and 5 μl of the diluted solution was used for each PCR reaction. The PCR reaction conditions were optimized for each set of primers (Table 2). Contaminations of total RNA with genomic DNA were examined by attempting to amplify cytokine genes from total RNA by conventional PCR and no cytokine genomic DNA was detected in 1 μg of total RNA. Each of the above-mentioned groups of pregnant mice were accompanied by a group of age-matched pregnant but uninfected control mice.

Ten microliter of each PCR product was electrophoresed in a 1.2% agarose gel with ethidium bromide

Table 2 PCR conditions (sequences of the oligonucleotide primers used for PCR amplification of cytokine mRNA, number of PCR cycles and product size predicated)

Cytokine		Primer sequences	Annealing temperature (°C)	Cycles	Product size (bp)
GAPDH	3′ 5′	TCCACCACCCJGTTGCTGTA ACCACAGTCCATGCCATCAC	55	37	452
IFN-γ	3′ 5′	CGACTCCTTTTCCGCTTCCTGAG TGAACGCTACACACTGCATCTTGG	60	37	460
TNF-α	3′ 5′	CCAAAGTAGACCTGCCCGGACTC ATGAGCACAGAAAGCATGATCCGC	60	37	691
IL-6	3′ 5′	CACTAGGTTTGCCGAGTAGATCTC ATGAAGTTCCTCTCTGCAAGAGACT	56	37	638
IL-4	3′ 5′	GCTCTTTAGGCTTTCCAGGAAGTC ATGGGTCTCAACCCCCAGCTAGT	54	37	399

Primer sequences were obtained from CLONTECH Laboratories, Inc., 4030 Fabian Way, Palo Alto CA 94303 USA.

staining for 25 min at 100 V and quantification of RNA was performed with an IPLab Gel Densitometer (Sigal Analytical Corp., Vienna, VA). The relative intensity of bands for each cytokine mRNA was related to the intensity of the autoradiogram band used as the internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are expressed as the ratio of the optical density (OD) value of the PCR product of each cytokine to the OD value of products of GAPDH according to the following formula: (OD of cytokine/OD of GAPDH) $\times 100$. The OD value for the blank control (distilled water) was set at zero.

Statistical analysis. Student's t-test was used to analyse differences in parasite loads or levels of cytokines between the various groups of mice. $P<0.05$ was accepted as significant.

RESULTS

The abundances of cytokine mRNA transcripts in BALB/c and C57BL/6 pregnant mice infected with a virulent RH *T. gondii* strain. High levels of *T. gondii* loads were detected in placentas, lungs and spleens from BALB/c and C57BL/6 pregnant mice in the early (1W4D) and late (2W4D) pregnant stages (Fig. 1A). To examine the correlation between parasite loads and cytokine responsiveness, mRNA transcripts encoding IFN-γ, TNF-α, IL-6 and IL-4 were tested in 1W4D and 2W4D BALB/c and C57BL/6 pregnant mice (Fig. 1).

High levels of IFN-γ mRNA were observed after infection in placentas, lungs and spleens of 1W4D BALB/c and C57BL/6 pregnant mice. Also high levels of IFN-γ were observed in lungs of 2W4D BALB/c mice and in spleens of 2W4D BALB/c and C57BL/6 mice after infection. In placentas of the RH-infected BALB/c and C57BL/6 pregnant mice, IFN-γ mRNA was detected at a higher level in the early stage (1W4D) than in the late stage (2W4D) of pregnancy ($P<0.05$). However, there was no significant difference in the infectivity of placentas between 1W4D and 2W4D pregnant BALB/c and C57BL/6 mice (Fig. 1A).

Significant levels of TNF-α mRNA were detected in placentas, lungs and spleens from 1W4D and 2W4D BALB/c and C57BL/6 pregnant mice after infection. IL-6 mRNA was induced at low levels in placentas, lungs and spleens of the RH-infected BALB/c and C57BL/6 pregnant mice. In the late stage (2W4D) of pregnancy, levels of IL-6 mRNA in placentas, lungs and spleens of BALB/c and C57BL/6 mice were much lower than those in the early stage (1W4D). 1W4D C57BL/6 pregnant mice had higher IL-6 levels in placentas and lungs than 1W4D BALB/c pregnant mice ($P<0.05$).

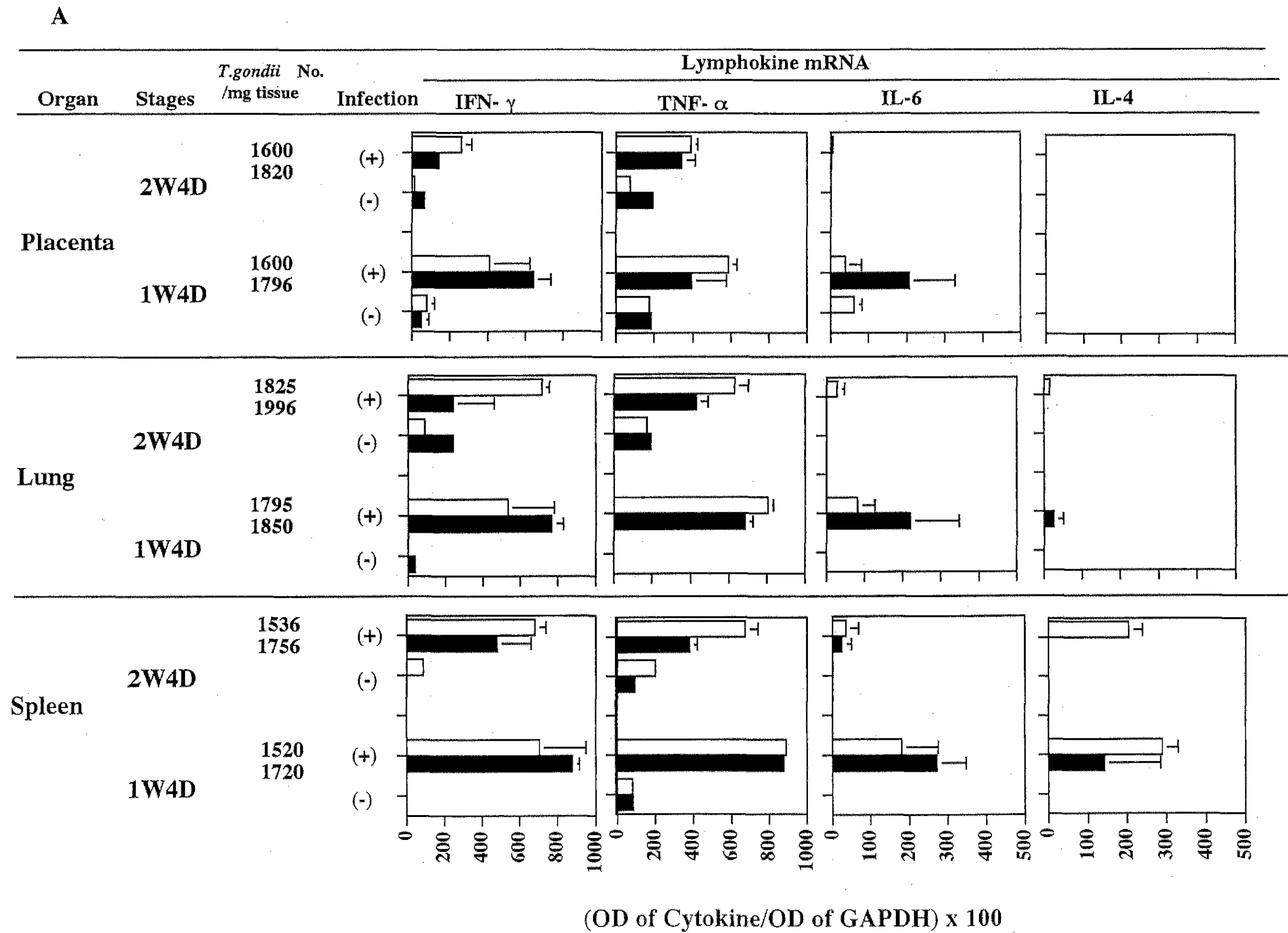
In the RH-infected BALB/c and C57BL/6 pregnant mice, no detectable level of IL-4 was observed in placentas and lungs of the early and the late stages of pregnancy. Significant levels of IL-4 were only observed in spleens from 1W4D BALB/c and C57BL/6 pregnant mice and 2W4D BALB/c pregnant mice.

At the late stage infection with the virulent RH strain, IFN-γ and TNF-α mRNA but not either IL-6 or IL-4 mRNA were dominant cytokines detected in placentas of both BALB/c and C57BL/6 mice (Fig. 1B).

The abundances of cytokine mRNA transcripts in BALB/c and C57BL/6 pregnant mice infected with an avirulent Fukaya *T. gondii* strain. A marked difference in susceptibility to the avirulent strain Fukaya infection between 1W11D BALB/c and 1W11D C57BL/6 pregnant mice was observed (Fig. 2). To examine the involvement of cytokines in the genetic basis of *T. gondii* infectivity in BALB/c and C57BL/6 pregnant mice, mRNA levels of IFN-γ, TNF-α, IL-6 and IL-4 in placentas, lungs and spleens were assayed (Fig. 2).

Levels of IFN-γ mRNA in placentas, lungs and spleens were higher in 1W4D BALB/c pregnant mice than in 1W4D C57BL/6 pregnant mice ($P<0.05$). This correlates with susceptibility to infection in 1W11D pregnant BALB/c and C57BL/6 mice (Fig. 2). The infection rate in placentas and lungs of 1W11D C57BL/6 pregnant mice was higher than that in 1W11D BALB/c pregnant mice ($P<0.05$) (Fig. 2). However, parasite loads in the spleen did not differ significantly between 1W11D BALB/c and C57BL/6 mice. Significant or low level of IFN-γ was not observed in placentas of 1W11D and 2W4D BALB/c and C57BL/6 pregnant mice, indicating that cytokines produced in the placenta of 1W4D BALB/c and C57BL/6 pregnant mice are transient and are not sufficient to protect placentas of C57BL/6 mice from Fukaya infection at a late stage of pregnancy. Levels of IFN-γ in lungs and spleens did not differ significantly between 1W11D BALB/c and C57BL/6 pregnant mice, and levels of IFN-γ were higher in lungs than in spleens in the two strains of mice. Thus the markedly lower *T. gondii* loads in 1W11D BALB/c pregnant mice than in 1W11D C57BL/6 pregnant mice can be attributed to other genetic differences but not to protection by IFN-γ. The fact that the IFN-γ level in the placenta of the Fukaya-infected BALB/c and C57BL/6 mice was higher in the early stage (1W4D) than in the late stage (1W11D or 2W4D) of pregnancy aggrees with the observations of the RH infection as shown in Fig. 1.

Levels of TNF-α in placentas, lungs and spleens



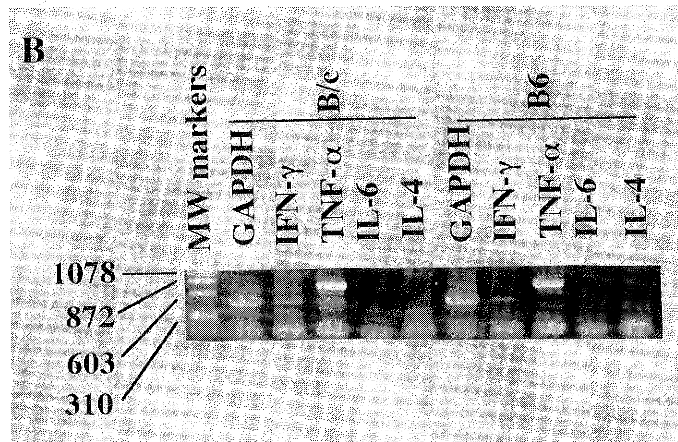


Figure 1 (A) Cytokine mRNA levels in virulent *T. gondii*-infected pregnant mice. Time courses of change in the abundance of mRNAs encoding IFN-γ, TNF-α, IL-6 and IL-4 in the placenta, lung and spleen of BALB/c (□) and C57BL/6 (■) pregnant mice after infection with RH strain. At each time point, experimentally-infected group of pregnant mice (+) was accompanied by a group of age-matched uninfected pregnant mice (−). Lymphokine mRNAs were assayed by RT-PCR and the data is expressed as described in Materials and Methods. (B). Cytokine mRNA levels in placentas of virulent *T. gondii*-infected pregnant mice. Abundance of IFN-γ, TNF-α, IL-6 and IL-4 mRNA in the placenta of BALB/c (abbreviated as B/c) and C57BL/6 (abbreviated as B6) pregnant mice infected at two-week after pregnancy with RH strain and tested on day 4(2W4D) after infection. MW markers are Hae III digest of ϕx174.

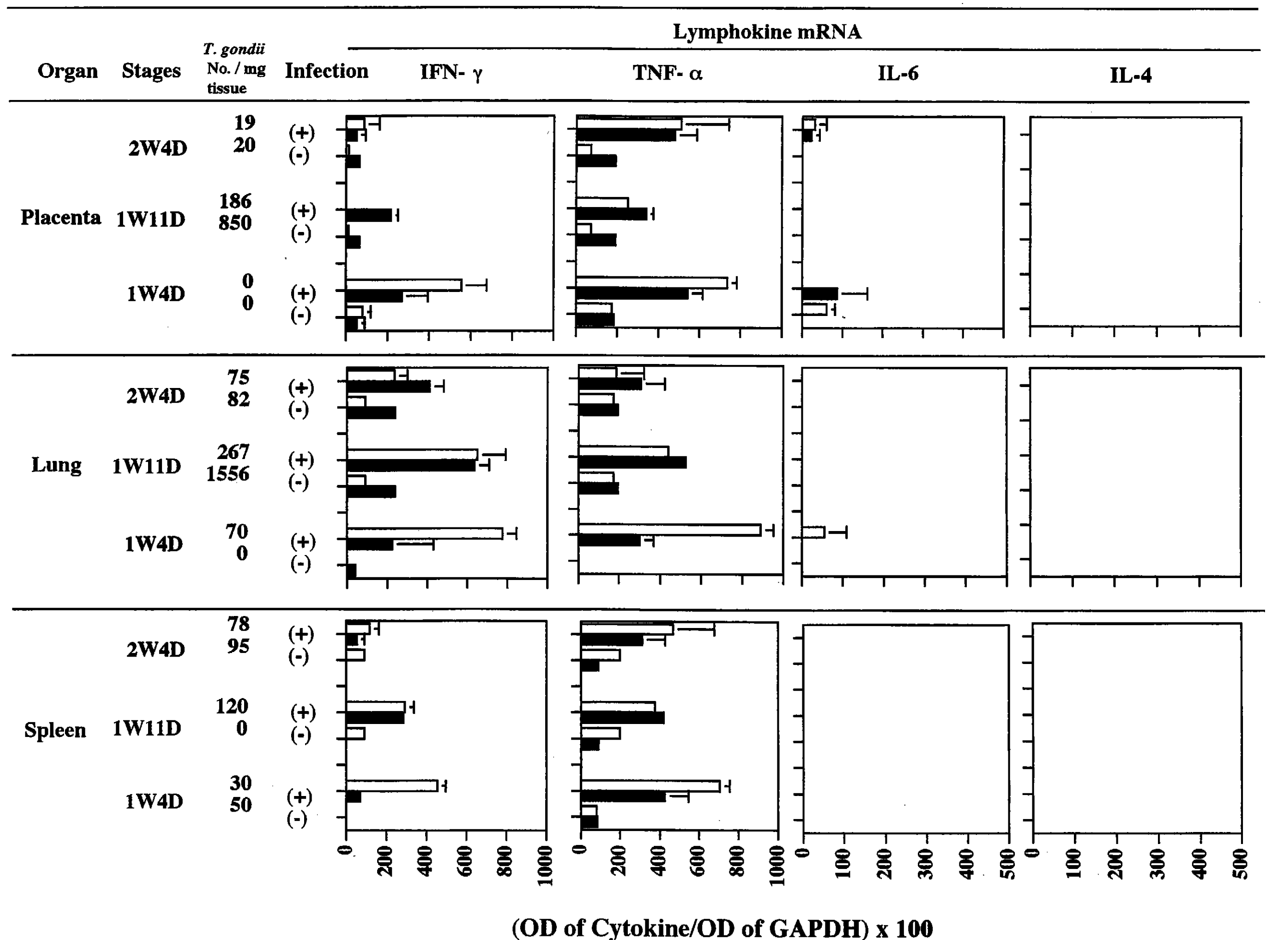


Figure 2 Cytokine mRNA levels in avirulent *T. gondii*-infected pregnant mice. Time courses of change in the abundance of mRNAs encoding IFN-γ, TNF-α, IL-6 and IL-4 in the placenta, lung and spleen of BALB/c (□) and C57BL/6 (■) pregnant mice after infection with Fukaya strain. At each time point, the experimentally-infected group of pregnant mice (+) was accompanied by a group of age-matched uninfected pregnant mice (−). Lymphokine mRNAs were assayed by RT-PCR and the data is expressed as described in Materials and Methods.

were higher in 1W4D (BALB/c pregnant mice than in 1W4D) C57BL/6 pregnant mice ($P<0.05$). TNF-α production paralleled the production of IFN-γ. However, in placentas, lungs and spleens of 2W4D and 1W11D pregnant mice, no significant difference in the TNF-α level was observed between infected BALB/c and C57BL/6 pregnant mice.

No significant or very low levels of IL-6 mRNA were detected in placentas, lungs and spleens of infected or uninfected BALB/c and C57BL/6 pregnant mice. No significant IL-4 mRNA level was detected in any of placentas, lungs or spleens from either strain of pregnant mice (Fig. 2).

Discussion

An acute *T. gondii* infection during pregnancy may disrupt the delicate hormone-influenced maternal-fetal immunological balance in favor of antiparasitic proinflammatory cytokines such as IFN-γ and TNF-α (Roberts *et al.*, 1995). As shown in Figs. 1 and 2, at the mRNA level, using lungs and spleens as references, IFN-γ and TNF-α, but not IL-6 or IL-4, were predominant in the immune response of the placenta, lung and spleen of BALB/c and C57BL/6 pregnant mice during *T. gondii* infection. On the other hand, IFN-γ and TNF-α have deleterious abortogenic effects, leading to fetal demise in otherwise normal pregnancy (Krishnan *et al.*, 1996). We observed lower pregnancy ratio and higher

abortion ratio of fetuses in the Fukaya-infected pregnant mice than in non-infected pregnant mice (data not shown), suggesting that a beneficial Thl antiparasite response can prove deleterious to successful gestation as it can influence both placental maintenance and preimplantation events (Krishnan *et al*., 1996). It has been shown that the Thl response to *Leishmania major* increases the risk of pre- and post-implantation pregnancy loss (Krishnan *et al*., 1996). Positive correlations have also been reported between abortions and the expression of IFN-γ, IL-2 and TNF (Tangri and Raghupathy, 1993). One of the mechanisms of fetal damage in response to cytokine production may be the induction of natural killer activity by IL-2 and IFN-γ (Wegmann *et al*., 1993). Placenta cells from mice undergoing fetal abortion were less able to suppress natural killer reactivity as compared to normal placental cells (Chaouat *et al*., 1985).

We previously analyzed the mRNA levels of cytokines (IFN-γ, TNF-α, IL-6 and IL-4) of brains from BALB/c and C57BL/6 non-pregnant mice infected by the avirulent strain Fukaya, and the results indicate that endogenous IFN-γ and TNF-α are not sufficient for inhibition of *T. gondii* growth *in vivo* (Luo and Yano, 1996). As with IFN-γ, many studies have demonstrated a protective role for TNF-α in toxoplasmosis (Gerard *et al*., 1993; Marchant *et al*., 1994), although there also exists some evidence that overproduction of TNF-α may be detrimental by increasing mortality (Black *et al*., 1989). From our data shown in Figs. 1 and 2 (1W4D), TNF-α production occurs in parallel with IFN-γ production, which may be consistent with the previous report that TNF-α functions as an endogenous modulator of macrophage activation by triggering IFN-γ-primed cells to express antitoxoplasmal activity (Sibley *et al*., 1991). In Fig. 2, IFN-γ and TNF-α levels in placentas and lungs of 1W4D BALB/c and C57BL/6 inversely correlated well with the infectivity of Fukaya strain in 1W11D pregnant mice, however, no difference in the level of IFN-γ or TNF-α in the 1W11D mice was observed between BALB/c and C57BL/6 pregnant mice. These results suggest strongly that IFN-γ and TNF-α production in the early stage of pregnancy (1W4D) may play a very important role and have protective immunity against parasites in the placenta and other organs in *T. gondii*-infected BALB/c pregnant mice. These speculations seem reasonable when parasite loads and cytokine production in 1W4D pregnant mice were compared to those in 2W4D pregnant mice infected with the Fukaya *T. gondii* stain.

It has been demonstrated that IL-6 enhances intracellular replication of *T. gondii* and reverses IFN-γ mediated activation of murine peritoneal macrophages, and that certain interactions between these two cytokines may be at the level of TNF-α triggering (Beaman *et al*., 1994). Previous studies have detected the presence of high circulating levels of IL-6 in a lethal *T. gondii* infection in mice (Beaman *et al*., 1991) as well as induction of IL-6 mRNA in the brains of mice infected with *T. gondii* (Hunter *et al*., 1993). We have reported that Thl cytotoxic T lymphocytes specific for *T. gondii*-infected melanoma cells derived from a patient with toxoplasmic chorioretinitis secreted high levels of IL-6 (Yang *et al*., 1995). However, we observed no significant (2W4D) or very low levels (1W4D) of productions of IL-6 in placentas, lungs and spleens of *T. gondii*-infected BALB/c and C57BL/6 pregnant mice (Figs. 1 and 2). Like IL-6, IL-4 has been suggested to be involved in disease exacerbation. IL-4 impedes the induction and effector function of the subpopulation of Thl cells which regulate cell-mediated immunity (Le *et al*., 1990). In our experiment, Thl dominancy in the *T. gondii*-infected pregnant mice was more impressive when IL-4 mRNA levels were examined (Figs. 1 and 2). The differences in the levels of IL-6 and IL-4 production between infections with the RH and the Fukaya strains may be explained by the differences between *T. gondii* strain properties, size of inoculum and/or infection route.

Further study, including pathological investigations into the transplacental spread of *T. gondii* and study of the immunological status of infected and control placentas of animals and the characteristics of the T lymphocytes involved in the process of placental destruction may provide further insight into the pathogenesis of congenital toxoplasmosis.

ACKNOWLEDGEMENT

This work was supported by a Grant-in-aid for Scientific Research from the Ministry of Health and Welfare, a grant from the ministry of Education, Science, Sports and Culture, Japan and Ohyama Health Foundation, Tokyo, Japan.

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