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Short communication

Relationship between type 1/type 2 immune responses and occurrence of vertical transmission in BALB/c mice infected with *Neospora caninum*

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Abstract

To examine the relationship between occurrence of vertical transmission and type 1/type 2 immune responses induced by *Neospora caninum* infection in BALB/c mice, pregnant (group 1p) and non-pregnant mice (group 1np) were inoculated with 2×10^6 of the *N. caninum* parasites and then we examined the vertical transmission rate and production of IFN-γ and IL-4. We also studied chronically infected mice, which were bred at 4 weeks or more after infection (group 2), and mice inoculated during pregnancy and re-bred at 4 weeks or more after delivery (group 3). In groups 1p, 2 and 3, vertical transmission was observed in 27.4, 41.4, and 50% of the offspring, respectively. The serum IFN-γ level increased on days 1 and 5 post-inoculation (p.i.) in groups 1p and 1np, while no increase level was observed in groups 2 and 3 during pregnancy or after delivery. When the mice in groups 2 and 3 were re-inoculated, all mice showed a transient increase in serum IFN-γ on day 1 post-re-inoculation. The serum IL-4 level in both of groups 1p and np increased in a similar manner following infection. In group 3, the serum IL-4 level was somewhat higher than that in group 2 after re-inoculation. The anti-*N. caninum* antibody IgG1 titer in group 3 increased on day 10 post-re-inoculation. These results suggest that the mice infected during pregnancy may acquire a weaker immune response to the parasite than mice infected when they are not pregnant, and that mice infected during pregnancy may show an enhanced type 2 immune response in the recrudescence of the infection.

Keywords: Immune regulation; *Neospora caninum*; Vertical transmission

Neospora caninum not only causes severe neuromuscular disease, but also abortion, stillbirth and congenital infection in livestock and companion animals (review by Dubey and Lindsay, 1996). Though the major mode of transfer is vertical transmission, and the parasite has been seen to be transmitted from mother to fetus via the placenta during successive pregnancies (Anderson et al., 1995;

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Cole et al., 1995; Long and Baszler, 1996), oral transmission through ingestion of oocysts or tissue cysts of the parasite is also possible (Basso et al., 2001; Lindsay et al., 1999; McAllister et al., 1998). It has been demonstrated that type 1 responses to play an important role in protection against intracellular pathogens, whose down regulation during pregnancy may be reflected in an increase in frequency of vertical transmission (Williams et al., 2000). In murine neosporosis, Long and Baszler (2000) hypothesized that induction of maternal type 1 responses against *N. caninum* could prevent vertical transmission and demonstrated that modulation of type 2 cytokines giving anti-IL-4 monoclonal antibodies before pregnancy can reduce the frequency of vertical transmission of *N. caninum*. The mechanisms of vertical transmission in the chronic phase and repeated abortion are, however, still unknown. Recently, we observed a high frequency of vertical transmission in BALB/c mice infected with *N. caninum* when the mice had been inoculated during pregnancy and then re-bred in the later stages of infection (Omata et al., 2004). In this case, the transplacental transmission may have been due to the reactivation of the parasite or down-regulation of protective immunity in the mice. In the present study, to clarify the immune response when mice are infected during pregnancy and then become pregnant again after giving birth, we examined the production of IFN-γ, as the type 1 cytokine, and IL-4, as the type 2 cytokine, in BALB/c mice which had been infected with *N. caninum* either during pregnancy or in the chronic phase of the infection.

Eight-week-old female and male BALB/c mice were purchased from Japan CLEA (Tokyo, Japan). Male and females were put together and the females were examined daily for formation of a vaginal plug. The day that a vaginal plug was found was designated as day 0 of pregnancy. Bovine angio-endothelial cells (BAE cells) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (D-MEM 10% FBS).

Tachyzoites of *N. caninum* derived from sheep (Kobayashi et al., 2001; Koyama et al., 2001), which has few tissue cyst forming ability and maintained by continuous passage in BAE cell cultures were isolated by filtration using a 3 μm polycarbonate filter (Nuclepore; Coster Scientific Corporation, Cambridge, MA) and suspended in phosphate-buffered saline (PBS). The parasite concentration in the suspension was adjusted with PBS.

In order to examine immune responses, pregnant mice (group 1p) and non-pregnant mice (group 1np) were inoculated intraperitoneally with 2×10^6 *N. caninum* tachyzoites between days 7 and 19 of gestation in the pregnant mice. Another group of mice (group 2) was inoculated with the same dose of tachyzoites. A blood sample of approximately 100 μl was obtained from the supraorbital vein of each mouse on days 1, 3, 5, 7 and 10 post-inoculation (p.i.). The serum of each sample was stored at −80 °C until use. For the purpose of comparing the frequency of vertical transmission and immune responses in the chronic phase, the mice in group 2 were housed together for a 3 days period between days 28 and 100 post-challenge to allow mating, and mice in group 1 were re-housed on day 28 or later following delivery (group 3). To examine the secondary immune responses of groups 2 and 3, the mice were re-inoculated with *N. caninum* on day 40 p.i. The offspring in all groups were killed by cervical dislocation on the day of birth. The brain, mesenteric lymph nodes and spleen of each neonate and dam were removed and then examined by PCR for the presence of parasite DNA. The PCR examination procedure used an *N. caninum* oligonucleotide primer and was based on the technique described by Yamage et al. (1996).

Serum IFN-γ and IL-4 levels was measured using an ELISA kit (ENDOGEN Inc., Cambridge, MA) according to the manufacturer's instructions. All experiments were performed in triplicate and repeated at least twice. The data from each experiment were evaluated using the Student's *t*-test and the level of significance was taken as 95%.

To evaluate the humoral immune response, individual sera were analysed for *N. caninum* specific IgG1 and IgG2a by ELISA (Long et al., 1998). Briefly, approximately 4×10^5 *N. caninum* with PBS containing 2% paraformaldehyde was coated onto 96-well plates, which had been pretreated with poly-l-lysine (0.1 mg/ml Sigma Chemical Co., St. Louis, MO) overnight at 4 °C. To block non-specific binding of antibodies, the plates were coated with PBS containing 3% bovine serum albumin and again left overnight at 4 °C. Then, each serum sample was run as two-fold serial dilutions starting at 1:100 and incubated at room

Table 1
Transmission rates for mice infected with *N. caninum* and mated at 4 weeks or more after infection

Group	No. of transmitting/ total dams	No. of positive/total neonates (rate of transmission, %)
1	7/8	12/44 (27.4)
2	5/7	12/29 (41.4)
3	3/3	14/28 (50)

temperature for 2 h. After washing with 0.025% Tween-20 in PBS, and horse-radish peroxidase conjugated anti-mouse IgG1 or IgG2a (Zymed Laboratories, Inc., San Francisco, CA) were added at dilutions of 1:1000 and incubated at room temperature for 1 h. The peroxidase reaction was visualized using ABTS as the substrate. After allowing the color to develop for 30 min, the o.d. of each well was determined by an electronic plate reader at 415 nm.

Table 1 shows the numbers of mice from which infection was transmitted and the transmission rates for the neonates in each group. In group 1p, mice were inoculated between days 6 and 17 of gestation, and 7 of the 8 dams transmitted the infection to 12 out of a total of 44 offspring. In group 2, 5 of the 7 mice transmitted the infection to 12 out of a total of 29 offspring. In group 3, 3 out of 3 mice transmitted the infection to 14 out of a total of 28 offspring.

As shown in Fig. 1a, in mice in groups 1p and 1np, there was a transient increase in the serum IFN-γlevel on days 1 and 5 p.i. Also, the serum IFN-γ level in the mice of group 1p on day 1 p.i. (5.30 ± 1.88 ng/ml) was lower than that in the mice of group 1np (7.15 ± 1.93 ng/ml), although the difference was not statistically significant. Looking at Fig. 1b, the mice in group 1np showed a transient increase in serum IL-4 level on days 3, 5 and 14 p.i. The mice in group 1p showed an increase in serum IL-4 level on days 7 and 10 p.i.

As for the chronic phase of the infection, no IFN-γ level was detectable in any of the mice in groups 2 and 3 throughout the pregnancy and delivery period (data not shown). As shown in Fig. 2, mice showed elevated serum IL-4 levels ranging from 50 to 270 pg/ml and they continued to be high until the end of the experiment, with the exception of the level in mice of group 3 on day 14 p.i. (380 ± 50 pg/ml). As seen from Fig. 3a, when the mice in groups 2 and 3 were re-inoculated, all of the mice in both groups showed transient increases in serum IFN-γ level on day 1 p.i., and then IFN-γ decreased to non-detectable levels and stayed there until day 10 p.i. Interestingly, serum IL-4 levels in the mice of group 3 were somewhat higher than those in mice of group 2, throughout the experiment (Fig. 3b).

Upon measuring anti-*N. caninum* specific IgG1 and IgG2a antibody level in groups 2 and 3, as shown in Table 2, in all of the mice in group 3, IgG1 titers were lower than IgG2a titers before re-inoculation. While,

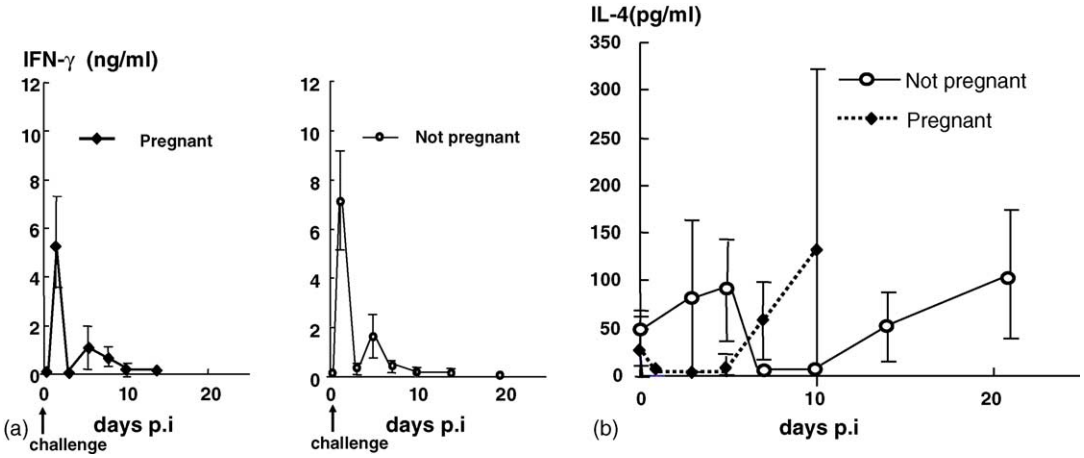

Fig. 1. Serum interferon-γ and interleukin-4 levels in mice inoculated with *N. caninum* when pregnant and not pregnant. (a) Interferon-γ and (b) interleukin-4.

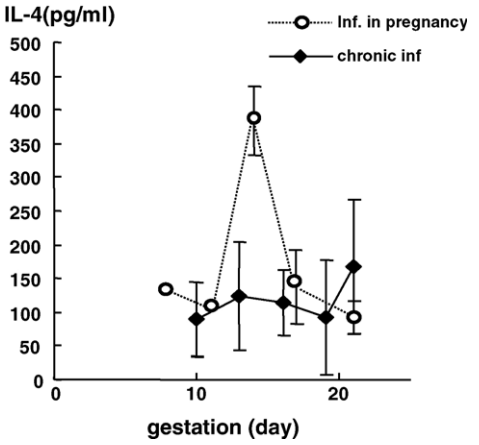

Fig. 2. Serum interleukin-4 levels in mice inoculated with *N. caninum* during pregnancy and re-bred on day 40 p.i.

on day 5 post-re-inoculation, all mice showed higher titers for both antibodies.

In the present study, a transient increase in serum IFN-γ was observed in both non-pregnant, and pregnant mice following infection. Although the IFN-γ level in the pregnant mice was lower than that in the non-pregnant mice, the difference was not significant. This suggests that physiological conditions under pregnancy have no influence on the increase in serum IFN-γ level following infection. Regarding previous studies related to our theme, though a similar transient increase in serum IFN-γ level was observed in mice infected with the JPA1 strain of *N. caninum* (Shibahara et al., 1999), mice

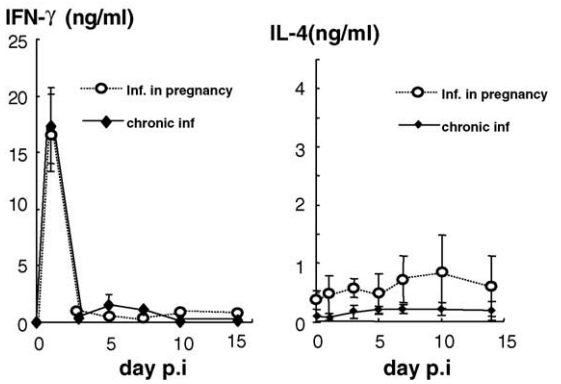

Fig. 3. Serum interferon-γ and interleukin-4 levels in mice re-inoculated with *N. caninum* when pregnant and not pregnant.

Table 2
Anti-*N. caninum* IgG subclass antibody activity in mice re-inoculated with *N. caninum*

Mice	Pre-re-inoculation		Day 5 post-re-inoculation	
	IgG1	IgG2a	IgG1	IgG2a
Group 2				
No. 1	6400	6400	6400	6400
No. 2	6400	6400	6400	6400
Group 3				
No. 1	800	6400	6400	6400
No. 2	1600	6400	6400	6400
No. 3	800	6400	6400	6400

infected with *T. gondii*, showed extremely elevated serum levels of type 1 cytokines (Mordue et al., 2001; Gavrilescu and Denkers, 2001). Thouvenin et al. (1997) reported that cultured spleen cells from pregnant mice infected with *T. gondii* produced more IFN-γ and more NO than non-pregnant mice, and that the type 2 response was weak. We consider that one possible explanation for the differences in IFN-γ production between *N. caninum* and *T. gondii* infection is that *N. caninum* has lower pathogenicity, making IFN-γ production induction weaker. Bearing this out, few pathological effects were observed in mice infected with *N. caninum* (Koyama et al., 2001), and though immuno-deficient mice infected with *N. caninum* exhibited relatively high serum IFN-γ levels, this may have been due to macrophages and natural killer cells, as infection proceeded (Shibahara et al., 1999). In the present study, vertical transmission was also observed in groups 2 and 3, and these mice produced no serum IFN-γ during pregnancy. These results indicate that vertical transmission in the chronic phase of infection is not accompanied by production of IFN-γ in the serum, and suggests two possibilities. One is that very few of the parasite multiply and that transplacental transmission occurs without immune responses. The other is that *N. caninum* in the maternal tissues may suppress type 1 responses, or enhance type 2 responses. In our previous study, we speculated that *N. caninum* infection during pregnancy may increase its numbers in the maternal tissues, or cause protective immune responses to be incomplete, for example by affecting the balance between types 1 and 2 responses. Therefore, becoming pregnant may produce a high rate of parasite re-activation or down-regulate

protective immune reactions and invoke trans-placental transmission (Omata et al., 2004). If the down-regulation of the protective immune reactions is maintained in mice infected during pregnancy (i.e., group 3), type 1 cytokine production in the mice may be at a lower level than in mice infected when not pregnant (i.e., group 2), when the mice were re-inoculated with *N. caninum*. To test these speculations, we re-inoculated the mice of groups 2 and 3 with *N. caninum* and measured the serum IFN-γ and IL-4 levels. A slight amount of serum IFN-γ production was observed in both groups on day 1 post-re-inoculation. Interestingly, on day 5 post-re-inoculation, the IL-4 serum level in group 3 was somewhat higher than that in group 2, and there was also an increase in the anti-*N. caninum* IgG1 antibody titer in group 3. These findings suggest the possibility that mice infected during pregnancy keep down-regulating type 1 cytokine production, resulting in a high rate of vertical transmission. Long and Baszler (2000) examined the effect of IL-4 on vertical transmission during pregnancy by modulating IL-4 levels in mice using monoclonal antibodies, and demonstrated that a decrease in vertical transmission was associated with lower levels of IL-4 and higher levels of IFN-γ. Further, systemic and placental type 1 cytokines are known to be associated with early embryonic death (Munn et al., 1998) and certain cytokines, especially IFN-γ and tumor necrosis factor-α, have been implicated in fetus loss due to placenta tissue damage (Long and Baszler, 1996; Williams et al., 2000). Another possibility is that cytokine production in neosporosis occurs in the lesions or affected organs, and provides a cytokine level sufficient for effective immune functions. Thus, study of causal relationship between vertical transmission mechanisms and cytokine production may also need to focus on local immune responses, such as those in the spleen and placental tissues. In this regard, a study on the role of associated cytokines in congenital infection is now under-way.

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