Post-parturitional changes in the proportion of CD4+ and CD8+ T lymphocytes in *Toxocara canis*-infected mice and their offspring

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ABSTRACT: The aim of this study was to determine the post-parturitional changes in the proportion of the splenic CD4+ and CD8+ subpopulation of T lymphocytes after the birth of *Toxocara canis* infected C57Bl/6 mice and their offspring in relation to the intensity of infection. In mothers infected on the day of mating the proportion of CD4+ T lymphocytes showed an increase for the period of 21 days after the birth in comparison with the control group of the mothers. Conversely, the proportion of CD8+ T lymphocytes showed a significant decline throughout the whole course of the observation. In the offspring of infected mothers the initial increase of CD4+ after the birth turned into a significant decrease in comparison with the offspring of the healthy mothers. The reduction of CD8+ T lymphocytes was detected in the offspring of the infected mothers almost throughout the whole period of observation. The CD4+/CD8+ ratio showed a considerable increase in infected mothers and their offspring in comparison with the control groups. *T. canis* larvae in the muscle of offspring were found for the first time on the fifth day after the birth and the number of larvae showed a moderate increase. The results refer to the changes in the immune regulatory mechanisms in the *T. canis* infected mothers and the high level of miscarriages after infection.

Keywords: CD4+ and CD8+ T cells; *Toxocara canis*; mice; pregnancy; offspring; immune response

The changes in the number and ratio of CD4+ and CD8+ T lymphocytes in the cell immune response are typical phenomena in larval toxocarosis (Kayes, 1997). Both subpopulations play an important role in regulatory and cytotoxic immune mechanisms and in resistance not only in case of toxocarosis, but also in other nematodoses (Cox, 1992; Rottman *et al*., 1997). The helper CD4+ T lymphocytes are essential for the activation, differentiation, and isotype regulation of B lymphocytes and are of a significant help in antigen presentation. After the antigen stimulation they release a whole range of cytokines, as regulatory mediators that determine the development of immune response into Th1 or Th2 type (Sasmaz, 1995). On the other hand, the cytotoxic CD8+ T lymphocytes are responsible for the destruction of structures carrying an antigen on their surface. They also participate in the inhibition of the immune response and regulate eosinophilia (Owhashi *et al*., 1998).

The larval toxocarosis of pregnant hosts is specific for the transplacental and lactogenic transfer of *Toxocara* spp. larvae and for the transfer of the products of the mother's immune system to the offspring. It is not only antibodies that can be transmitted prenatally and postnatally, but also soluble circulating antigens, cells and cytokines. The changes in cell-immunity and mainly in representation of both subpopulations of T lymphocytes associated with pregnancy can increase responsiveness to parasitic infection (Carlier and Truyens, 1995). Larval toxocarosis during pregnancy may cause the decrease of the litter size, which can be explained by the reduction of moth-

Supported by the Slovak Scientific Agency – VEGA (Project No. 2/4179/04) and partially supported by the Slovak Agency for Science and Technology – APVT (Grant No. 51-004702).

er's fertility after transplacental transfer of larvae (Akao *et al*., 1990).

In spite of a relatively well researched immune response at larval toxocarosis the changes in the number of T cell subsets and their ratio in *T. canis* infected mother after the birth as well as in their offspring are recondite.

The aim of this study is to determine the proportion of the splenic CD4+ and CD8+ T lymphocytes after the birth of *T. canis* infected mother as well as in their offspring, during the period of 3 weeks after birth.

MATERIAL AND METHODS

Animals. Experiments were carried out on 54 inbred female C57Bl/6 mice, at the age of 2 months, weighing 25–30 g and their offspring. The animals were kept under standard laboratory conditions at a 12-hr light regime (12 hrs light and 12 hrs dark), at 19–21°C, 50–60% relative humidity and fed *ad libitum* on a commercial pellet food mixture and water.

Isolating and maturing of *Toxocara canis* eggs. The eggs recovered from the uteri of *T. canis* females were isolated from naturally infected puppies and incubated in 0.1 N H_2SO_4 at room temperature until infective larvae developed.

Experimental design. In order to monitor the postpartum changes in the proportion of CD4+ and CD8+ subpopulations of T lymphocytes, the females were infected on the day of mating. The presence of a vaginal mucus plug was taken as day 1 of pregnancy.

Experimental groups: the mice infected with 1 000 eggs of *T. canis* per mouse (Group 1) and their offspring (Group 2).

Control groups: uninfected mothers (Group 3) and their offspring (Group 4).

The spleens of the infected mothers and their offspring were collected before mating and infection (day 0), on the day of birth and after birth on day 1, 3, 5, 7, 10, 14 and 21. The samples from the control groups were collected simultaneously. On each given day 3 mice were euthanized by CO_2 and their spleens analysed individually.

Flow cytometry. Splenic lymphocytes were washed twice with Dulbecco's-PBS at pH 7.2 (Sigma) and once with RPMI 1640 medium (Sigma). Erythrocytes were lyzed in 0.84% NH_4Cl. Pure lymphocytes were resuspended to a final concentration of 1×10^6 cells per 1 ml. After incubation at 4°C for 30 minutes directly with rat anti-mouse CD4 FITC (Sigma) and rat anti-mouse CD8a PE labelled monoclonal antibodies (Sigma) 4 μl per tube cell samples were washed three times in 0.1% sodium azide-PBS and analysed by flow cytometry. Finally, data for 10 000 cells falling within FSC and SSC gates set for lymphocytes were collected from each sample with a FACScan flow cytometer (Becton Dickinson Biosciences). All data files were analysed with CellQuest software.

Larval recovery. In order to detect the presence of migrating larvae in the infected mice their brains were collected into petri dishes, washed with saline, dried on filter paper and finely minced. The larvae were isolated by Baermann's method. Samples of muscle were digested according to Velebny *et al.* (1992) and larvae were counted using a light microscope.

Statistical analysis. The experimental results were evaluated statistically by Student's *t*-test. The differences among the groups were considered significant at the values of $P < 0.05$ and $P < 0.001$.

RESULTS

CD4+ and CD8+ T lymphocytes in infected mothers and their offspring after the birth

Mothers. In uninfected mothers an increase in proportion of T cell subpopulations until day 21 after delivery was observed: in CD4+ from 30 to 47% ($P < 0.05$) and in CD8+ from 22 to 48% ($P < 0.001$). In infected mothers (Group 1) CD4+ T cells decreased prior to giving birth. In comparison with the control group (Group 2), the proportion of CD4+ showed an increase immediately after the delivery and continued to rise until it reached the maximum on day 5. The increased values remained above the values of the uninfected control group until the end of the experiment. In contrast, the CD8+ T cells were significantly ($P < 0.01$) reduced throughout the entire course of the study (Figures 1 and 2).

The ratio CD4+/CD8+ in uninfected mothers (Group 3) was balanced almost during the whole course with the mean value of 0.9 from the day of delivery up to day 21 *post partum*. In infected mothers the ratio increased 2 and 2.5 times with CD4+ dominating in comparison with the uninfected control mice (Figure 3).

Offspring. In offspring of uninfected mothers the values of both subpopulation increased until day 21

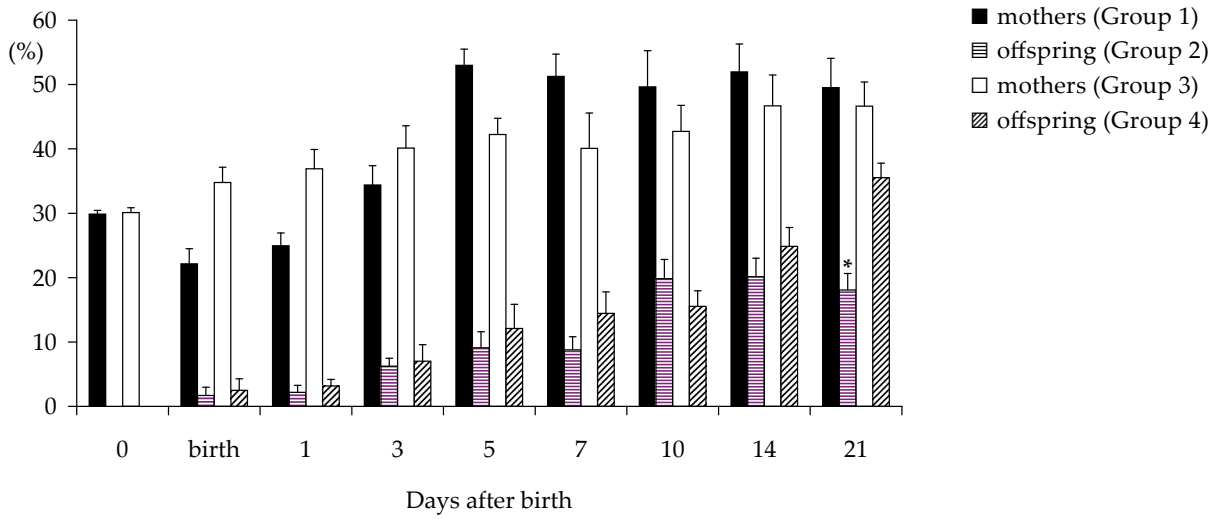

Figure 1. Occurrence of CD4+ T lymphocytes in *Toxocara canis*-infected and uninfected C57Bl/6 female mice and their offspring. Results are expressed as the mean percentage of lymphocytes ± SD (n = 3/group). Differences with statistically significant decrease compared with control group *$P < 0.05$

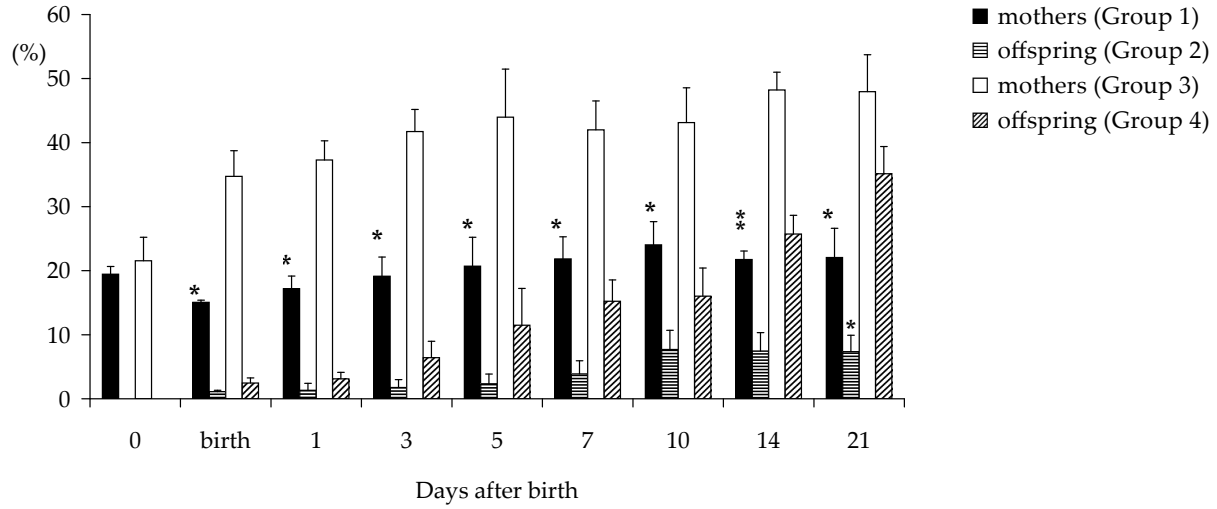

Figure 2. Occurrence of CD8+ T lymphocytes in *Toxocara canis*-infected and uninfected C57Bl/6 female mice and their offspring. Results are expressed as the mean percentage of lymphocytes ± SD (n = 3/group). Differences with statistically significant decrease compared with control group *$P < 0.05$; **$P < 0.001$

after delivery from 2 to 35% ($P < 0.001$) (Figures 1 and 2). The course of curved line of CD4+ T cells in offspring of infected mothers (Group 3) was nearly identical with that of the uninfected control (Group 4) until day 14. The turnover came about on the day 21, when increase was changed with reduction. On the other hand, occurrence of CD8+ T lymphocytes in offspring (Group 3) survived in relatively low level during the entire course of the experiment, in comparison with the control (Group 4) (Figures 1 and 2). In offspring of infected mothers the ratio CD4+/CD8+ increased 3.9 times (Figure 3).

Larval recovery and litter size

The larvae of *T. canis* were detected in the muscle of the offspring on day 5 after birth (6.6 ± 1.4) and their number continued to rise during the course

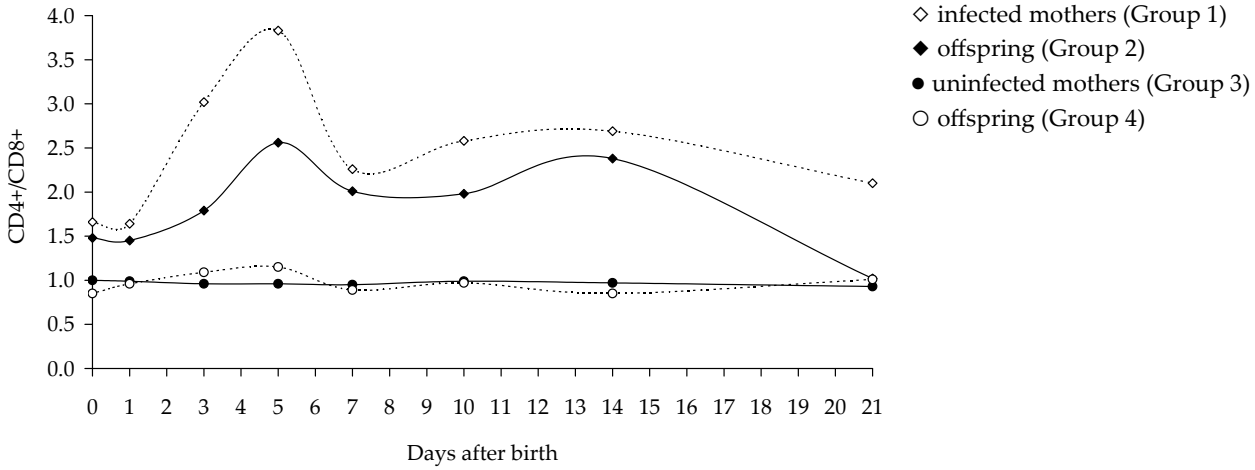

Figure 3. Proportion of CD4+ /CD8+ T cells in *Toxocara canis*-infected and uninfected female mice and their offspring

of the experiment. In mothers, considerably higher mean numbers of larvae were observed (304 ± 39.2) and they varied only moderately in the next days (Table 1). A significant ($P < 0.05$) difference was found in litter size of infected mothers when comparing with the uninfected mothers. Up to 54% of miscarriages belonged to the group of infected females (Table 2).

Table 1. Number of *Toxocara canis* larvae in C57Bl/6 female mice infected with 1 000 ova on the day of mating and their offspring after the birth

Days after birth	Mothers (Group 1)	Offspring (Group 2)
	mean ± SD[a]	
0	376 ± 22.2	0
1	369 ± 24.4	0
3	339 ± 25.6	0
5	304 ± 39.2	6.6 ± 1.4
7	297 ± 29.3	8 ± 1.7
10	299 ± 26.8	10.8 ± 1.9
14	278 ± 43.7	12.2 ± 1.6
21	283 ± 19.9	14.9 ± 1.9

[a]mean number ± standard deviation; n = 3/group

DISCUSSION

The cellular immune response in *T. canis* infection depends on two of the most important subpopulations of T cells with the membrane markers of CD4+ and CD8+ (Kayes, 1997). The changes in their numbers and proportion ratio in the blood and in the organs when infected by larval toxocarosis may influence the production of serum cytokines and therefore disturb the immune balance. Their importance in connection with larval toxocarosis has not been clarified so far and there are only few papers dealing with this issue (Takamoto *et al*., 1995; Owhasi *et al*., 1998).

The changes in the ratio of both subpopulations in the offspring during the period after birth clearly showed the evidence of age dependency in both

Table 2. Delivery of *Toxocara canis*-infected mice

Experimental mice	Mothers					Newborn	
	total number	delivery	%	pregnancy loss	%	total	mean ± SD[a]
Infected Group 1	24	11	45.8	13	54.1	56	5.13 ± 1.2*
Uninfected Group 3	18	18	100	0	0	118	6.56 ± 1.3

[a]mean number ± standard deviation in one gestation; n = 3/group

*significant of differences between infected and uninfected mice $P < 0.05$

uninfected and infected mice, i.e. the cell percentage increased in proportion to age. We recognised more expressive age dependency of CD4+ in comparison with CD8+. This result corresponds to that of other authors examining other animals (dog, pig) (Joling *et al*., 1994; Greeley *et al*., 1996; Faldyna, 1998). When assessing the results, we have to consider the factor of age dependency, which can measurably distort the results. This phenomenon can also be accompanied by a simultaneous increase of total T cell population.

Pregnancy and a simultaneous infection are important risk factors for mothers and their embryo and can affect both T cell subpopulations. During gestation and after the birth several changes occur in the immune system. Among these there is the influenced number and function of both CD4+ and CD8+ T cells. The number of CD4+ T cells decrease, generally towards the end of the gestation, however, CD8+ increase (Landers *et al*., 1991). Contrary to this observation, our experiments did not show a suppression of CD4+ and an increase of CD8+ T cells described during gestation in healthy mothers or their offspring in the postpartum period. On the contrary, the cytotoxic CD8+ subpopulation significantly decreased in the infected mothers. We can suppose that the reduction of these cells in infected mothers after the delivery, as well as in their offspring, may negatively affect the complex cytotoxic immune mechanisms. It could be one of the reasons that contribute to the increased responsiveness towards repeated infection with *T. canis* after the birth. The reduction of cytotoxic CD8+ probably originated during the pregnancy but we cannot eliminate the immunosuppressive effect of *T. canis* larvae in the infected hosts, which was also observed by other authors (Yamashita *et al*., 1993; Soltys *et al*., 1996; Wnukowska and Dzebenski, 2001).

From the presented results it can be concluded that the infection of mothers during gestation considerably influenced the CD4+/CD8+ ratio after the birth. The changes in ratio of T lymphocyte subpopulations are manifested mainly in cases of immunoregulation malfunctioning and their detection has become an important diagnostic tool even in infectious diseases. There was a 2.5 times increase of CD4+ against CD8+. We can assume that the changes in this proportion of T cell subsets make other modifications in the defence mechanism of infected mothers. Unfortunately, it is not quite clear yet how these changes influence the total immune status of offspring after the birth.

The presence of *T. canis* larvae in the offspring of infected mice was observed for the first time on day 5 after the birth, which proves the lactogenic transmission. The transfer of larvae in paratenic hosts in most cases is via milk, whereas, the intrauterine migration is only sporadic (Tomasovicova *et al*., 1993). Transplacental transfer of larvae was observed i.e. in rats after high infective doses in the case of other helminthosis (trichinellosis) (Cosoroaba and Orjanu, 1998; Nunez *et al*., 2002). The intrauterine infection may result in immunotolerance, a persistent infection, foetal death or its survival. In our case, using C57BL6/J mouse strain we observed a significant difference in the amount of litter of infected mothers in comparison with the uninfected control group. According to the results of Akao *et al*. (1990) *T. canis* infection during pregnancy in different mouse strain (Balb/c) caused the decrease in the amount of the litters. The reason for that may have been apart from reduced fertility also foetal death and resorption caused by transplacental invasion of *T. canis* larvae. Up to 54% of the infected females aborted. It is the high frequency of miscarriages of infected mothers in our case that did not enable us to observe the immune responses in this group during pregnancy.

A more detailed study of this subject, particularly that of the complex mechanisms regulating the immune response after *T. canis* infection, would undoubtedly be of great benefit to neonatal medicine.

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Received: 03–03–31
Accepted after corrections: 04–01–13

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