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Studies on the development and role of some cell-mediated immune responses in experimental toxocarosis in mice

Natalia Wnukowska and Tadeusz H. Dzbeński*

Department of Medical Parasitology, National Institute of Hygiene, Chocimska 24, 00-791 Warsaw, Poland

Abstract

The studies were undertaken to investigate the development of some cell-mediated immune responses in experimental toxocarosis in mice and to assess the influence of these responses on the course of infection. Mice were infected orally with 350 eggs of *Toxocara canis* and reinfected with the same dose of parasites after 8 weeks. Groups of infected animals were killed each week of the experiments to obtain spleens, livers and brains for further studies. Lymphocytes from removed spleens were analysed by flow-cytometry for CD4 and CD8 expression and cultured *in vitro* to measure their responses to Concanavalin A and excretory-secretory (ES) antigen of *T. canis* in a lymphocyte transformation test. Pieces of livers were used to prepare paraffin sections to be stained later with haematoxylin and eosin, whereas whole brains of the infected animals were examined for the presence of parasite larvae. The results of the studies showed depression of T-cell responses to ConA in early stages of infection and significant increase in the blastogenic responses to the ES antigen from week 4 following infection. The depression of T-cell responses was accompanied by lowered CD4+/CD8+ ratio resulting from increased percentages of CD8+ T cells. Histopathological examination of liver sections revealed trapping of larvae in *T. canis* reinfected mice. The intensity of infection as measured by larval recoveries from the brains of mice increased gradually up to the 8th week of infection, but did not show significant changes after reinfection, testifying to the development of long-lasting protective immunity during primary infection.

Keywords

Toxocara canis, CMI responses, protective immunity

Introduction

Toxocarosis is a parasitic zoonosis due to the infection with larvae of either *Toxocara canis* or *Toxocara cati*. In the definitive host, i.e. in the domestic dog and cat, the parasites live as adults within the lumen of the small intestine, but in the accidental host, such as a man or a mouse, the parasites fail to develop to mature adult worms. When mature eggs are ingested by an accidental host the larvae hatch in the intestine, then enter a blood vessel and migrate through the liver and lungs to the left heart where they are eventually disseminated throughout the body, including vital organs such as the brain and eye.

Migrating *Toxocara* larvae induce a persistent immune response, characterised by eosinophilia, leucocytosis, hypergammaglobulinaemia and production of IgM, IgG and IgE antibodies. During somatic migration the larvae may become encapsulated within granulomas which are a form of cell-mediated immunity, however, at least some of them are capable to escape from the trap and continue the migration. Although the above mentioned immune responses are more pronounced in reinfections with toxocaral larvae it is not quite clear whether the evoked immunity is protective to the host at all (Despommier 2003).

The aim of the experiments described below was to investigate the development of some cell-mediated immune responses in experimental toxocarosis in mice, and to assess the influence of these responses on the course of reinfection.

Materials and methods

Experimental animals

Young adult CFW/Pzh mice of both sexes weighing 18–20 g were used throughout the experiments. The mice were infected orally with a dose of 350 mature eggs of *T. canis* and rein-

*Corresponding author: tdzbenski@pzh.gov.pl

fected with the same dose of parasites after 8 weeks. Groups of infected animals were sacrificed by cervical dislocation each week of the experiments to obtain spleens, livers and brains for further studies.

The experiments were performed according to the approval by the IV Ethics Committee for Animal Experimentation.

Parasites

Eggs of *T. canis* were squeezed out of the terminal portions of the uteri of dissected females and kept in 1% formalin solution at 27°C to reach maturity. The eggs used for infecting the animals were obtained from cultures kept no longer than 4 months. An infective inoculum was prepared by diluting suspension of eggs to obtain a dose of 350 mature eggs in a volume of 100 µl.

Preparation of excretory-secretory (ES) antigen of T. canis

The antigen was prepared essentially as described by de Savigny (1975). Larvae of *T. canis* were liberated from eggs by incubation in a solution of 7% sodium hypochlorite for 30 min at 37°C, then they were transferred to culture flasks (Nunc) filled with Eagle's minimum essential medium supplemented with penicillin (100 iu/ml), streptomycin (0.25 mg/ml) and nystatin (0.1 mg/ml). The larvae suspended in culture fluid were kept at 37°C in an atmosphere of 5% CO_2 in amounts of 10,000/ml of a fluid. Culture fluids containing toxocaral ES antigen were collected at weekly intervals and frozen at –20°C. Pools of 200–500 ml of the fluid were dialysed against distilled water, then freeze-dried.

Isolation of murine splenocytes

Spleens were mashed in PBS with all-glass tissue grinder, strained through double layer gauze and spun down for 10 min at 300 g. The red blood cells present in a pellet were lysed with a solution of ammonium chloride and the remaining splenocytes were washed by triple centrifugation in Eagle's medium, then checked for viability by trypan blue dye exclusion test and counted with haemocytometer.

Lymphocytes obtained from the spleens of age-matched, uninfected CFW/Pzh mice served as controls in the tests described below.

Lymphocyte transformation test

Splenocytes at a concentration of 2×10^6/ml were cultured in Eagle's minimum essential medium containing 10% heat-inactivated foetal bovine serum, L-glutamine and gentamycin in wells of 96-well microtitre trays. Blastogenic responses of the cells were stimulated with either Concanavalin A (ConA) or *T. canis* ES antigen. Concentrations of 2.5 µg ConA per ml of a culture fluid and 50 µg of the ES antigen per ml of the culture fluid were found to be optimal in initial experiments and were employed later in all experiments. Cultures of splenocytes and mitogen and splenocytes and antigen were kept at 37°C in a humidified atmosphere containing 5% CO_2 for 3 and 5 days, respectively. For the last 18 h the cultures were incubated with 1 µCi/microtitre well of tritiated thymidine (Amersham), then they were collected using a multiple automated sample harvester (Scatron) and processed in a plastic dishes with 3 ml of scintillation fluid (1l toluene, 0.1 POPOP, 4.0 PPO). Incorporation of tritiated thymidine was assessed in a liquid scintillation counter (Packard) and the results were expressed as counts per minute (cpm) or stimulation index – SI (difference between the cpm of stimulated and unstimulated lymphocytes divided by the cpm of unstimulated lymphocytes).

Flow cytometric analysis of spleen lymphocyte subpopulations

Viable splenic lymphocytes isolated as described above were adjusted to 1.5×10^6/ml and each 100 µl of the cell suspension were stained with 2 µl of fluorescein-conjugated rat anti-mouse CD4 (L3T4) (PharMingen) and 2 µl of phycoerythrin-conjugated rat anti-mouse CD8a (Ly-2) (PharMingen) for 30 min at 4°C, then washed with "cell wash" (Becton Dickinson), and resuspended in 1% buffered formalin. Samples were analysed on FACS Calibur flow cytometer with Cell Quest programme (Becton Dickinson) selecting 5×10^3 cells for the analysis.

Evaluation of the intensity of infection in vital organs (*brain*)

Groups of infected mice were killed by cervical dislocation at each week of the experiments then the brains were removed, crushed gently in a mortar and pestle and digested with a solution of 1.5% pepsin in 0.1 M hydrochloric acid for 1 h at 37°C. The resulting homogenate after being centrifuged at 210 g for 10 min was resuspended in PBS and checked for the presence of larvae under a microscope, then the number of larvae was counted.

Histopathologic examinations

Livers obtained from infected animals at 8th week of infection and reinfection were subjected to histopathologic examination. To achieve this end, small pieces of the removed livers were fixed overnight in 10% buffered formalin, then passed through a graded series of ethanol solutions, washed with acetone, immersed in xylene and finally embedded in paraffin. Sections were cut at a thickness of 4 µm, then they were deparaffinised and rehydrated for routine staining with haematoxylin and eosin.

Statistical analysis

Student t-test was used to compare two means when the assumptions of normal distribution and equal variances were not rejected. In other cases a non-parametric test of Mann-Whitney was employed. In the case of multiple comparison a non-parametric method of Kruskal-Wallis followed by Mann-Whitney test were employed. The criterion for significance was taken as $p<0.05$. The values of $0.05<p<0.1$ were considered borderline.

Results

Lymphocyte transformation test

Unstimulated spleen lymphocytes from infected mice incorporated spontaneously more tritiated thymidine than lymphocytes from uninfected animals (Table I). The difference between spontaneous transformation shown by lymphocytes from infected and reinfected mice was insignificant. ConA stimulation of lymphocytes from infected mice showed depression of blastogenic responses in the first 3 weeks of infection when results were expressed as counts per minute, and significant depression when they were measured by stimulation index (p = 0.023, 0.004, 0.038). The responses were not depressed during reinfection (Table I).

Stimulation of cultures of lymphocytes with *Toxocara* ES antigen did not demonstrate significant differences in cpm

Table I. Means of cpm and SI of spleen lymphocytes from mice infected with *Toxocara canis* and stimulated/unstimulated with ConA

wpi	No.	A	(SE)	p*	B	(SE)	p*	SI	(SE)	p*
0	26	392.6	(36.5)		96031.4	(12935.2)		234.3	(23.9)	
1	6	724.8	(135.0)	0.019	69557.8	(14687.2)	ns	98.7	(13.5)	0.023
2	6	649.3	(54.6)	0.004	47149.7	(11267.9)	ns	72.1	(17.0)	0.004
3	6	652.9	(102.6)	0.016	74892.0	(9851.0)	ns	118.3	(18.1)	0.038
4	6	529.9	(102.9)	ns	103170.3	(20416.6)	ns	240.2	(54.3)	ns
5	6	445.8	(68.1)	ns	125471.2	(10078.5)	ns	308.6	(40.0)	ns
6	6	542.9	(143.7)	ns	120088.9	(12883.8)	ns	262.2	(41.3)	ns
7	6	476.1	(76.2)	ns	124510.0	(11056.9)	ns	287.0	(38.1)	ns
8	6	426.4	(53.8)	ns	142725.3	(19502.1)	ns	345.8	(46.6)	0.091
9	6	1035.5	(163.1)	<0.001	124474.4	(17824.9)	ns	147.4	(40.6)	ns
10	6	753.1	(188.0)	0.03	94058.8	(17111.7)	ns	177.8	(69.3)	ns
11	6	502.3	(76.0)	ns	98710.4	(6163.9)	ns	232.7	(48.5)	ns
12	6	675.9	(83.8)	0.006	130483.0	(23028.7)	ns	197.1	(46.2)	ns
13	6	678.9	(121.8)	0.026	92927.9	(7752.5)	ns	174.6	(51.8)	ns
14	6	435.6	(96.1)	ns	95192.3	(11807.4)	ns	255.0	(47.1)	ns
15	6	413.3	(59.4)	ns	119311.1	(30421.6)	ns	269.5	(33.1)	ns
16	6	344.8	(56.2)	ns	71373.64	(10105.3)	ns	228.0	(32.8)	ns

wpi = week post infection; 0 = control group of lymphocyte cultures from uninfected mice; 1–8 = infection; 9–16 = reinfection; No. = number of examined mice (lymphocyte cultures); SE = standard error; A = cpm of lymphocytes from unstimulated cultures; B = cpm of lymphocytes from cultures stimulated with ConA; SI = stimulation index (B-A)/A; p* = significance of differences between values obtained from experimental and control cultures (Mann-Whitney test); ns = insignificant difference.

Table II. Means of cpm and SI of spleen lymphocytes from mice infected with *Toxocara canis* and stimulated/unstimulated with toxocaral ES antigen

wpi	No.	A	(SE)	p*	B	(SE)	p*	SI	(SE)	p*
0	32	314.4	(15.0)		1284.6	(64.2)		3.2	(0.2)	
1	6	525.8	(82.3)	0.01	1036.9	(131.3)	ns	1.0	(0.2)	<0.001
2	6	597.8	(115.8)	0.004	1380.1	(199.4)	ns	1.6	(0.4)	0.004
3	6	440.8	(53.3)	0.023	1507.3	(242.6)	ns	2.4	(0.3)	0.082
4	6	585.1	(134.3)	0.01	2245.8	(402.7)	0.008	3.3	(0.8)	ns
5	6	652.3	(166.1)	0.016	3261.9	(668.2)	<0.001	4.8	(1.0)	ns
6	6	669.7	(146.1)	0.002	3702.8	(709.4)	<0.001	4.9	(0.8)	0.041
7	6	482.7	(77.4)	0.013	3123.0	(502.9)	<0.001	5.6	(0.9)	0.013
8	6	637.6	(147.6)	0.006	4467.2	(1269.8)	0.001	5.5	(1.0)	0.011
9	6	910.9	(78.3)	<0.001	8693.1	(1775.1)	<0.001	9.0	(1.9)	<0.001
10	6	752.2	(150.6)	<0.001	7939.4	(1135.5)	<0.001	11.2	(2.8)	<0.001
11	6	600.7	(128.4)	<0.001	6237.7	(1122.2)	<0.001	9.9	(1.5)	<0.001
12	6	888.5	(180.3)	0.008	6910.4	(2222.2)	<0.001	8.4	(0.9)	<0.001
13	6	597.3	(105.5)	<0.001	4593.8	(1023.2)	<0.001	7.1	(1.6)	0.05
14	6	493.4	(43.6)	<0.001	3720.2	(897.9)	0.001	7.6	(2.5)	ns
15	6	422.9	(31.8)	0.007	3239.2	(972.3)	0.001	7.2	(1.7)	0.007
16	6	576.0	(125.7)	0.046	4755.8	(1617.3)	<0.001	7.8	(2.2)	0.02

wpi = week post infection; 0 = control group of lymphocyte cultures from uninfected mice; 1–8 = infection; 9–16 = reinfection; No. = number of examined mice (lymphocyte cultures); SE = standard error; A = cpm of lymphocytes from unstimulated cultures; B = cpm of lymphocytes from cultures stimulated with ES antigen; SI = stimulation index (B-A)/A; p* = significance of differences between values obtained from experimental and control cultures (Mann-Whitney test); ns = insignificant difference.

between the cultures set up within 3 weeks after infection and control cultures of lymphocytes from uninfected mice. Significant increase in the blastogenic response was noted starting from week 4 following infection (Table II), however, mean cpm value for cultures established during 4–8 week of infection was significantly smaller than the mean for reinfection cultures (p = 0.003). When the results of lymphocyte transformation under the influence of the ES antigen were expressed as SI, significant depression of the response were observed for 2 weeks following infection (Table II).

Flow cytometric analysis of spleen lymphocyte subpopulations

The effects of infection on splenocyte cell subpopulations are presented in Table III. The CD4+/CD8+ ratio was significantly lowered in the first 2 weeks of primary infection (p = 0.04,

Table III. Mean percentages and proportion of CD4+/CD8+ cells in the spleens of *T. canis* infected mice

wpi	No.	CD4+	(SE)	p*	CD8+	(SE)	p*	CD4+/ CD8+	(SE)	p*
0	16	35.30	(1.53)		23.15	(1.24)		1.57	0.09	
1	6	34.68	(4.67)	ns	27.46	2.74	ns	1.25	0.07	0.04
2	6	33.24	(3.06)	ns	30.90	2.32	0.01	1.11	0.11	0.002
3	6	42.08	(2.09)	0.027	26.36	3.16	n.i.	1.76	0.21	ns
4	6	42.86	(1.86)	0.008	26.55	1.94	0.083	1.66	0.14	ns
5	6	38.59	(5.96)	ns	28.07	3.31	ns	1.47	0.33	ns
6	3	39.30	(2.38)	ns	21.19	1.65	ns	1.86	0.06	ns
7	3	39.80	(2.15)	ns	19.13	0.61	ns	2.09	0.18	0.047
8	3	29.56	(1.88)	ns	18.61	3.02	ns	1.73	0.17	ns
9	3	30.31	(5.53)	ns	19.61	1.61	ns	1.57	0.28	ns
10	3	30.15	(3.69)	ns	17.16	2.47	0.085	1.78	0.17	ns
11	3	29.72	(3.47)	ns	15.81	1.97	0.008	1.91	0.22	ns
12	3	32.90	(0.51)	ns	16.54	1.28	0.014	2.01	0.13	0.064
13	3	37.91	(1.01)	ns	15.52	1.38	0.008	2.48	0.19	0.004
14	3	38.81	(1.62)	ns	21.62	2.72	ns	1.87	0.29	ns
15	3	40.61	(1.98)	ns	24.75	3.60	ns	1.70	0.22	ns
16	3	33.62	(2.10)	ns	17.42	5.02	ns	2.21	0.50	ns

wpi = week post infection; 0 = cells from control group of uninfected mice; 1–8 = infection; 9–16 = reinfection; No. = number of examined mice (spleens); CD4+ = mean percentage of CD4+ cells; CD8+ = mean percentage of CD8+ cells; SE = standard error; p* = significance of differences between values calculated for cells from experimental and control groups of animals (Mann-Whitney test); ns = insignificant difference.

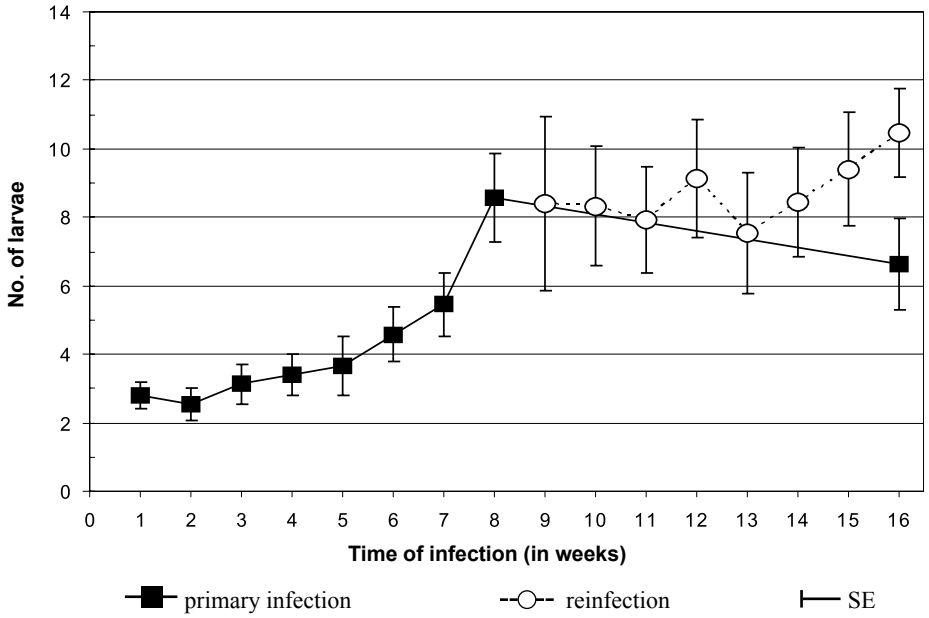

Fig. 1. Numbers of *T. canis* larvae recovered from brains of experimentally infected mice (means from 6 animals ± SE; weeks 8 and 16 – means from 24 animals)

p = 0.002), then returned to the values calculated for uninfected mice and raised significantly at weeks 7th of primary infection and 5th of reinfection. The decreased CD4+/CD8+ ratio resulted from an increased percentages of CD8+ T cells at the time (significant at week 2 of infection; p = 0.01).

Histopathological examinations

Histopathological examination of liver sections taken from mice in week 8 of infection and in week 8 of reinfection revealed numerous changes of similar character, however, those observed after reinfection were far more advanced and extensive. Typical changes observed at the end of primary infection consisted of multiple foci of cellular infiltrates composed mainly of polymorphonuclear neutrophils and eosinophils, which were located along the routes of migrating larvae. Prolonged search of sections of livers removed during primary infection failed to demonstrate parasite larvae, but they were easily found in sections taken after reinfection. Around the entrapped larvae inflammatory infiltrates consisting predominantly of eosinophils and progressing eventually towards well-structured granuloma were observed. Granulomas advanced in development showed peripheral fibrosis.

Larval counts in the brains

An intensity of infection as measured by larval recoveries from the brains of experimental mice increased gradually up to the 8th week of infection. The number of larvae found at the end of 8th week did not differ significantly from that demonstrated in control mice after 16 weeks from infection. In reinfected mice the number of larvae showed an insignificant increase (p>0.1; Student t-test) from week 8 to the end of observation period (Fig. 1).

Discussion

The results of the present studies have shown that mice infected with 350 mature eggs of *T. canis* and reinfected 8 weeks later with the same dose of parasites develop strong antitoxocaral immunity. The immune response was observed after initial depression of some activities of the immune system as indicated by lowered blastogenic response to the T-cell mitogen ConA in the first 3 weeks of infection. The lymphocyte transformation response of mice infected with *T. canis* was less than that of uninfected mice when results were expressed either as cpm or SI. Although the values of SI were significantly smaller at the time, it is the decrease in cpm that provides more convincing evidence of the immune depression because the values of SI were strongly influenced by high background of radioactivity. Similar objections may be raised against significance of lowered SI values of lymphocytes from cultures set up within 2 weeks of infection and stimulated with the ES antigen of *T. canis*.

The depression of T-cell responses to ConA in the early stages of experimental toxocarosis in mice was first reported by Yamashita *et al*. (1993) who used C3H/HeN *T. canis*-infected mice, and later by Aldawek *et al*. (2002) who worked with animals of C57BL6 strain. The results obtained by the authors mentioned above and our own investigations are in apparent contradiction with the reports of Kayes (1984) and Kayes *et al*. (1985) who found that ConA-induced T-cell responses were augmented in CBA/J infected mice. A possible reason for that discrepancy could be the difference in mouse strains used, because in later experiments Kayes *et al*. (1987) also observed impaired ConA responses using C57BL/6 mice. The mechanisms responsible for the depression are not clear although Yamashita *et al*. (1993) ascribed the phenomenon to the activities of suppressor macrophages. In our studies, while investigating the effects of infection on splenocyte cell subpopulations, we found significantly decreased ratio of CD4+/CD8+ in the first 2 weeks of primary infection. The ratio was decreased owing to an increase in the percentage of CD8+ T cells, a fact that was also noted in the work of Aldawek *et al*. (2002), and it is tempting to speculate that augmented CD8+ T lymphocyte subpopulation included suppressor CD8+ T cells. The role of these cells in an immune regulation was originally described in the 1970s and then it was forgotten for decades but recalled recently to life by findings of Noble *et al*. (2006) and stressed by Metwali *et al*. (2006) in their work on immunity to *Heligmosomoides polygyrus* in mice.

Cellular immune reactions to the ES antigen of *T. canis* measured by lymphocyte transformation test increased significantly from the 4th week of infection and showed an anamnestic response after reinfection. The above mentioned observation is compatible with the results of histopathological examination of liver sections which revealed trapping of larvae in *T. canis* reinfected mice. Inflammatory infiltrates consisting predominantly of eosinophils surrounded the larvae or their remnants, then encased them in fully grown granulomas showing fibrosis at the periphery. The phenomenon was described earlier by Kayes and Oaks (1978) and Sugane and Oshima (1983) who found, that far more larvae were trapped in the liver after reinfection than after the primary infection. In our studies we were unable to detect larvae in sections prepared at the end of primary infection, i.e. in the 8th week, but found them easily after reinfection. It has been suggested that infiltration of eosinophils around larvae obstructs their movement in the liver which renders them more vulnerable to killing and prevents their access to vital organs, such as the central nervous system.

To test the hypothesis we performed an experiment in which the number of larvae recovered from the brains of mice after 8 weeks of infection was compared with that after 16 weeks of primary infection and 8 weeks of reinfection. The experiment has shown that the numbers of larvae recovered from the brains at the above observation time points were similar, which allow to make the following conclusions: (1) the process of larval migration to the brain is practically accomplished by the end of 8th week from infection; (2) larvae can survive in the brain for at least 16 weeks; (3) larvae from reinfecting dose do not add significantly to the number of those

already present in the brain; (4) primary infection stimulates protective immunity which does not harm the larvae that have already found their way to the brain, but kills most parasites of subsequent infection.

The development of acquired immunity to *Toxocara* infection in mice has already been reported by Abo-Shehada *et al*. (1991) who found that animals which had been infected twice at 7-days interval showed reduction in brain larval recoveries compared with animals which had received the same number of larvae in one dose. However, the described immunity was short-lived, because the significant reduction in brain larval count on day 16 post-infection appeared insignificant when measured on day 35. Our studies seem to be the first report on a long-lasting protective immunity to *Toxocara* infection and provide some grounds for future research on vaccination against toxocarosis.

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