DOI 10.2478/s11687-006-0014-0

VERSITA

HELMINTHOLOGIA, 43, 2: 69 – 75, JUNE 2006

Immunological changes after multiple *Toxocara canis* infection of lambs

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Summary

To estimate the effect of lambs' immunoreactivity to multiple *Toxocara* infection, subpopulations of lymphocytes, determination of specific IgG levels, proliferation activity of splenic lymphocytes, and metabolic activity of granulocytes were performed. Five-month-old Valaška lambs were daily infected with 1000 embryonated *Toxocara canis* eggs for 23 days and immune responses were studied up to 49 days post infection (dpi). The number of leukocytes and neutrophiles was no significantly higher on 12 hrs pi in infected group. Absolute counts of eosinophiles were increased on 14 dpi and significantly increased on 21 d in infected animals. Greater numbers of CD2+ T lymphocytes were observed during the course of the experiment in the peripheral blood of infected animals. The level of CD4+ cells was lower 42 dpi, but the number of CD8+ cells was higher in the experimental animals. IgM positive B cells were significantly increased on 28 dpi, and monocytes on 12 hours, 14 and 35 dpi in infected animals. Production of anti- *T. canis* IgG was significantly enhanced on 28 dpi in infected lambs. Significant increased proliferative response of splenic T and B lymphocytes was found on 14 and 21 dpi. Metabolic burst of granulocytes demonstrated the decrease of percentage values from 3 dpi. Larvae of *T. canis* were microscopically observed in the peripheral blood from 14 to 35 dpi. The multiple reinfection of sheep with 1000 *T. canis* eggs caused eosinophilia, increased the proliferation activity of B- and T-cells and increased the production of *T. canis* specific antibodies. The cooperation of immune cells has directed to kill the larvae in the peripheral blood and trapping them in granulomas.

Key words: *Toxocara canis*; sheep; flow cytometry; immune response; paratenic host

Introduction

Larval toxocarosis is a serious and widespread zoonosis. Animals and people living in contaminated habitat are exposed to a noticeable risk from permanent infection mostly by a lower and repeated dose of *T. canis* eggs (Borošková *et al*., 1999).

The disease results in a significant modification of the immune system, which manifests in a higher lymphoproliferative activity, eosinophilia and specific antibody production. The immune response during toxocarosis tends to the rise of infection's resistance (Kayes *et al*., 1985). By Kayes (1984), primary *T. canis* infection of paratenic host not followed to the development of splenic immunosuppression to larval antigens, which are an important immune stimulator during the parasite migration.

On the other hand, single infection of mice with *T. canis* and *T. cati* temporarily suppressed functional activity of lymphocytes and caused the changes in specific antibody production and IL-2 production (Yamashita *et al*., 1993; Šoltýs *et al*., 1996). Gupta *et al*. (1990) supposed the presence of immunosuppressive substances to *T. canis* ES antigen. Immunosuppression was detected in pregnant bitches and infected puppies, respectively (Lloyd, 1983). Little attention has been paid to the host immune response after *T. canis* reinfection, which occurred in men and animal in a natural conditions and is responsible for the chronic course of toxocarosis.

The aim of this study was to study the modification of the immune response in the peripheral blood of lambs – paratenic host – to long term *T. canis* infection. Determination of haematological data, lymphocyte subpopulations, specific antibodies, proliferative activity of T and B lymphocytes, and metabolic activity of granulocytes from the peripheral blood was performed.

Material and Methods

Animals

Twenty-three five-month-old Valaška lambs were divided into an experimental group (n = 18) and a control group (n = 5). Before starting of the experiment the lambs were coprologically examined for the presence of parasite oocyts and eggs (flotation and sedimentation methods), and larvae

(Baerman method). The animals were treated with Sulfacox obtained from Pharmagal (Nitra, Slovakia) to remove detected coccidia. When the rectal faecal samples were proved to be negative for parasite eggs and larvae, the lambs were used in the experiment.
Each animal in the experimental group was infected orally with 1000 embryonated *T. canis* eggs every morning before feeding during 23 days of the experiment. The eggs of *T. canis* were mixed in bread crumb. *Toxocara canis* eggs were isolated from the worm uteri according to Costello (1961) and parasite was obtained from the intestine of some infected puppies. The control animals were not infected. The lambs of the experimental and control group were kept separately in two different places in pens, housed in a stable, and fed with a standard feed dose.

Experimental design
The lambs were infected during 23 days, and the total duration of experiment was 49 days. The blood of each animal was collected from *v. jugularis* 12 hrs after infection (0 dpi) and then at days 1, 3, 7, 14, 21, 28, 35, 42, and 49 days pi.
The blood was collected into heparinized tubes for examination of haematological data, the proliferative activity and metabolic burst assays, and into EDTA (0.1 ml 1.5 % ethylenediamine tetra-acetic acid per ml of blood; Lachema Brno, Czech Republic) for flow cytometry. Serum samples were obtained for the determination of IgG specific antibody levels.

Counting of total and differential leukocytes
Blood samples were transferred into Turk's solution (in dilution 199:1). The cells were counted by routine laboratory method. Differential cell counts were made on blood smears after staining with a Hemacolor kit (Merck, Germany) by counting 100 cells per slide. Absolute leukocyte counts were computed as follows: WBC count x % of differential types of leukocyte.

Haematological data
Determination of erythrocytes was made by routine laboratory method with Hayem solution (Slanina *et al*., 1985).
The values of haematocrit was performed using the assay described by Winthrob (Slanina *et al*., 1985)
The amount of haemoglobin was measured spectophotometrically by Hrubiško *et al*. (1981) using of Sevatest Standard Hb (Imuna, Šarišské Michaľany, Slovakia).
The values of red blood cells – MCV, MCH, and MCHC were computed from the number of erythrocytes, values of haematocrit and haemoglobin (Slanina *et al*., 1985).

Flow cytometry
An indirect immunofluorescent method and whole blood staining were used. Mouse anti-ovine primary monoclonal antibodies (MAbs) are summarized in Table 1. They were obtained from Veterinary Medicine Research and Development, Inc., USA (CD2, CD4, IgM) and Serotec, UK (CD8 and Mf). FITC-conjugated $F(ab')_2$ fragment of anti-mouse immunoglobulins (Code No. F 0479, Dako, GB) was used as secondary antibody.
Whole blood in EDTA (100 µl) was added into the tube and incubated with 50 µl prediluted primary monoclonal antibody 15 min at room temperature in dark Lysis of erythrocytes with 2 ml lysing solution per tube (NH_4Cl 8.26 g, $NaHCO_3$ 1 g, NaEDTA 0.037 g in 1 l distilled H_2O) was followed during 10 min at room temperature. The tubes were centrifuged (250 g, 5 min) and the cells were washed twice in PBS. The pellets were mix with 25 µl secondary antibody (dilution 1:50) and incubated 30 min at 4˚C in the dark. After being stained, the cells were washed twice with PBS and resuspended in 0.2 ml of fixative solution (1 % paraformaldehyde in PBS).

Analysis of stained cells
Flow cytometric analysis was performed using FACScan (Becton Dickinson, Germany). Gates were drawn around the lymphocytes based on 90º and forward-angle light scatter. Fluorescence data were collected on at least 10 000 lymphocytes. The results are therefore expressed as the relative percentage of the lymphocyte population, which was positive for specific MAbs. The absolute number of lymphocytes was calculated by multiplying absolute counts of lymphocytes and relative percentage of lymphocytes.
The obtained data were statistically evaluated by two-tailed paired Student *t*-test.

Determination Toxocara-specific IgG production by ELISA
Sera were examined by the modified indirect ELISA method using ES antigen according to Havasiová-Reiterová *et al.* (1995).

Assaying the proliferative activity of splenic lymphocytes in vitro
Concanavalin A (final concentration 3 $\mu g.ml^{-1}$) was used as a polyclonal activator in T-cells and lipopolysaccharide (final concentration 6 $\mu g.ml^{-1}$) was used for B-cells (both activators, Sigma, Germany), according to the method of Pagé *et al*. (1988).

Metabolic burst of phagocytes
Metabolic activity of granulocytes was performed by flow cytometry. The commercial kit BURSTTEST (Orphegen-Pharma, Germany) was used.

Isolation of T. canis larvae from peripheral blood of infected lambs
The blood was collected from *v. jugularis* immediately after killing of experimental lambs in volume of 500 ml per animal. The blood was haemolysed with H_2O and subsequently it was artificially digested (1 % pepsin, 1 % HCl for 2 hours at 37°C). Samples of digestive blood were allowed to settle for 2 hours. The sediment was checked over for the presence of *T. canis* larvae using a stereomicroscope.

Results

The absolute count of leukocytes, lymphocytes and neutrophils is shown in Tab. 2.

The total count of leukocytes was no significantly increased on 12 hrs post infection (pi), and slightly higher values of leukocytes were observed during throughout experiment in infected lambs. An increase in neutrophils, decrease in lymphocytes on 12 hrs pi was observed. Absolute counts of neutrophils in experimental lambs were slightly higher at 7 dpi and from 35 dpi to the end of the experiment. On the other hand, no significantly lower values of lymphocytes were increased from 7 dpi to the end of the experiment.

Table 1. Primary monoclonal antibodies used

Specificity	MoAbs	Isotype	Dilution
CD2	36F-18	IgG2a	1:50
CD4	74-12-4	IgG2b	1:50
CD8	MCA837F	IgG2a	1:25
IgM	PIG45A	IgM	1:25
Mf	MCA919	IgG1	1:25

Eosinophilia was demonstrated from 14 dpi to the end of experiment (Fig. 1) with significance on 21 dpi ($P < 0.05$).

The absolute monocytes count (Fig. 2) was significantly higher on 12 hrs pi ($P < 0.05$), 14 dpi ($P < 0.01$), and 35 dpi ($P < 0.05$).

Red blood cell counts showed lower values in infected lambs from 1 dpi, and hematocrit from 14 dpi with significance ($P < 0.05$) to the end of experiment (Tab. 3). Because the level of haemoglobin (Tab. 3) and calculated values (MCV, MCH, MCHC) demonstrated no changes (data not shown), normocytic normochromic anaemia was indicated.

Examination of T lymphocytes showed an increase in the absolute counts of the CD2+ subpopulation from 7 dpi (Fig. 3), and CD8+ from 14 dpi, but lower levels of CD4+ to 42 dpi (Tab. 4). Examination of B lymphocytes by IgM+ expression demonstrated significant increase on 28 d of experiment ($P < 0.05$), and macrophages (Mf+) on 35 d of experiment (Tab. 4).

The production of specific anti- *T. canis* antibodies was increased from 14 dpi (Fig. 4) with significance on 28 dpi.

Table 2. Absolute count of white blood cells

	Mean values of absolute count of the white blood cells - $G.l^{-1}$ ($1.10^9.l^{-1}$) ± SD					
	Leukocytes		Lymphocytes		Neutrophils	
Days pi	C	I	C	I	C	I
0	7.5 ± 0.8	11.6 ± 3	4.9 ± 0.5	3.4 ± 0.3	2.5 ± 1.0	8.0 ± 3.0
1	8.6 ± 1.9	8.9 ± 1.2	5.4 ± 0.6	5.4 ± 0.7	3.2 ± 1.2	3.4 ± 1.3
3	7.8 ± 1.9	7.8 ± 1.4	5.2 ± 0.7	4.3 ± 0.2	2.5 ± 0.4	3.2 ± 1.0
7	7.8 ± 1.3	8.5 ± 1.3	5.9 ± 0.6	6.4 ± 0.3	2.1 ± 0.4	2.6 ± 1.0
14	8.1 ± 2.6	9.2 ± 2.2	5.0 ± 0.5	5.7 ± 1.0	3.0 ± 1.4	2.9 ± 1.2
21	9.1 ± 3.5	10.1 ± 2	4.0 ± 0.7	5.5 ± 0.4	5.0 ± 2.4	4.5 ± 1.7
28	7.9 ± 0.5	7.4 ± 1.2	4.8 ± 0.4	5.2 ± 0.7	3.0 ± 1.0	2.3 ± 1.3
35	8.4 ± 1.0	10.5 ± 3	5.4 ± 0.5	5.3 ± 0.6	3.0 ± 0.3	5.0 ± 2.3
42	8.6 ± 1.7	8.9 ± 1.1	3.7 ± 0.5	3.8 ± 0.9	4.9 ± 0.7	5.6 ± 1.5
49	7.3 ± 2.5	11 ± 3.5	4.5 ± 0.9	6.9 ± 0.6	2.7 ± 1.2	4.3 ± 2.5

C – control; I – infected

Table 3. Haematological values of red blood cells (RBC), haemoglobin (Hb) and packed cell volume (PCV) with significant changes - *P < 0.05

Days pi	Haematological values – mean ± SD					
	RBC ($T.l^{-1}$)		Hb ($g.dl^{-1}$)		PCV ($l.l^{-1}$)	
	C	I	C	I	C	I
1	9.46 ± 0.35	9.22 ± 0.51	17.93 ± 2.77	19.49 ± 4.38	0.32 ± 0.01	0.32 ± 0.02
3	9.28 ± 0.39	8.91 ± 0.53	15.96 ± 0.87	16.14 ± 2.11	0.31 ± 0.01	0.32 ± 0.03
7	9.28 ± 0.37	8.95 ± 0.50	16.09 ± 2.01	15.39 ± 0.77	0.32 ± 0.02	0.33 ± 0.03
14	9.25 ± 0.38	8.74 ± 0.56*	14.29 ± 0.74	13.04 ± 0.56*	0.32 ± 0.02	0.31 ± 0.01
21	9.20 ± 0.48	8.92 ± 0.36	15.50 ± 2.21	15.60 ± 1.90	0.33 ± 0.02	0.31 ± 0.01
28	9.43 ± 0.49	8.71 ± 0.63	17.64 ± 1.63	15.34 ± 1.64	0.35 ± 0.02	0.32 ± 0.02
35	9.42 ± 0.47	8.64 ± 0.55	17.99 ± 1.64	18.2 ± 1.44	0.35 ± 0.02	0.31 ± 0.02
42	9.37 ± 0.46	8.93 ± 0.04	14.45 ± 0.77	15.33 ± 0.58	0.34 ± 0.03	0.31 ± 0.01
49	9.34 ± 0.52	8.87 ± 0.03	17.56 ± 3.46	15.35 ± 0.99	0.34 ± 0.03	0.32 ± 0.01

C – control; I – infected

A significant increase in proliferative activity of splenic T lymphocytes ($P < 0.01$) was observed from 14 dpi (Fig. 5) and splenic B lymphocytes from 21 dpi ($P < 0.05$; Fig. 6). Flow cytometric measurement of metabolic burst of granulocytes demonstrated the decrease of percentage values in infected animals (Fig. 7).

In the peripheral blood the larvae were microscopically found from 14 dpi (8 ± 2.8 larvae) to 35 d (2 ± 2.8 larvae) of the experiment, with maximum on 28 dpi (13 ± 4.2 larvae) (Tab. 5).

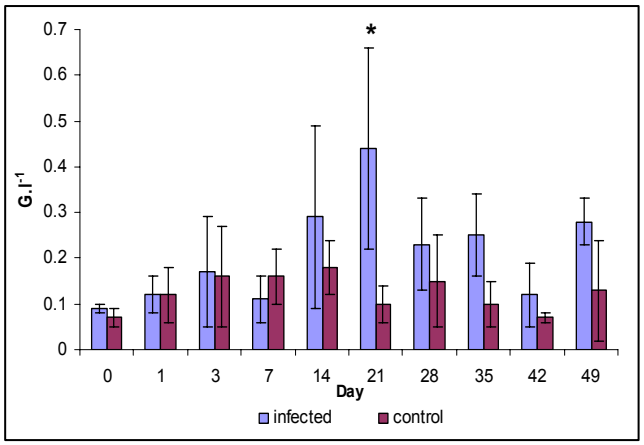

Fig. 1. Absolute counts of eosinophils in the peripheral blood ($G.l^{-1} – 1.10^9.l^{-1}$; average ± SD; *P < 0.05)

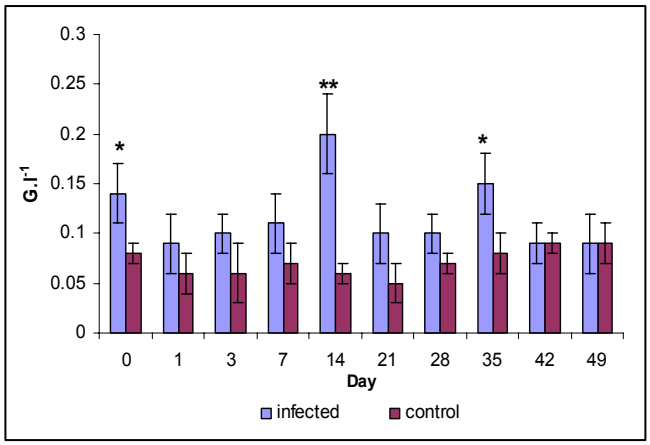

Fig. 2. Absolute counts of monocytes in the peripheral blood ($G.l^{-1} – 1.10^9.l^{-1}$; average ± SD; *P < 0.05; ** P < 0.01)

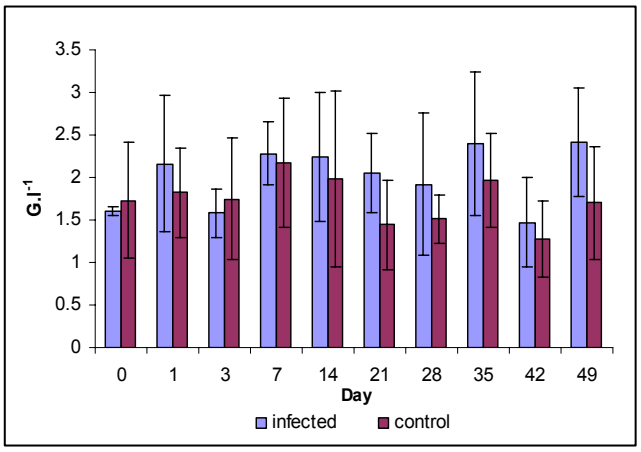

Fig. 3. Absolute counts of CD2 positive cells in the peripheral blood ($G.l^{-1} – 1.10^9.l^{-1}$; average ± SD)

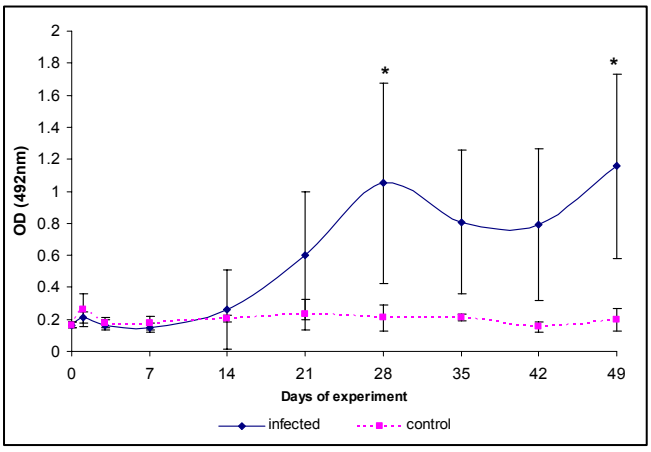

Fig. 4. Production of the specific IgG to *T. canis* (*P < 0.05)

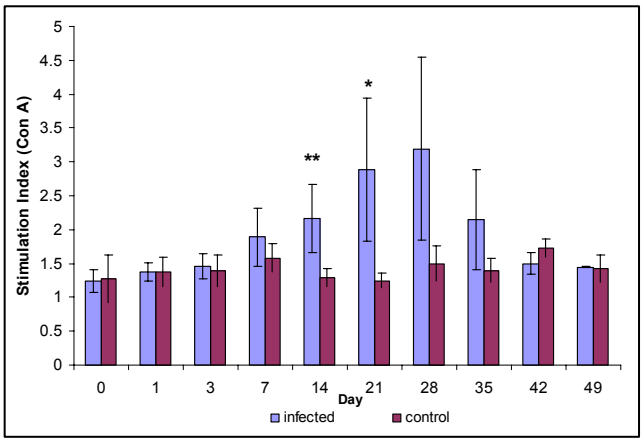

Fig. 5. Proliferative activity of splenic T-lymphocytes to Concanavalin A (**P < 0.01, *P < 0.05)

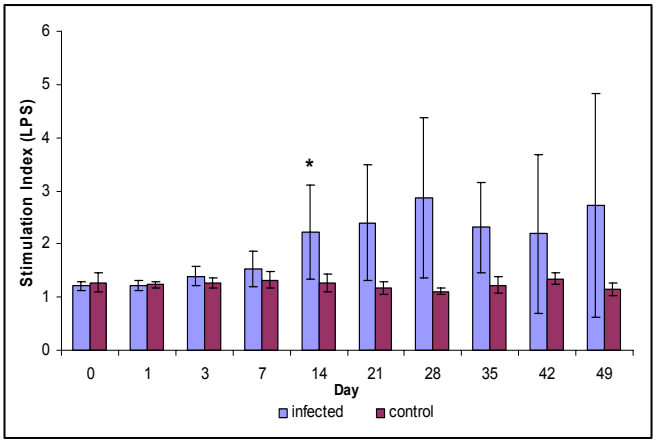

Fig. 6. Proliferative activity of splenic B-lymphocytes to LPS (*P < 0.05)

Table 4. Absolute count of the lymphocyte subpopulation (C- control, I – infected). Significantly different from the control group (*P < 0.05, **P < 0.01)

	Mean values of absolute count ($G.l^{-1} – 1.10^9.l^{-1}$) ± S.D.							
	CD4		CD8		IgM		Macrophages	
Days pi	C	I	C	I	C	I	C	I
0	0.05 ± 0.03	0.04 ± 0.01	0.89 ± 0.35	0.89 ± 0.07	0.06 ± 0.03	0.03 ± 0.01	0.06 ± 0.02	0.03 ± 0.01
1	0.08 ± 0.04	0.06 ± 0.02	0.72 ± 0.29	0.82 ± 0.30	0.08 ± 0.04	0.06 ± 0.01	0.09 ± 0.06	0.05 ± 0.02
3	0.07 ± 0.02	0.04 ± 0.01	0.91 ± 0.47	0.72 ± 0.18	0.05 ± 0.02	0.03 ± 0.02	0.09 ± 0.04	0.06 ± 0.01
7	0.17 ± 0.06	0.14 ± 0.04	0.92 ± 0.35	0.93 ± 0.2	0.08 ± 0.03	0.07 ± 0.01	0.13 ± 0.05	0.12 ± 0.03
14	0.11 ± 0.04	0.10 ± 0.04	0.84 ± 0.35	1.08 ± 0.48	0.17 ± 0.07	0.13 ± 0.07	0.07 ± 0.03	0.06 ± 0.03
21	0.05 ± 0.03	0.06 ± 0.03	0.78 ± 0.28	1.02 ± 0.32	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.02
28	0.28 ± 0.15	0.12 ± 0.04	0.78 ± 0.22	0.85 ± 0.46	0.13 ± 0.06	0.25 ± 0.05*	0.04 ± 0.01	0.05 ± 0.02
35	0.09 ± 0.04	0.05 ± 0.01	1.11 ± 0.26	1.08 ± 0.32	0.16 ± 0.05	0.20 ± 0.06	0.02 ± 0.00	0.03 ± 0.01*
42	0.11 ± 0.04	0.14 ± 0.04	0.70 ± 0.19	0.76 ± 0.27	0.27 ± 0.11	0.18 ± 0.08	0.16 ± 0.04	0.15 ± 0.05
49	0.25 ± 0.07	0.39 ± 0.09	0.85 ± 0.33	1.31 ± 0.07	0.21 ± 0.08	0.30 ± 0.29	0.08 ± 0.01	0.18 ± 0.01**

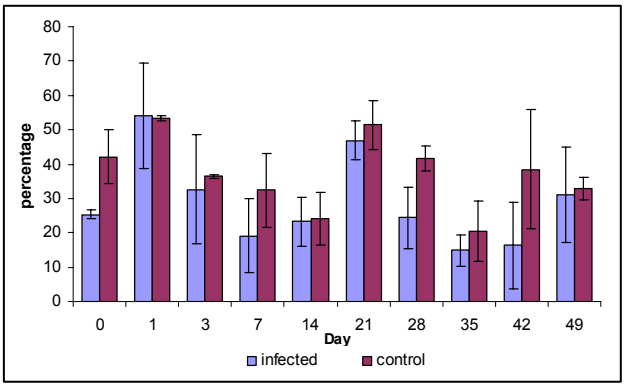

Fig. 7. Percentage values of metabolic burst of granulocytes (average ± SD)

Table 5 The number of *T. canis* larvae isolated from 500 ml of the peripheral blood of infected lambs

Days p.i.	Number of *T. canis* larvae in peripheral blood (500 ml) of infected lambs (mean ±SD)
1	0.0 ± 0.0
3	0.0 ± 0.0
7	0.0 ± 0.0
14	8.0 ± 2.8
21	6.0 ± 2.8
28	13.0 ± 4.2
35	2.0 ± 2.8
42	0.0 ± 0.0
49	0.0 ± 0.0

Discussion

A wide spectrum of paratenic hosts can be infected with larval toxocarosis either naturally or experimentally. These include: humans (Havasiová *et al*., 1993), mice (Samanta, 1989; Aldawek *et al*., 2001), cats (Parsons *et al*., 1989), chickens (Glickman *et al*., 1987), quail (Maruyama *et al*., 1994), and sheep (Hayate *et al*., 1973; Aldawek *et al*., 2002). The migration of *T. canis* larvae through the liver and lungs in sheep induced a variety of immune reactions in affected tissues (Medveďová *et al*., 1994; Revajová *et al*., 2002). The relative contribution of immunological factors as a barrier to *T. canis* infection or protection against reinfection is uncertain. This study observed the changes in immune reactions during *T. canis* reinfection of lambs with small doses of embryonated eggs to reflect the natural situation on pasture infected by farm dogs.

Haematological examination including determination of red and white blood cells showed normocytic normochromic anaemia and mild leukocytosis. Anaemia had a haemorrhagic character as a result of oozing of erytrocytes into the tissue during parasite migration. Leukocytosis of infected animals has arisen out the increase of neutrophils from 0 to 7 dpi, lymphocytes from 7 to 28 dpi, and eosinophils from 14 dpi to the end of experiment. Despite of higher values of granulocytes in the peripheral blood of infected lambs, their respiratory burst (metabolic activity) was lower compared to controls. Little attention has been given to exploration of host cell reactive oxidants during helminthosis, and little is known about reactive oxygen metabolites in relation with toxocarosis. Some studies have been made regarding on singlet oxygen production not only by macrophages, but also by neutrophils and eosinophils during some helminthosis (schistozomosis, nippostrongylosis, echinococosis). However, these cells are present in the highest concentration in granulomas, trapping the helminth larvae, and they are responsible for granuloma formation, as well as demonstration of hypersensitive reactions. In granulomas, the above mentioned cells adhere to the larval tegument, where their cytotoxic mechanisms are the result of respiratory (metabolic) burst (Karelin *et al*., 1992; McCornick *et al*., 1996).

Proliferative activity of splenic T and B lymphocytes showed significant increase at the same time, 14 dpi. Whereas the decrease of T cell activity was observed at the end of experiment, B cell activity demonstrated permanently higher levels. The stimulation time of the proliferation of T

and B cells was not different from a single high-dose *T. canis* infection of lambs (Revajová *et al.*, 2002). Proliferation of lymphocytes during cell mediated immunity is one of the important indices that displayed their functional state, i.e. ability to react with specific or nonspecific polyclonal activators (Šterzl, 1993). No substitutive function both of them during larval toxocarosis is known and play important role in stopping of growth and larval migration of parasite (Lloyd, 1987). In other tissue helminthosis - trichinellosis - the stimulation of proliferative responses of T and B lymphocytes during larval migration was also observed (Dvorožňáková *et al*., 2005). Dvorožňáková (1998) states, that *Toxocara* infection stimulated this activity, i.e. induced growth, division and arising of a new T and B lymphocytes. Thereafter there is increased migration of immunocompetent and other cells into the places of parasite localization.

Persistent antigenic stimulation throughout chronic parasitic infection very often leads to a polarisation of T cell subset populations and extreme immunoregulatory states in the host (Sher & Coffman, 1992). Moreover, the resolution of helminth infection in the gastrointestinal tract is controlled by T cell subsets (Allen & Maizels, 1996). Administration of 1000 *T. canis* eggs to sheep in our experiment caused the increase of CD2+ T lymphocytes in the peripheral blood from 7 dpi to the end of experiment. This increase was mainly due to CD8+ cells, because the values of CD4+ were decrease to 42 dpi. Although Revajová *et al*. (2002) demonstrated higher values of CD4+ T cells during single dose infection with 10 000 *T. canis*-infective eggs and showed their primary role in inducing immune response to toxocarosis, Vervelde *et al*. (1996) ascertained their considerable decrease after primary infection with coccidia, what might explained the CD4+ cells decrease. Multiple infection of mice with 1000 infective *T. canis* eggs resulted also in the decrease of CD4+ cells in spleen and in the peripheral blood (Aldawek, 2002). The migration of these cells into the parasite-localized tissue may be connected with this decrease. CD8+ T cells are effector cells of immune response, and their massive increase was observed during the acute phase of our infection (14 – 28 dpi), as a reaction to excretory-secretory antigen of *T. canis* larvae. The increase of these cell subsets in the blood and spleen was observed after primary and secondary infection with *T. canis* in mice. A high presence of splenic CD8+ T subpopulation was also found in trichinellosis during larval migration (Dvorožňáková *et al*., 2005). Probably these cells and their cytokines are important in antiparasitic defence.

Cell mediated immunity is very closely connected with the humoral immune response. Multiple infection in our experiment showed increase of specific anti-*Toxocara* IgG antibodies from 14 dpi with significance on 28 and 49 dpi, in the time, when the most of larvae were found in the peripheral blood. Despite a large amount of specific antibodies only some of them have a protective or destructive effect (Lloyd, 1987). Romasanta *et al*. (2004) found in canine toxocarosis that the-IgG_2 secretion was stimulated firstly and when infection progressed this IgG_2 production decreased and IgG_1 levels increased. These findings agree with Deplazes *et al*. (1995) who demonstrated that IgG_1 and IgG_2 antibodies in dogs are antagonistically regulated. Dvorožňáková *et al*. (2002) demonstrated in experiments with excretory/secretory (ES) and somatic (S) antigens in mice, that immunization with ES antigen accelerated cell mediated immunity and higher ability of immunocompetent cells to reduce the counts of *Toxocara* larvae as soon as two weeks pi. In our work *T. canis* larvae were migrating in peripheral blood of lambs even for 2 weeks after the end of the multiple infection.

In conclusion, the multiple reinfection of sheep with 1000 *T. canis*-infected eggs showed participation of both, non specific and acquired immunity, cell mediated and humoral immune response, with the aim of elimination, stopping the migration and trapping of parasite in the host organism.

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RECEIVED MARCH 14, 2006

ACCEPTED MAY 2, 2006