

Selenium supplementation enhances the protective response to *Toxocara canis* larvae in mice

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SUMMARY

The effect of oral and intraperitoneal supply of sodium selenite on the immune response to, and the course of T. canis larvae infection in mice were determined. The number of worms in the host tissue was reduced but the migratory route of larvae was not affected. Selenite (Se) supplementation influences Se retention in the liver, enhanced IL-5 and eosinophil responses and evoked IL-6 production in mice infected with T. canis. The enhanced protection in mice given Se intraperitoneally was associated with high levels of parasite-specific IgE, and enhanced concentration of Th1-related cytokines such IL-12p70, TNF- α and IFN- γ . In mice given Se orally, the predominant cytokines produced were IL-10, MCP-1 and IL-6 and these mice had lower protection. In conclusion, Se supplementation increases production of specific cytokines in mice infected with T. canis and increases protection against infection.

Keywords cytokines, mice, selenium, *Toxocara canis* larvae

INTRODUCTION

The dog roundworm *Toxocara canis* infection is a widespread zoonotic disease. *Toxocara canis* eggs dispersed by dogs or foxes have been identified in contaminated soil samples in both rural and urban areas (1). Mice infected with this parasite provide a laboratory model in which the immune response to visceral *larvae migrans* can be characterized. Larvae can damage tissue by provoking cells to produce reactive oxygen species (2). The overproduction of oxygen species may be reduced by the action of antioxidant enzymes, particularly glutathione peroxidase (GPx). GPx in the gastrointestinal tract constitute a defence against hydroperoxides (3,4).

The effectiveness of the immune system depends on trace elements which may act as antioxidants and as cofactors for cytokine regulation (4). Selenium (Se) is a trace element which influences both innate and acquired immune responses (5–7). The immune function of Se arises from selenocysteine. It acts as a redox centre in GPx which can effectively scavenge hydroperoxides and lipoperoxides to prevent tissue and cell injury (8,9). Se could affect enzyme activity in different organs and increase the level of cytokine production (10). Se deficiency inhibits both cellular and humoral responses (11). During Se deficiency the severity of *Trypanosoma cruzi* infection in mice was enhanced (12) but resistance to *Heligmosomoides polygyrus* infection in mice was reduced (13). In experimental animals Se supplementation has been associated with increases in natural killer cell activity, T cell proliferation, delayed-type hypersensitivity and vaccine-induced immunity (14). An important question is whether alternations on micronutrient intake modify the course of nematode infection and accompanied immune reactions.

In our studies we tested how Se influences immune responses to *T. canis* infection. We used the same dose of sodium selenite delivered in drinking water or by intraperitoneal injection. We tested whether Se supplementation before primary *T. canis* infection would enhance protective response and reduce the number of larvae in mice. We

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Received: 16 January 2008

Accepted for publication: 2 April 2008

characterized the dynamics of the immune response during larval migration through host tissue. As hallmarks of the immune response to *T. canis* infection, the percentage of eosinophils and neutrophils in the blood, the concentration of Th1 and Th2 cytokines and IgG1 and IgE specific to *T. canis* antigens in serum were measured.

MATERIALS AND METHODS

Parasite and mice

Toxocara canis was isolated from the intestine of naturally infected red foxes during postmortem examinations. Adult female worms were sectioned and the gravid uterus was emptied. Eggs developed in 21 days at room temperature. The second stage larvae were hatched *in vitro* from eggs in standard conditions (15). Male Swiss mice, which weighed 25–30 g at the age of 10 weeks, were infected with 1000 embryonated eggs of *T. canis*. Animals were housed and maintained in a 12 : 12 h light and dark cycle and were allowed *ad libitum* access to water and commercial pellet food. The experiment was conducted in accordance with the guidelines of the Local Ethical Committee.

Experimental protocol

Mice were divided into six groups; infected with *T. canis* (*T. canis*), infected with *T. canis* and given with sodium selenite in drinking water (*T. canis* + SeA), injected intraperitoneally (*T. canis* + SeIP), uninfected and given with sodium selenite in drinking water (SeA), uninfected and injected intraperitoneally (SeIP) and uninfected nontreated (Control). Mice were given sodium selenite at a dose of 0.06 µg per mouse in drinking water for 5 days. Seven days afterwards, mice were infected with *T. canis*. Alternatively, mice were given intraperitoneally with 0.06 µg sodium selenite injected two times within a break of 2 days. The second injection was given seven days before infection with *T. canis*. Mice were killed 1, 3, 5 and 7 weeks post-infection (WPI). Five mice were sampled at each date to determine the percentage of neutrophils and eosinophils in the blood smears, *T. canis*-specific IgG1 and IgE responses in serum, spleen, lung and liver weight ratio and number of worms. Three mice chosen randomly from each group of five were examined to evaluate cytokine response. Leukocyte differential counts of infected and uninfected mice and treated and untreated mice were provided on the same days. The level of cytokines and antibodies was determined in serum of three uninfected control mice.

Toxocara canis larvae were isolated after digesting the brain, lungs and muscle tissue in pepsin solution (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) using the method described by Köhler (16). Spleens, lungs and livers

were dissected and weighed. The relative organ ratios were calculated (17) as follows:

$$\frac{\text{organ weight/body weight(g) in infected animals}}{\text{organ weight/body weight(g) in controls}}$$

From the tail vein, 5 µL of blood was taken and smeared on a glass, air dried and stained with a May Grunwald-Giemsa technique. Two hundred cells are then counted and classified.

Mice were anaesthetized with carbon dioxide, and decapitated. Blood was collected from the heart and left 2 h at room temperature. Subsequently, the blood clot was discarded, and samples were centrifuged at 3000 g for 5 min. Serum was frozen at –70°C until used to determine cytokines and *T. canis* specific IgG1 and IgE.

Determination of Se content

The liver and kidneys were dissected from each mouse and weighed. Sections were selected for examination and one gram from the same part of the organs was removed and sampled. Se contents were determined fluorometrically using 2, 3 diamionaphthalene after digestion using a mixture of perchloric acid and nitric acid (18).

Determination of IgG1 and IgE specific to *Toxocara canis* antigen

Infective larvae were sonicated in PBS, (136 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4, pH 7.4) containing 0.001 M sodium EDTA, 0.001 M PMSF (phenylmethylsulphonyl fluoride) and 0.0001 M TPCK, as an inhibitor of chymotrypsin. The suspension was centrifuged, the supernatant was dialysed against 0.001 M PBS solution pH 7.2 at 4°C, concentrated and the protein concentration was measured in Bradford assay. The larval homogenate was stored at –70°C.

The specific antibody response was measured by ELISA against somatic *T. canis* larvae. The homogenate was used at 0.5 µg per microtitre plate well. Specific IgG1 antibodies in the samples were screened by incubation with sheep antimouse IgG1 biotinylated antibodies (Serotec, Oxford, UK). Specific IgE was measured by ELISA using rat antimouse IgE labelled with biotin (Serotec) against the somatic homogenate from *T. canis* larvae.

Cytokine measurement

The concentration of cytokines was measured in serum using the BD Mouse Inflammation CBA Kit (BD Biosciences, San Diego, CA, USA) (19). The quantitative measurements of IL-12p70, TNF-α, IFN-γ, IL-10, monocyte chemoattractant

protein-1 (MCP-1) and IL-6 protein levels were performed with FACS in a single sample. Fifty microlitre of sample collected from three individual mice diluted 1 : 2 were mixed and used in the test. Concentrations of cytokines were calculated using standards and plotting results against a standard curve.

IL-5 was measured in serum by sandwich ELISA, using a pair of monoclonal rat IgG1 antimouse IL-5 (TRFK-5/ biotinylated-TRFK-4) antibodies. Quantification of cytokine was determined by reference to recombinant murine standards (Pharmingen, San Diego, CA, USA). Cytokine production was estimated in triplicate for each mouse.

Statistical analysis

The percentage of blood eosinophils and neutrophils, concentration of cytokines, specific IgE and IgG1 in serum, the worm numbers, spleen, lung and liver weight ratio and the course of *T. canis* infection in mice were evaluated in the GLM procedure (MINITAB). The procedure tested differences among days and between groups, infected with *T. canis* vs. uninfected or Se given in the drinking water or by intraperitoneal injection. Statistical significance was calculated using Student's *t*-test and is shown on the graphs. All values are expressed as mean $\pm$ SEM. A *P*-value < 0.05 was considered as statistically significant.

RESULTS

Se concentration in the liver

The level of Se in untreated mice ranged from 0.13 to 0.47 $\mu\text{g/g}$ of wet tissue. After supplementation in the drinking water, the level of Se more than during infection doubled then gradually decreased to the control untreated level ($P < 0.0001$, Figure 1a). There was no difference in the concentration of Se given alimentary to uninfected and infected mice.

In mice supplemented intraperitoneally the level of Se was four times greater than in nonsupplemented mice ($P < 0.0001$) and gradually reduced over time also in uninfected mice. Supplementation of mice with Se intraperitoneally resulted in a greater and longer accumulation of Se in the liver than in mice supplemented orally ($P < 0.0001$). The infection with *T. canis* did not influence an accumulation of Se given alimentary but retained the trace element after intraperitoneal Se injection.

Se concentration in the kidneys

The kidney level of Se in untreated mice ranged from 0.46 to 0.69 $\mu\text{g/g}$ of wet tissue (Figure 1b). The concentration of Se in *T. canis* infected mice was at the control level. Se given

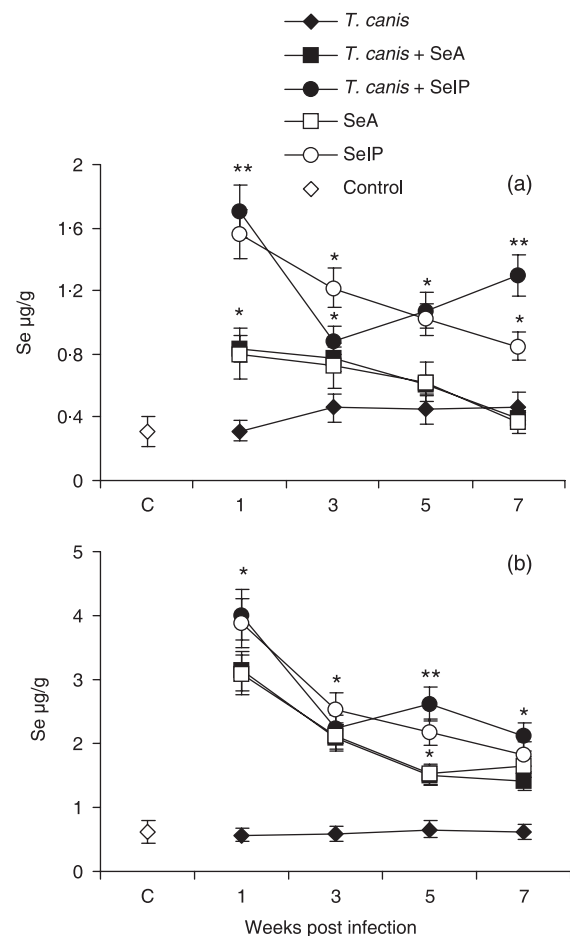


Figure 1 A comparison of the selenium (Se) concentration in the liver (a) and kidneys (b) of mice given with Se alimentary (SeA) or intraperitoneally (SeIP) and infected with *T. canis*. The way of supplementation impacts the level and dynamic of Se retention in mice uninfected and infected with *T. canis*. Each data point represents the means $\pm$ SE of five mice. * $P < 0.05$ comparing to the results of the uninfected control (C) mice. ** $P < 0.05$ mice given Se alimentary vs. injected intraperitoneally. The present results are representative of two experiments.

alimentary both in uninfected and infected mice was six times greater than in untreated *T. canis* infected mice. The level of Se in mice injected intraperitoneally was higher than in mice treated alimentary ($P < 0.001$). The kinetics of Se removing both from mice given alimentary and intraperitoneally was similar ($P < 0.0001$). *T. canis* infection did not change the level of Se in the kidneys.

Larvae counts

Se reduced the level of infection. Fewer larvae were recovered from supplemented mice (Figure 2); in mice treated intraperitoneally 60% of larvae survived in comparison to

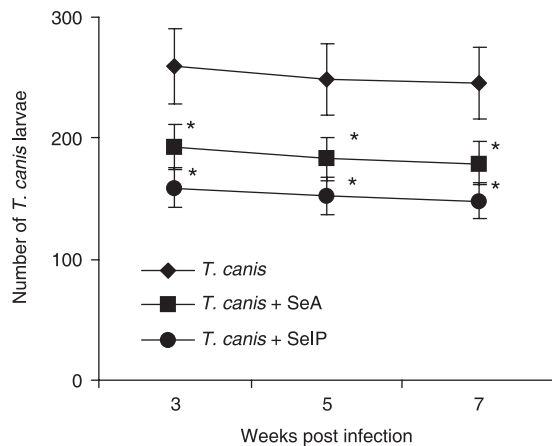


Figure 2 Number of *T. canis* larvae recovered from mice in different points of infection. Treatment of mice with Se reduces the level of subsequent infection with *T. canis* larvae. Each data point represents the means $\pm$ SE of five mice. * $P < 0.05$ comparing to the results of the untreated infected mice. The present results are representative of two experiments.

untreated mice ($P < 0.0001$). The number of worms was reduced only at the beginning of infection. During ongoing infection, the number was mostly unchanged in all groups. The distribution of larvae in the body of mice was not influenced by Se supplementation.

The spleen, lungs and liver weight ratio

In mice infected with *T. canis* the spleen weight ratio (Figure 3) increased within 3 weeks of infection and during next 4 weeks was reduced ($P < 0.0001$). The elevated lung weight ratio was observed from three WPI ($P < 0.0001$). Administration of Se did not significantly alter the weight ratio of spleen and lungs. Supplementation with Se increased the liver weight ratio of mice infected with *T. canis* ($P < 0.0001$).

Leukocyte composition in blood smears

In control uninfected mice the percentage of neutrophils increased during 7 weeks of observations. *Toxocara canis* larvae induced changes in the leukocyte composition (Figure 4). The percentage of neutrophils and eosinophils was higher in the blood of infected mice than in uninfected mice ($P < 0.001$). Supplementation of mice with Se reduced the percentage of neutrophils in the blood ($P < 0.002$) of infected mice. The response of eosinophils was different. Treatment of uninfected mice with Se enhanced the percentage of eosinophils ($P < 0.0001$). In mice infected with *T. canis* the percentage of these cells was not affected by Se treatment but the kinetics of the response was altered ($P < 0.0001$, Figure 4).

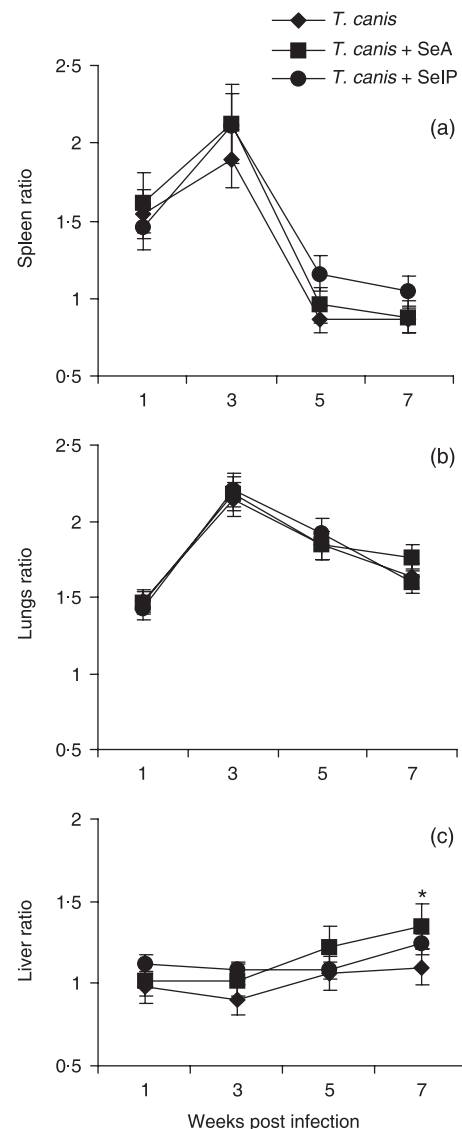


Figure 3 The relative weight ratio of spleen, lungs and liver in relation to adequate control groups. After infection of mice with *T. canis* the weight of spleen increased up to 3 WPI. There was no change in the ratio of spleen weight after supplementation with Se (a). In mice infected with *T. canis* the weight of lungs increased and supplementation with Se did not affect the ratio of lungs weight in all groups (b). The ratio of liver weight was higher in mice treated with Se (c). Each data point represents the means $\pm$ SE of five mice. * $P < 0.05$ comparing to the results of the first week of infection.

Cytokine responses

Se given into uninfected mice induced some cytokine response (Figure 5). Se given alimentary reduced IL-12p70 concentration, temporary increased TNF- α and IFN- γ concentration with different kinetics of response and in the opposite way to IFN- γ slightly raised IL-10 production.

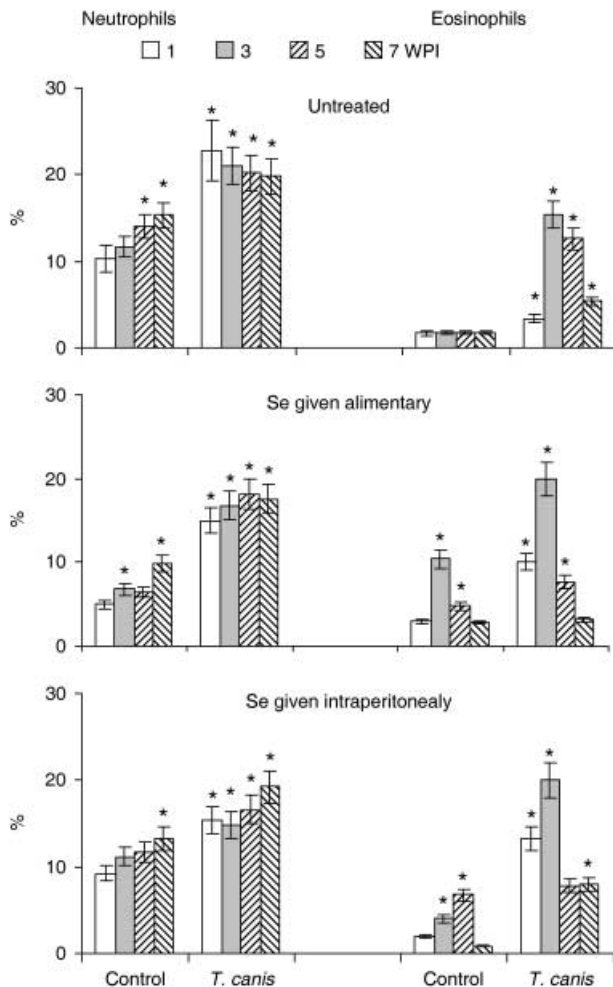


Figure 4 Evidence of changing in the percentage of neutrophils and eosinophils in the blood of mice untreated and treated with Se. The percentage of neutrophils enhanced over time. *Toxocara canis* infection increased the percentage of neutrophils and eosinophils in the blood. Selenium increases the percentage eosinophils in the blood of uninfected mice, affects kinetics of neutrophil and eosinophil responses in mice infected with *T. canis*. Two hundred cells were identified in the blood smear of five mice. Each data point represents the means $\pm$ SE. * $P < 0.05$ comparing to the results of adequate control groups.

Se given intraperitoneally enhanced IL-12p70, TNF- α , IFN- γ and IL-5 production with different kinetics of response ($P < 0.0001$), was not effective in induction of MCP-1 and IL-6 production but reduced IL-10 level.

Changes in the concentration of cytokines during infection were statistically significant ($P < 0.0001$). At 3 weeks of *T. canis* infection, IL-12p70 production was inhibited ($P < 0.0001$), TNF- α production in nontreated mice increased and then fell. Both methods of supplementation with Se enhanced TNF- α production in comparison to untreated

mice ($P < 0.0001$). The increase in IFN- γ concentration was observed in all examined groups of mice ($P < 0.0001$) but the kinetics of response were distinct after Se administration ($P < 0.001$). *Toxocara canis* infection reduced IFN- γ response. Infection of mice with *T. canis* evoked steady enhancement in MCP-1 ($P < 0.001$), IL-10 ($P < 0.0001$) but not IL-6 concentration. The increase in the level of these cytokines were significant in comparison to nonsupplemented mice ($P < 0.0001$) and between groups supplemented in the drinking water and intraperitoneally ($P < 0.0001$). Mice supplemented with Se in the drinking water produced higher levels of MCP-1, IL-10 and IL-6 than mice treated intraperitoneally but mice treated with Se intraperitoneally produced more IL-12p70, TNF- α and IFN- γ ($P < 0.0001$). *Toxocara canis* infection reduced IFN- γ and enhanced IL-10 concentration.

The highest increase in IL-5 concentration was observed 1 WPI (Figure 5). The level of IL-5 gradually decreased but was still elevated 7 WPI ($P < 0.001$). In mice infected with *T. canis*, treatment enhanced IL-5 production more than 10-fold in both supplemented groups (drinking water; $P < 0.0001$; intraperitoneal injection; $P < 0.002$). The method of Se delivery did not influence the level of IL-5 but did affect the dynamics of IL-5 production between 3 and 7 WPI ($P < 0.0001$).

Antibodies response

Infection of mice resulted in steady increase ($P < 0.0001$) in IgG1 level (Figure 6). The concentration of IgG1 also increased in mice given Se in the drinking water ($P < 0.0001$), but the level of IgG1 was lower than in untreated mice ($P < 0.012$). The production of IgG1 increased moderately when mice were injected with Se intraperitoneally ($P < 0.0001$). However the concentration was reduced in comparison to none treated ($P < 0.038$) and mice given Se in the drinking water ($P < 0.0001$). Supplementation with Se of mice infected with *T. canis* reduced specific IgG1 response in serum.

During infection, the level of IgE increased about five times and mice supplemented intraperitoneally produced the most IgE ($P < 0.0001$, Figure 6).

DISCUSSION

Our studies show that administration of sodium selenite resulted in reduced numbers of L2 stage *T. canis* recovered from mice. Animals supplemented with Se before infection create incomplete protection against the parasite. The degree of protection depended on the route of administration and the amount of Se in the liver and kidneys of infected mice. The outcome of immune activation was dependent on the

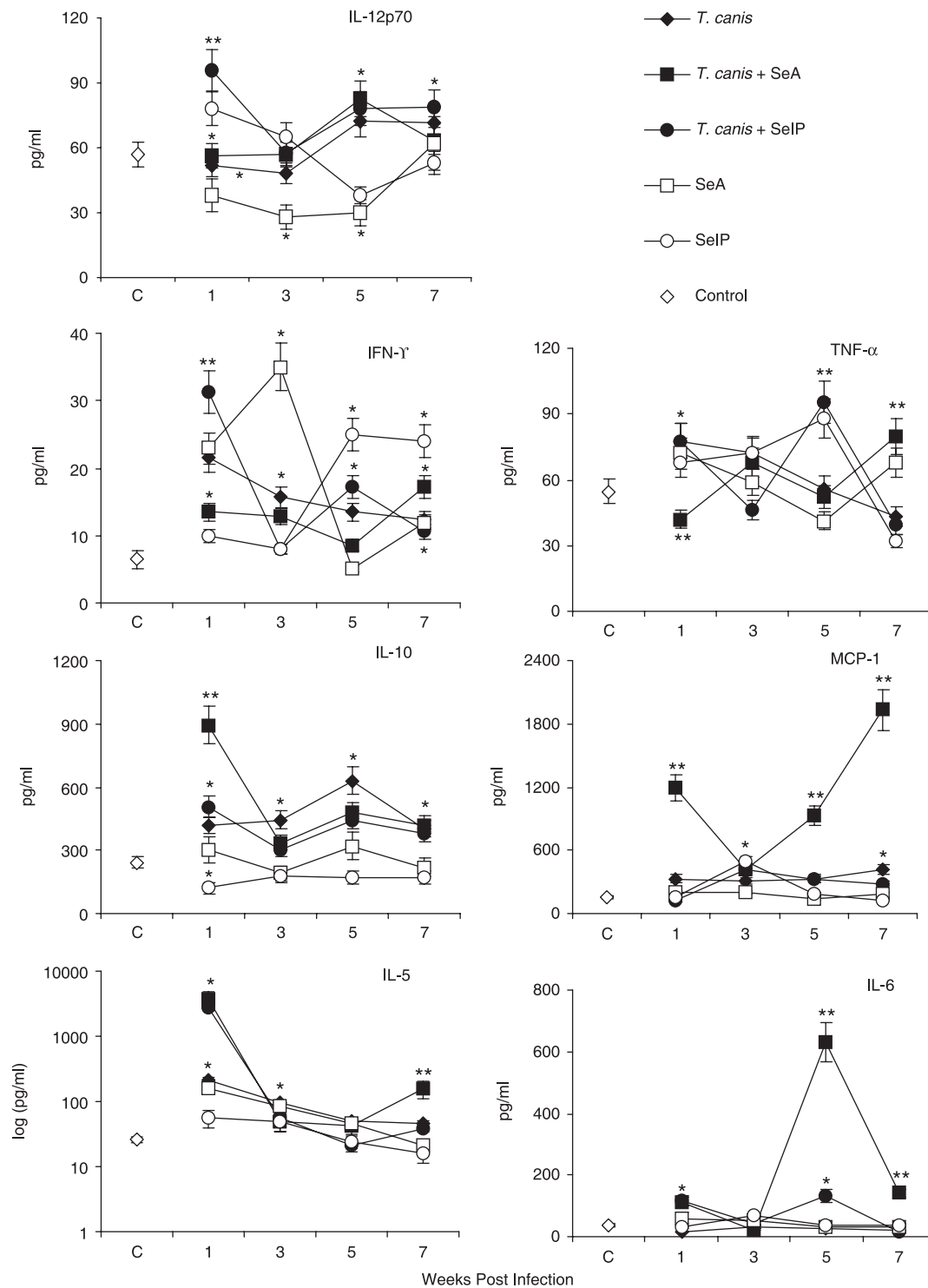


Figure 5 Effect of Se supplementation on cytokine response. Se injected intraperitoneally (SeIP) enhanced production of IL-12p70, TNF- α and IFN- γ . Infection of mice with *T. canis* induced production of MCP-1 and IL-10 which were elevated in mice supplemented with Se alimentary (SeA). Se given in drinking water induced huge IL-6 production after 5 weeks. Se administered intraperitoneally (SeIP) or alimentary (SeA) provokes temporal increase in IL-5 concentration in the blood of mice uninfected and infected with *T. canis*. The control level of cytokines from untreated mice is shown as C point. Each data point represents the means $\pm$ SE of three mice. * $P < 0.05$ comparing to the results of uninfected control. ** $P < 0.05$ mice given Se alimentary vs. injected intraperitoneally.

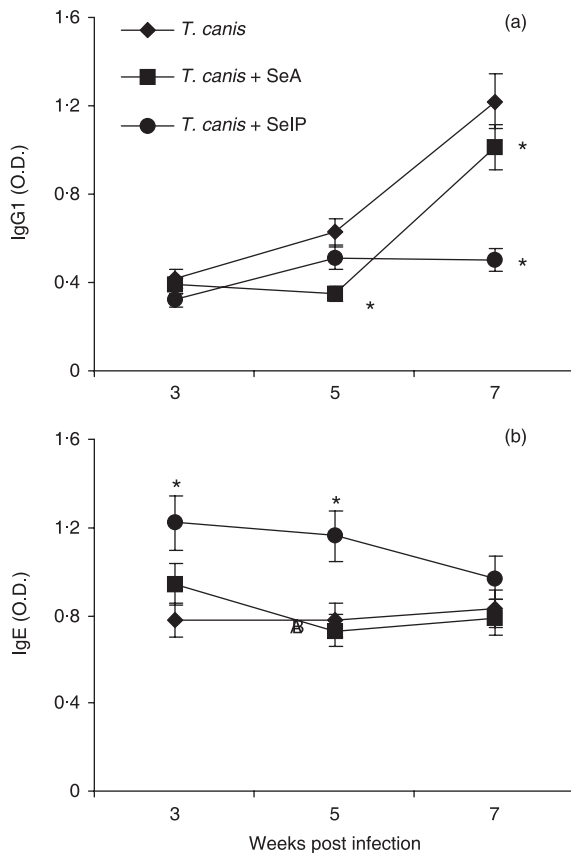


Figure 6 Evidence of Se supplementation on IgG1 and IgE specific response to *T. canis* larvae antigens. IgG1 response is reduced in mice treated with Se. Intraperitoneally administration of Se (SeIP) more effectively reduced IgG1 production. The concentration of IgE specifically recognized larvae somatic antigens is the highest in mice treated intraperitoneally with Se. Each data point represents the means $\pm$ SE of five mice. * $P < 0.05$ comparing to the level of response in mice infected with *T. canis*.

amount of Se given to animals (20). Larvae were destroyed mainly during the first 3 weeks of infection. Mice treated with Se intraperitoneally reduced the parasitic load as more than 40% of larvae were killed when compared to non-supplemented mice. The number of larvae established at the beginning of infection remained mostly unchanged during infection. The greatest number of worms was identified in the brain, liver and muscle (data not shown). Although granulomatous inflammation occurred in the liver, lungs and musculature, dormant larvae survive for a prolonged period (21).

Different methods of Se supplementation did not affect the migratory route of larvae but influenced the level and kinetics of IL-12p70, TNF- α , IFN- γ , MCP-1, IL-10 and IL-6 responses in serum. In mice supplemented orally, the highest concentration of Se in the liver enhanced IL-10 level

and later MCP-1 when Se concentration dropped. These results are consistent with idea that IL-10 and MCP-1 are important elements of the immune response (22). Se also significantly enhanced production of IL-6 (23).

Intraperitoneal injection of sodium selenite resulted in a steady concentration of Se in the liver and enhanced Th1-related cytokines such as IL-12p70, IFN- γ and TNF- α concentration. Tissue trauma evoked by moving larvae may stimulate production of cytokines including IL-6, TNF- α and IFN- γ by immune and nonimmune cells (24).

Production of IL-5 during the first week of infection corresponded to the level of infection. The higher protection may be correlated with higher production of IgE but not IgG1. In mice, production of IL-5 induced by *T. canis* larvae results in a massive eosinophilia that has no apparent effect on the larvae but is deleterious to the host (25). Perivascular and peribronchial cellular infiltrates in the lungs injured by larvae passing through the tissue comprise mainly eosinophils and lymphocytes (26). Production of IL-5 by CD4⁺ T cells and by CD4⁺CD8⁻ cells in the lung (27) occurs predominately during the first week of infection in mice (28). Eosinophils in the blood peaked at day 10 and 3 WPI (29). In our studies the highest level of IL-5 appeared in the blood 1 week post-*T. canis* infection. Mice treated with Se both orally and intraperitoneally produced 20-fold more IL-5 1 week post-infection and noninfected mice were also temporary induced to IL-5 production.

Se treatment did not affect spleen and lungs size but increased the liver weight ratio. A diffuse *T. canis*-induced pulmonary inflammation and eosinophil and macrophage-rich hepatic *granulomas* are the most prominent pathological features in toxocariasis. Changes in the liver weight ratio may be connected with Se retention. Se is known to affect the activity of both extracellular and intracellular antioxidative enzymes and therefore regulates the level of free radicals (30). Hepatic injury was expressed in the overproduction of proinflammatory cytokines including IL-6 and TNF- α (4). The deposition of Se in the liver may prevent injury induced both by larvae and eosinophils (31) or support the regenerative process reflected in the growing mass of the liver. IL-6 is required for normal liver regeneration (32) and our studies showed that Se supplementation enhanced IL-6 production.

Changes in TNF- α production may differentially affect neutrophil adhesion and improve the inflammatory response (33,34). A tight correlation between tissue lesions caused by larvae migration and increased plasma levels of proinflammatory and chemotactic cytokines in bronchoalveolar lavage fluid was established (35). In mice supplemented orally, the kinetics of TNF- α production was distinct from nonsupplemented animals. The greatest changes appeared in MCP-1, and IL-6 levels which were raised after Se treatment. Also IL-10 concentration was elevated with the highest

production one week post-infection. In previous studies the exposure of mice to sodium selenite in the drinking water stimulated splenic macrophages to produce cytokines and TNF- α (36). Sodium selenite given intraperitoneally increased IL-12p70, TNF- α and IFN- γ production.

In comparison to mice infected with *T. canis* alone or treated with Se orally, a higher level of specific IgE and reduced IgG1 production was observed in mice treated intraperitoneally. The elevated level of IL-12p70 after supplementation may mirror stimulation and activation of antigen presenting cells. *Toxocara canis* infection inhibited production of IL-12 in macrophages (37). Stimulation of peritoneal cells with Se may restore the nematode inhibitory effect as IL-12p70, TNF- α and IFN- γ production increased. Elevation of these cytokines may significantly accelerate protective response against larvae.

The protective effect was also identified in mice supplemented orally. The huge increase in MCP-1 and IL-10 concentration may indicate immune activation at the mucosal surface. Up-regulation of MCP-1 influenced production of Th2 cytokines in nematode infection (38). IL-10 produced by Th2 or T reg cells acts as a regulator of inflammatory responses and inhibited activation of macrophages controlling responses of the innate immune system (39). By acting on antigen-presenting cells and inhibiting Th1 cell differentiation and action, IL-10 modulates the T cell-dependent response (40). Mice supplemented orally may more effectively regulate immune responses. IL-6 involved in modify the polarization of CD4⁺ T cells would strongly support Th2 phenotype response (41), however, the level of IgE or IgG1 production and Th1 related cytokines are reduced with only slightly weaker degree of protection.

In conclusion, Se supplementation influences Se retention in the liver and increases cytokine production in mice infected with *T. canis*.

ACKNOWLEDGEMENTS

The research was supported through the AR Szczecin intramural grant (BW/HB/03/02). We thank Professor M. J. Stear for advice and help in writing the manuscript.

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