

Protective Immunity Against Challenge Infection with *Trichinella spiralis* in the Rat

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Protective immunity and immune response were studied in rats after primary infection with *Trichinella spiralis*. The parameters measured include serum antibody isotype responses, mesenteric lymph node (MLN) cell and spleen cell proliferation responses *in vitro*, and the levels of interferon (IFN)- γ , interleukin (IL)-2, IL-4 and IL-10 as markers of the T-helper (Th) subset. Protective immunity was assessed by the degree of expulsion of adult worms from the rat intestine. Protective immunity against adult worms after challenge infection was 99.80%. Although IFN- γ , IL-2, IL-4 and IL-10 in both the MLNs and spleen were detected, IFN- γ and IL-2 levels were higher in the spleen than in the MLNs, and IL-4 and an increased amount of IL-10 was released in the MLNs as compared to the spleen. The levels of the specific immunoglobulins (Ig) G, IgM, IgG1 and IgG2a on week 6 after primary infection, and on day 7 after challenge infection, were higher as compared to levels in uninfected rats that only received a challenge infection ($P < 0.001$), whereas the antibody level of IgG1 was significantly elevated from that of IgG2a ($P < 0.001$). These results demonstrate that Th1 type responses predominated. In addition, as Th2 type cytokines were also produced, it is proposed that the protective immunity by primary infection was also related to the Th1 type responses.

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Introduction

Trichinosis, the commonest parasitic infection of muscle, is caused by infection with the nematode *Trichinella spiralis* (*T. spiralis*). The epidemiology of trichinosis is highly complex, due to a very low host specificity exhibited by the parasite, resulting in many zoonotic infections. Human infection is initiated from the ingestion of raw or poorly cooked pork, bear, walrus, or horsemeat, or from meat obtained from other mammals (carnivores and omnivores) containing viable, infective larvae. The disease has variable clinical manifestations, ranging from an asymptomatic to fatal presentation. Human responses to trichinosis mainly include stomachache, and achy muscles and joints. Trichinosis is also a common infection worldwide and it has been a main public health concern in many countries. There are still some human trichinosis outbreak reports in the world [1-3]. In

the United States, trichinellosis has caused hundreds of preventable cases of illness and occasional deaths. Even though it is seldom seen in the United States due to regulations regarding the feeding of domestic animals and meat-processing inspections, there is still a continued occurrence of cases among consumers of wild game meat and noncommercial pork [4]. More targeted public education is needed to reduce further the incidence of this disease. It is therefore essential to understand the immune responses that are produced in the host after infection with *T. spiralis*. As there is still a chance of infection due to particular dietary habits, it is necessary to understand clearly the relationship between protection against *T. spiralis* infection and cytokine production or antibody reaction in the host during a secondary infection. This understanding will provide very important information for vaccine development.

Most studies have been performed on the protective immunity in

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mice and the detection of parasitic-specific antibodies in humans, rats or mice [5-11], but the results are controversial. Grecnis et al. have demonstrated that protective immunity to *T. spiralis* infection in mice is associated with the activation of Th2-type cells within the mesenteric lymph nodes (MLNs) with a relative absence of Th1-type cells [12]. Hermanek et al. have shown that B10 cnddot BR mice, in which single infections produce a Th1 response to *Trichuris muris* and develop no protective immunity, can mount a protective T-helper-2 (Th2) response and expel *Trichuris muris* when concurrently infected with the 'Th2-inducing' nematode *T. spiralis* [13]. Ramaswamy et al. found that the presence or absence of IFN-gamma secretion rather than IL-4 alone, determines whether a T cell subset has protective activity against *T. spiralis* infection in rats [14]. However, the changes of humoral and cellular immune responses after primary and secondary infection have not been examined in detail. Little is known about the relationship between the role of Th cells and the cytokine responses and protective immunity during infection. In the present study, we have studied the protective immunity induced by primary infection with *T. spiralis* prior to challenge infection using a rat model. In addition, we have examined lymphocyte proliferation and secretion of cytokines by cells purified from primarily infected rat spleens after stimulation with *T. spiralis* excretory-secretory antigen (ES Ag). In order to evaluate the functional significance of a Th1 or Th2 cytokine response, we have monitored the levels of the specific antibodies IgG, IgG1, IgG2a and IgE. Taken together, our results suggest the association of Th1 or Th2 type cytokines and antibody responses in rat provide protective immunity against reinfection.

Materials and Methods

Parasites and experimental animals

Sprague-Dawley rats (Female, 8 wk old, outbred) were used for *in vivo* maintenance of *T. spiralis* through several passages. Muscle larvae were recovered by digestion with pepsin-HCl, followed by filtration through layers of gauze. Procedures involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies.

Preparation of antigens

Excretory-secretory antigen (ES Ag) was obtained by culturing living muscle larvae in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with antibiotics at 37 °C and 5% CO₂. Samples from the culture medium were collected. After centrifugation at 4 °C, 10,000 rpm for 30 min, the supernatant was used as ES Ag. The protein concentration of antigens was determined using the DC Protein Assay kit (Bio-Rad, Hercules, CA USA) and samples were stored at -70°C until used.

Primary infection and challenge infection

Six rats were infected with 1000 infective worms of *T. spiralis*, each via the orogastric tube under the effects of light ether anesthesia. For challenge infection, rats were infected with 500 infective larvae. Control rats also received the same infective larval burden. The time of challenge infection was chosen on the sixth week after primary infection.

Adult worm recovery and protection assay

The adult worm recovery from the intestine was performed on day 7 after challenge infection. The entire length of the small intestine was removed, opened longitudinally, placed in saline solution and incubated for 4 h at 37 °C. The solution was poured into a petri dish, and the number of worms released was counted using a dissecting microscope. Protective immunity was determined by a comparison of worms recovered from primarily infected and control rats.

Cell proliferation assay

Mesenteric lymph nodes and spleens were collected on week 6 after primary infection containing uninfected rats for lymphocyte proliferation and cytokine production. Spleens and mesenteric lymph nodes were excised from the rats and cell suspensions were aseptically prepared by squeezing the spleens between two sterile glass slides. Spleen lymphocytes were purified by the use of percoll and the lymphocytes from the spleen and the MLNs were cultured at a final number of 5×10^4 cells in RPMI 1640 containing 2 mM glutamine, 25 mM HEPES, 100 units penicillin/streptomycin, 5×10^{-5} mM 2-mercaptoethanol, 1 mM sodium pyruvate, 1% MEM non-essential amino acid solution (NEAA) and 10% fetal bovine serum (FBS) (Invitrogen., Grand Island, NY USA). Cultures were maintained in 96-well flat bottom culture plates for 3 days at 37°C in 5% CO₂. Cells were stimulated with ES Ag and phytohemagglutinin (PHA) at 10 µg/ml final concentration. Each test was performed in triplicate. Cells were pulsed for the last 16 h with 0.5 µCi/well [³H] thymidine (Amersham, Arlington Heights, IL USA) and were then harvested on glass fiber filters with the use of a semi-automatic cell harvester (Skatron, Lier, Norway). Incorporated radioactivity was measured in a liquid scintillation counter (LKB 1214 Racbeta) and was expressed as the background subtracted geometric mean (CPM).

Cytokine analysis

For detection of cytokine production, triplicate culture of 5×10^4 cells as described above were incubated with *T. spiralis* ES Ag and PHA in 96-well culture plates at 37°C in 5% CO₂ for 3 days. The supernatants of three wells were pooled following centrifugation and were stored at -20°C until used. Levels of IFN-γ, IL-2, IL-4 and IL-10 were determined in culture supernatants by use of the rat OptEIA set (Pharmingen, San Diego, CA USA). The lower limit of detection for all cytokines was 0.5 pg/ml.

Sera collection

Blood was collected from each rat at the time of culture of the mesenteric lymph nodes and spleen and on day 7 after challenge infection that followed adult worm recovery. The blood was allowed to clot at room temperature and was stored at 4°C. After removal of the clot, sera were centrifuged at 3,000 rpm for ten min and stored at -20°C until needed.

Enzyme-linked immunosorbent assay (ELISA)

Solid phase ELISA was performed in a microtiter plate as previously described. Plates for IgG, IgM, IgE, IgG1 and IgG2a were coated with 5 µg/ml *T. spiralis* ES Ag. Sera dilution was 1:100 for IgG, IgM, IgE and 1:25 for IgG1 and IgG2a. Enzyme conjugate dilution was 1:1,000. Peroxidase-conjugate goat affinity purified antibody rat IgG (Cappel, Durham, NC USA), peroxidase-conjugate goat affinity purified antibody rat IgM (Cappel), peroxidase-monoclonal mouse anti-rat IgG1, IgG2a (Zymed., South San Francisco, CA USA) and biotin-mouse anti-rat IgE (Zymed) were used. 1:1000 dilutions of streptavidin alkaline phosphatase conjugate and alkaline phosphatase substrate kit (Bio-Rad) were used for the detection of IgE. Plates were scanned at a wavelength of 490 nm for IgG, IgG1, IgG2a and IgM detection, and at a wavelength of 420 nm for IgE detection. Each sample was performed in duplicate; the ELISA reader (Molecular Devices, Menlo Park, CA, USA) was zeroed using a blank control plate.

Statistical analysis

Intestinal worm burden and serum antibody levels were recorded for individuals within the primary and control group. Statistical comparison of the data was carried out using the ANOVA and Npar1-way Kruskal-Wallis test of the PC-SAS system. A value of $P < 0.05$ was considered as statistically significant. Cellular immune responses were performed using pooled cells from five rats and were not subject to statistical analysis.

Results

Resistance to reinfection after primary infection

For observation of the resistance to reinfection, rats were primarily infected with infective muscle larvae of *T. spiralis*. On day 7 after the challenge infection, adult worms were recovered from the intestine. Resistance (99.80%) was estimated by a comparison of worm burdens from primarily infected rats with worm burdens from control rats. A significant difference was observed as compared to the control rats (Table 1).

Lymphocyte proliferative responses

Lymphocyte proliferative responses were examined from mesenteric lymph nodes and spleens collected at week 6 after primary infection. Lymphocytes were stimulated with specific *T. spiralis* ES antigen and PHA. PHA seems to trigger similar growth response mechanisms as the antigen. In the present study, we used PHA as a polyclonal mitogen and compared its use with *T. spiralis* ES Ag. As a result, significant proliferative responses from the MLNs and spleen were observed as compared to responses for the uninfected rats. Lymphocytes from the MLNs showed the highest increase when stimulated with both *T. spiralis* ES Ag and PHA. Proliferation of lymphocytes from the spleen also was increased by treatment with both stimulators, as compared to the proliferation of lymphocytes from the spleen of uninfected rats. However, the proliferative responses of spleen lymphocytes were lower than the proliferative responses of lymphocytes in the MLNs (Figure 1).

Cytokine production

Cytokine production was detected from the spleen and MLNs when stimulated with *T. spiralis* ES Ag and PHA. Similar levels of IFN- γ and IL-2 were observed in both the MLNs and spleen. Production of IFN- γ and IL-2 in the spleen showed a significant increase when stimulated with *T. spiralis* ES Ag (Figure 2 and 3).

Table 1. Protective effect in primarily infected rats against challenge infection

Group	No. of rats	No. of larvae used in the primary infection	No. of larvae used in the challenge infection	No. of worms recovered (Mean \pm SD)	Immunity (%)
Control	6	0	500	185.8 \pm 58.00	
Primarily infected rats	6	1000	500	0.3 \pm 0.45	99.80

Immunity (%) =

Challenge infection was performed on week 6 after primary infection.

Adult worm recoveries from rat intestine were performed on day 7 after challenge infection.

Immunity (%) = $\frac{A - \text{Mean no. of worms recovered from primary infected group}}{\text{Mean no. of worms recovered from control group (=A)}} \times 100\%$

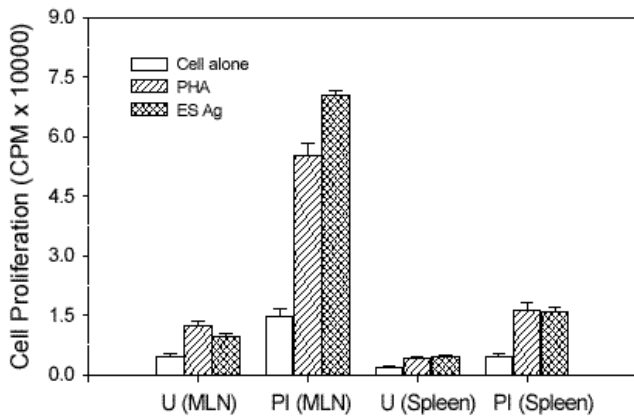


Figure 1. Lymphocyte proliferative responses. Comparisons of the *in vitro* effects of PHA and ES Ag on lymphocyte proliferation (mean \pm SD) from the MLNs and spleen of uninfected and primarily infected rats. U, uninfected; PI, primary infection

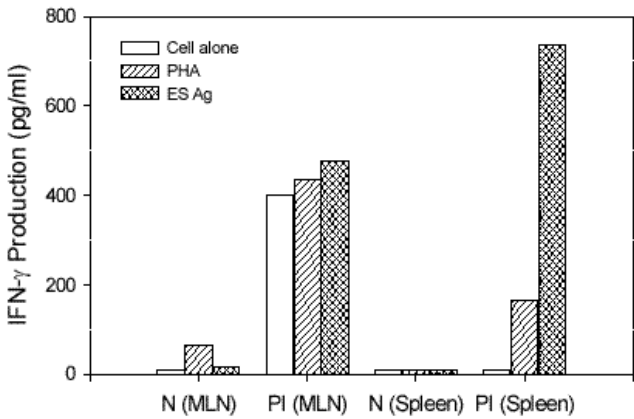


Figure 2. Production of IFN- γ . Comparisons of the *in vitro* effects of PHA and ES Ag on IFN- γ production by lymphocytes obtained from the MLNs and the spleen of uninfected and primarily infected rats. In this experiment, lymphocytes that were pooled from 5 rats were utilized. U, uninfected; PI, primary infection

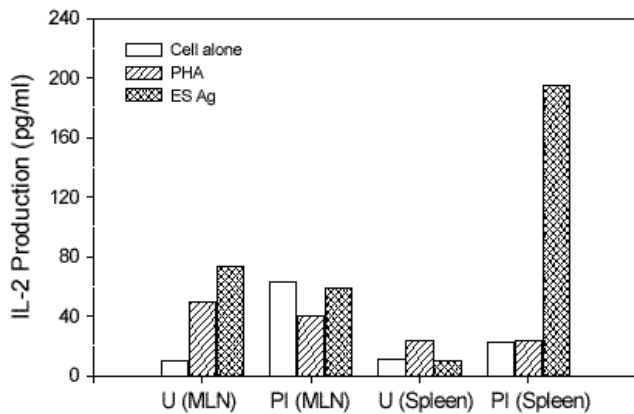


Figure 3. Production of IL-2. Comparisons of the *in vitro* effects of PHA and ES Ag on IL-2 production by lymphocytes obtained from the MLNs and the spleen of uninfected and primarily infected rats. In this experiment, lymphocytes that were pooled from 5 rats were utilized. U, uninfected; PI, primary infection

However, levels of IFN- γ and IL-2 were not significantly different as compared to the levels from uninfected cell alone and cells stimulated with PHA. Levels of IL-4 and IL-10 showed significantly increases as compared to levels of the cytokines in uninfected rats when stimulated with *T. spiralis* ES Ag (Figure 4 and 5).

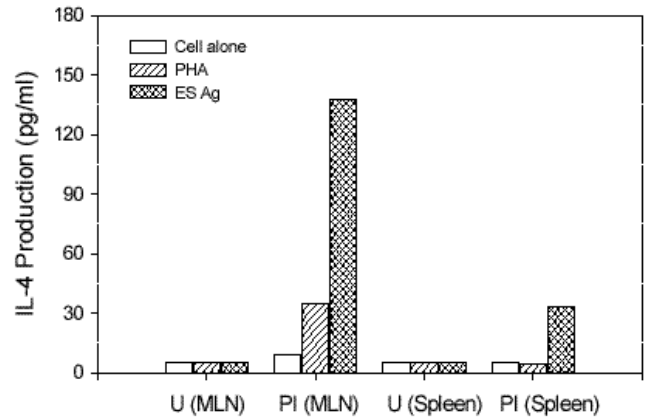


Figure 4. Production of IL-4. Comparisons of the *in vitro* effects of PHA and ES Ag on IL-4 production by lymphocytes obtained from the MLN and the spleen of uninfected and primarily infected rats. In this experiment, lymphocytes that were pooled from 5 rats were used. U, uninfected; PI, primary infection

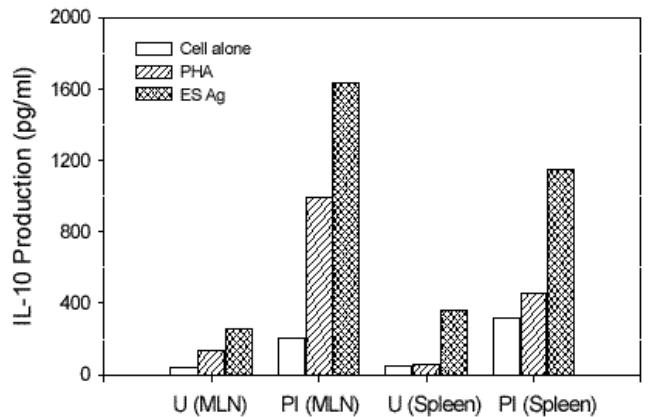


Figure 5. Production of IL-10. Comparisons of the *in vitro* effects of PHA and ES Ag on IL-10 production by lymphocytes obtained from the MLN and the spleen of uninfected and primarily infected rats. In this experiment, lymphocytes that were pooled from 5 rats were utilized. U, uninfected; PI, primary infection

IgG, IgG1, IgG2a, IgM and IgE responses

Levels of IgG, IgG1, IgG2a, IgM and IgE antibody responses were detected at week 6 after primary infection and on day 7 after challenge infection. Significant increases of IgG and IgM antibody levels were detected as compared to levels in uninfected rats ($P < 0.001$, Figure 6). Antibody levels of IgG and IgM increased on day 7 after challenge infection when compared with before challenge ($P < 0.05$). The IgE antibody level was elevated as compared with uninfected rats on day 7 after challenge infection but no significance was observed

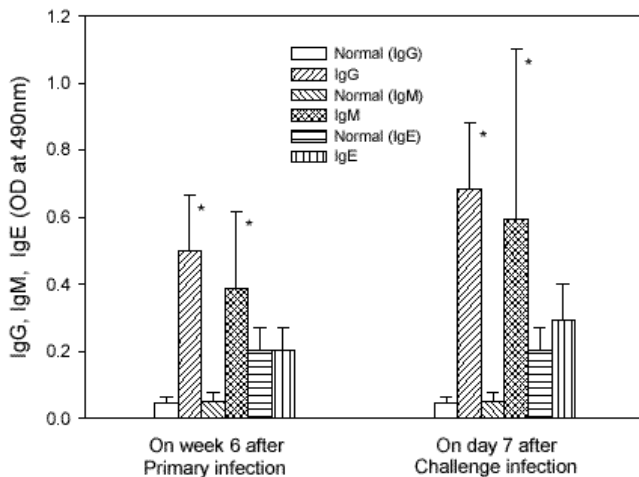


Figure 6. IgG, IgM and IgE responses. The levels of IgG, IgM and IgE antibody titers (mean \pm SD) on week 6 after primary infection and on day 7 after challenge infection. * $P < 0.001$, significantly different from controls

($P < 0.05$). In addition, significant elevation of the levels of IgG1 and IgG2a were detected as compared to uninfected rats ($P < 0.001$, Figure 7). Antibody levels of IgG1 were significantly increased from that of IgG2a after primary infection and on day 7 after challenge infection ($P < 0.001$, Figure 7). IgG1 showed a closer relation to resistance to reinfection than IgG2a. No significant changes of IgG1 and IgG2a antibody levels were seen between week 6 after primary infection and day 7 after challenge infection.

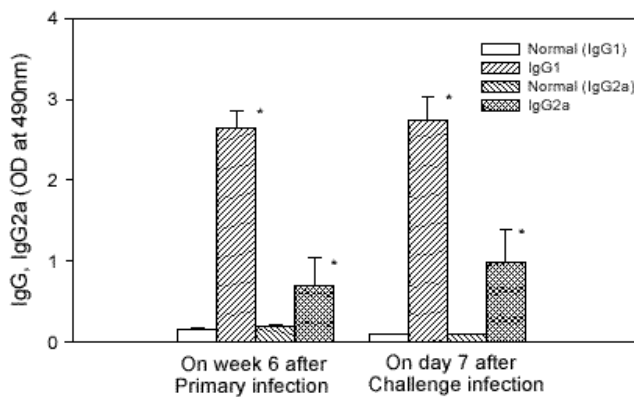


Figure 7. IgG1 and IgG2a responses. The levels of IgG1 and IgG2a antibody titers (mean \pm SD) after primary infection and after challenge infection. A significant difference was observed between IgG1 and IgG2a ($P < 0.001$). * $P < 0.001$, significantly different from controls

Discussion

In the present study, immunity to reinfection with *T. spiralis* was shown to develop in a rat model. The resistance was expressed as reduced adult worm recovery from the intestine. These results are consistent with previously published data [15-17]. Moreover, we focused on adult worm expulsion in relation to serum antibody isotype

response, lymphocyte proliferation and cytokine production in primary infected rats. The present study showed that more than 99% of adult worms were expelled and this was associated with Th1 or Th2-type cytokine responses and parasite specific antibody production. Many studies have reported on the implication of Th1 versus Th2 responses in protective immunity in mouse models, as both molecular and cellular immunology studies were facilitated by the availability of sensitive assays for the measurement of cytokine levels and the production of transgenic and knockout mice. However, for some infective agents, susceptibility or resistance and the nature of the immune response described in mice is different from the response observed in other experimental animals. A previous study demonstrated that a different T-helper subset was involved for a different host with different infection duration, even though the parasite (*T. spiralis*) was the same. In mice, IgG1 is considered to be controlled by Th2 cytokines whereas IgG2a is known to be controlled by Th1 cytokines. However, in the rat, IgG1 is a Th1 cytokine-driven antibody subclass, while IgG2a, IgG2b and IgE arise during a Th2-type response [18, 19]. Rat IgG1 antibodies act as highly effective blocking IgE antibodies over a wide concentration range. Rat IgG2a antibodies are able only to inhibit degranulation of cells [18, 19].

In a previous study, we reported that immunization of rats with ES Ag of *T. spiralis* Korean isolate without any adjuvant elicits effective protective immunity against challenge infection, and Th2 type IL-10 and IL-4 were the predominant cytokines produced [20]. However, the present study showed the production of cytokines IFN- γ , IL-2, IL-4, and IL-10 increased. A significant increase in the level of IgG, IgG1, IgG2a and IgM antibodies were detected, but no significant level of IgE antibody was detected. The significant elevation of IgG, IgG1, IgG2a and IgM levels in the present study support the involvement of Th1 and Th2-type responses in protective immunity against *T. spiralis*. Also significant IgG1 and IgG2a production may lead to a down regulation of IgE production. In the present study, a significant level of IgG1 antibody as compared to the level of IgG2a antibody ($P < 0.001$, Figure 7) and a significant increase in the level of IgM antibody was detected after primary infection and after challenge infection in rats as compared to uninfected rats. When related to cytokine production, the Th1-type response predominated in protective immunity, with participation of the Th2-type response. This finding was similar to previously published results [21]. IgM levels on day 7 after challenge infection increased significantly as compared to IgM levels in uninfected rats (Figure 4). This result suggests Th1-type associated IgG1 might be related to *T. spiralis* infection and is also closely associated with *T. spiralis* adult worm expulsion, a finding consistent with a published study [22]. In mice, IFN- γ promotes IgM and IgG2a production and IL-4 promotes production of IgG1, IgE and IgA. IgG subclasses in mice and rats have quite distinct biological properties [18]. Th1 T cells mediate macrophage activation, stimulate production of IgG1 opsonizing and complete-fixing antibodies and produce IFN- γ , IL-2 and TNF. Th2 T cells provide help for B cells, stimulate the production of IgG2a antibodies, produce IL-4, IL-5, IL-6, and IL-10 and down regulate macrophage function [23].

Until now, the association between T-helper subset and IgG subclass remained unclear. In contrast with published findings [18, 19], Gracie and Bradley [24] and Cetre et al. [25] showed that IgG2b and IgG2c were Th1-type isotypes and IgG1 and IgG2a were Th2-type isotypes. In the present study, no significant levels of IgG2b and IgG2c were detected (data not shown). The result of present study was consistent with the findings of previous studies by Cuturi et al. [18] and Philips et al. [19].

In conclusion, our results support the view that protective immunity can be induced after primary infection and protective immunity was mainly associated with Th1-type response, with participation of Th2-type response. The present study provides new information and complements earlier reports with *T. spiralis* about the correlation between induced protective immunity and T-helper subset. These findings indicate the importance of the use of appropriate immunization strategies to induce the correct T-helper-cell set (Th1) in the development of vaccines against trichinosis.

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