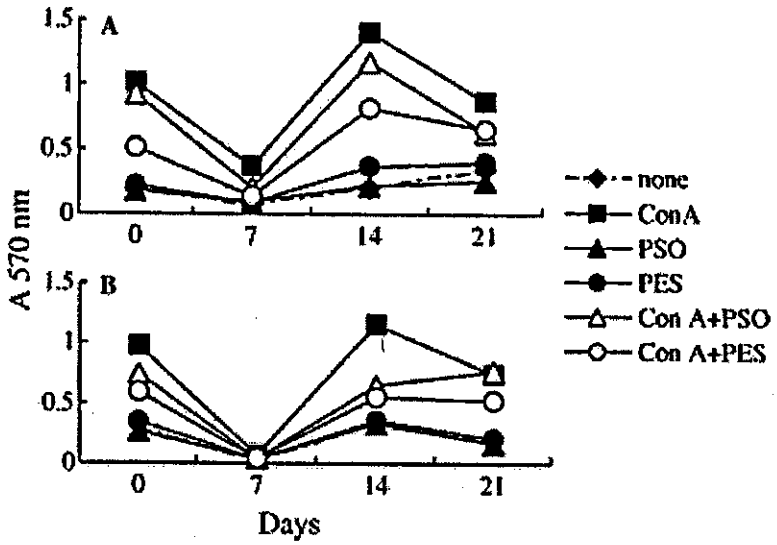


**Fig. 3** Time course of *E. multilocularis* specific lymphocyte responses and the effects of parasite antigens on dog peripheral blood mononuclear cells (PBMC). Dog PBMC were stimulated with concanavalin A (*ConA*) or parasite antigens, or co-stimulated with ConA and either of the parasite antigens. Cellular proliferation at 72 h was measured by MTT assay. Data are presented as mean values of duplicate samples of each dog (*A* dog 1, *B* dog 2). *None* Non-stimulated (medium only), *PSO* protoscolex somatic antigen, *PES* protoscolex excretory-secretory antigen

## Lymphocyte proliferation tests of lymphocytes at 21 DPI

The blastogenic response against the parasite antigen was observed only in PP cells from dog 1 with PES stimulation (SI=4.65); however, no responses were observed in other cells stimulated with either PSO or PES (Fig. 4, Table 1). In contrast, co-stimulation of the cells with ConA and the protoscolex antigens resulted mostly in the reduction of the responses of ConA mono-stimulation, and maximum reduction of 33.5% was observed in MLN cells of dog 1 co-stimulated with ConA and PES.

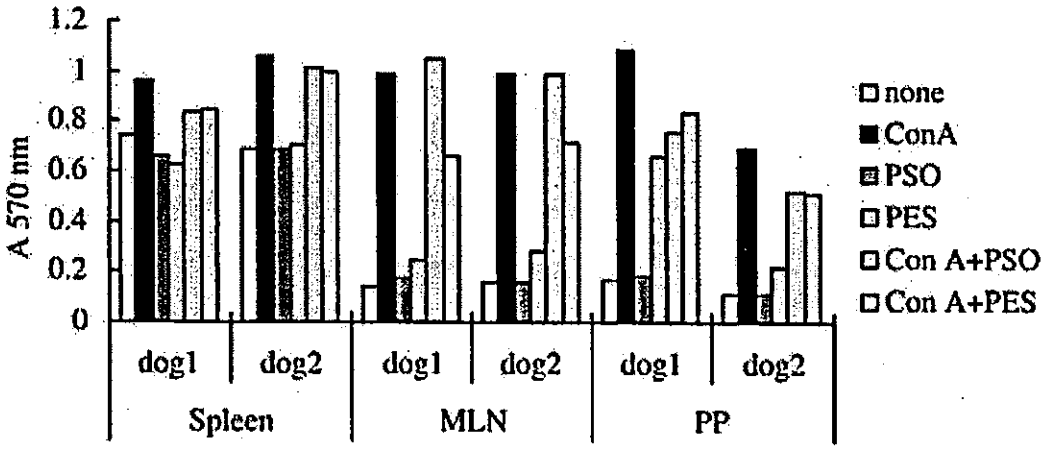


**Fig. 4** *E. multilocularis* specific lymphocyte responses and the effects of parasite antigens on dog lymphocytes at 21 days post-infection. Lymphocytes from spleen, mesenteric lymph node (*MLN*) and Peyer's patches (*PP*) at 21 days post-infection were stimulated with concanavalin A (*ConA*) or parasite antigens, or co-stimulated with ConA and either of the parasite antigens. Cellular proliferation at 72 h was measured by MTT assay. Data are presented as mean values of duplicate samples. *None* Non-stimulated (medium only), *PSO* protoscolex somatic antigen, *PES* protoscolex excretory-

secretory antigen

**Table 1** Parasite specific lymphocyte blastogenesis in *Echinococcus multilocularis* infected dogs at 21 days post-infection. Data are presented as stimulation indices (SI=OD of stimulated cells/OD of non-stimulated cells). PSO protoscolex somatic antigen, PES protoscolex excretory-secretory antigen. *Asterisk* indicates SI $\geq$ 2, which is considered as significant

| Stimuli | Dog | Spleen | Mesenteric lymph node | Peyer's patches |
|---|---|---|---|---|
| PSO | 1 | 0.93 | 1.16 | 1.29 |
| | 2 | 0.96 | 1.06 | 0.82 |
| PES | 1 | 0.87 | 1.63 | 4.65* |
| | 2 | 0.98 | 1.89 | 1.58 |

# Discussion

Regarding the parasite antigen generated lymphocyte blastogenesis, our data are mainly consistent with those reported by Al-Khalidi and Barriga (*1986*) and Deplazes et al. (*1994*) in dogs infected with *E. granulosus* (at 29 and 35 DPI, respectively), in which they observed little blastogenesis against parasite antigens in PBMC, whereas those in lymphocytes of local lymph nodes were observed in some dogs. In our other experiment using a prednisolone-non-treated rodent definitive host model, lymphocyte blastogenesis against parasite antigens were restricted in PP cells (Kato et al. *2005*). In the present study, parasite antigen oriented blastogenesis was observed only in PP cells from one of two dogs. These findings imply the local stimulation of the parasite antigens in the immune system of the intestine where the parasites reside. Further studies are needed to validate this hypothesis.

On the other hand, parasite antigens also suppressed the ConA-induced proliferative responses in all lymphocytes. The suppressive effect was observed before infection (0 DPI) and continued to the end of the experiment (Fig. 3), indicating that it would be available for parasite survival not only at the beginning of the infection, but also for maintenance of the infection and, moreover, for reinfection. Parasite antigens possibly also contribute to the disturbance memory cell generation. It might be crucial for parasites to suppress host immunity, especially at the beginning of infection.

A possible reason for the depression of ConA-induced proliferative responses of PBMC at 7 DPI (Fig. 3) could be the increased level of PES or a change in the lymphocyte profile, which may be caused by the infection. It is inferred that each lymphocyte profile may have a different responsibility against ConA (a mitogen). In *E. granulosus* infection (at 29 DPI), an enhanced ConA-induced proliferative response and suppressed LPS- and PPD-induced proliferative responses of PBMC were reported (Al-Khalidi and Barriga *1986*; Barriga and Al-Khalidi *1986*). The reason for the opposite result with ConA-induced PBMC proliferation obtained in the present study may be the difference of the time (29 DPI vs before 21 DPI) or the difference of parasite (*E. granulosus* vs *E. multilocularis*). More detailed analyses on the effect of the parasite antigens on lymphocyte responses are needed to clarify this point.

Immune modulation of *Echinococcus* spp. was reported in mice: the hydatid disease model. In mice intraperitoneally inoculated with *E. mutilocularis* protoscoleces, suppressed spleen cell proliferation against ConA and parasite antigens occurred by $CD8^{dull}$ suppressor cells (Kizaki et al. *1991*) or by modified peritoneal macrophages (Rakha et al. *1991*). This macrophage modification was mediated by a increased level of nitric oxide (Dai and Gottstein *1999*). Furthermore, Rakha et al. (*1991*) separated the murine macrophage inhibition factor(s) from *E. mutilocularis* protoscolex culture supernatant. According to Walker et al. (*2004*), the antigen secreted by *E. mutilocularis* metacestodes (designated Em492 antigen) was carbohydrate rich and modified the peri-parasitic cellular environment. Carbohydrate-rich *E. granulosus* protoscolece antigen $E4^+$ suppressed ConA-driven spleen cell proliferation, which involved IL-10 secretion in infected and immunized mice (Dematteis et al. *2001*). From preliminary analysis, it was also confirmed that PES has plenty of carbohydrate molecules. It is hypothesized that the carbohydrate molecule of *E. mutilocularis* antigens affects the host local immune system by suppressing lymphocyte responses.

The elevation of parasite specific serum antibodies or antibodies secreted from PP cells (IgG, IgA, IgM, and IgE) was demonstrated in dogs infected with *E. granulosus* (Barriga and Al-Khalidi *1986*; Gasser et al. *1993*; Deplazes et al. *1994*) and in a rodent definitive host model (Inohara et al. *1996*). Generally, IgG1 antibody is associated with infections with extracellular pathogens (Th2 responses) and IgG2 is associated with infections with intracellular pathogens (Th1 responses) (Deplazes et al. *1995*). Interestingly, in the present study, IgG2 response to PES was detected earlier than that of the IgG1 response, indicating Th1 dominance in the early phase of infection.

Barriga and Al-Khalidi (*1986*) reported a significant increase in parasite-specific fecal IgA levels at 35 DPI in *E. granulosus* infection. In the present study, the OD of parasite specific fecal IgA showed large daily variations, which were attributed to the daily change in fecal volume and condition. Further research on the role of mucosal IgA in *E. multilocularis* infection is required.

The most plausible explanation for the antibody production despite suppressed lymphocyte responses would be antibody production by T-cell-independent B1 cells, not by T-cell-dependent B2 cells or the incomplete suppression of lymphocytes by parasitic antigens. T-independent antibodies are produced by carbohydrate-rich antigens. In murine hydatidosis, carbohydrate antigen ($E4^+$) of *E. granulosus* protoscoleces and that of the *E. multilocularis* laminated layer [Em2 (G11)] induced T-independent antibody production (Baz et al. *1999*; Dai et al. *2001*). Thus, a high antibody response against carbohydrate-rich PES suggests that most of the antibodies are T-independent antibodies. T-independent antibodies may act as immune perplexing rather than protecting; however, there was also some evidence that T-independent antibodies protected hosts from pathogens (Szomolanyi-Tsuda and Welsh *1998*; Al-Qaoud et al. *1998*). Even though T-independent antibodies to PES do not contribute to worm expulsion directly, they may affect parasite growth or maturation. Detailed analyses are required for confirmation of the role of parasite-specific antibodies in *E. multilocularis* definitive hosts.

In conclusion, our results indicate that *E. multilocularis* infection/antigens may modulate the local immune system probably in order to support parasite survival. Blocking of immune suppression derived from the parasite might be important for preventing infection in the definitive hosts. Further studies on local immunity in the definitive hosts and parasite antigens are necessary to elucidate host-parasite interactions.

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Original Paper

# Immune responses to oral infection with *Echinococcus multilocularis* protoscoleces in gerbils: modified lymphocyte responses due to the parasite antigen

**Naoko Kato[1, 2] ✉, Nariaki Nonaka[1], Yuzaburo Oku[1] and Masao Kamiya[1, 3]**

(1) Laboratory of Parasitology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, 060-0818 Sapporo , Japan

(2) Department of Tropical Medicine and Parasitology, Dokkyo University School of Medicine, 321-0293 Tochigi, Japan

(3) OIE Echinococcosis Reference Laboratory, c/o Center for Advanced Science and Technology (CAST), Hokkaido University, 001-0021 Sapporo, Japan

✉ **Naoko Kato**
**Email:** nkato@dokkyomed.ac.jp
**Phone:** +81-282-872134
**Fax:** +81-282-866431

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**Abstract** Immune responses to oral infection with *Echinococcus multilocularis* protoscoleces in Mongolian gerbils were investigated. Gerbils not treated with prednisolone expelled most of the parasites within 3 days post-infection and induced parasite-specific intestinal IgA secretion after the oral inoculation with protoscoleces. In contrast, prednisolone-treated gerbils harbored notable numbers of parasites, and the parasite-specific intestinal IgA secretion was lower. In gerbils not treated and orally inoculated with protoscoleces (infected group), parasite-specific antibody levels in sera and intestinal washings were elevated, but blastogenesis against protoscolex antigens was observed only in cells from Peyer's patches at 14 days post-infection. Concanavalin A-induced proliferative lymphocytes from both infected and naive gerbils were suppressed by adding protoscolex somatic antigen. These data indicate that while parasite-specific antibody responses were observed, lymphocyte proliferative responses were suppressed by parasite infection/antigens in the Mongolian gerbil, prednisolone-untreated rodent definitive host model.

## Introduction

The zoonotic cestode *Echinococcus multilocularis* has a life cycle composed of a metacestode (larval) stage in rodent intermediate hosts and an adult tapeworm stage in carnivore definitive hosts. Human alveolar hydatidosis is caused by the accidental ingestion

of parasite eggs deposited in the feces of infected carnivores. In order to reduce the risk of human disease, baiting campaigns against wild foxes with anthelmintic praziquantel have been tried successfully in Europe (Schelling et al. *1997*; Tackmann et al. *2001*; Hegglin et al. *2003*) and Japan (Tsukada et al. *2002*). However, since reinfection seems to occur easily after deworming, repeat administration is required for the effective control of the infection. Thus, immunoprophylaxis of *Echinococcus* infection to definitive hosts is of great interest as an alternative approach to deworming. Although some reports suggest the existence of acquired resistance against the tapeworm stage of *Echinococcus* in the definitive hosts in experimental (Herd et al. *1975*; Heath *1986*; Gemmel et al. *1986*) and field studies (Tackmann et al. *1998*; Morishima et al. *1999*; Yimam et al. *2002*), the mechanisms of resistance are not yet clear. Despite the need, there are only a few studies on the immune responses to *E. granulosus* infection in the definitive hosts (Al-Khalidi and Barriga *1986*; Barriga and Al-Khalidi *1986*; Deplazes et al. *1994*), and little work has been done on *E. multilocularis*.

Research on the carnivore definitive hosts (dogs or foxes) is accompanied by biohazards and animal welfare problems. To overcome this, a rodent alternative definitive host model has been developed using steroid-treated Mongolian gerbils (*Meriones unguiculatus*) (Kamiya and Sato *1990*a) and golden hamsters (*Mesocricetus auratus*) (Kamiya and Sato *1990*b). This system enables us to monitor and handle infected animals easily. Parasite sexual maturation and egg production can be achieved only when rodents are treated with steroids such as prednisolone; however, worms can survive in the intestine for approximately 7 days even in prednisolone-untreated animals (Matsuo et al. *2000*). Although the mechanism of parasite survival in the prednisolone-treated alternative definitive host has not yet been determined, the broad and complex effects, in addition to the immune suppression by prednisolone, influence parasite survival. Because of the striking suppressive effects of corticoids, especially on GALT (Roy and Walsh *1992*; Padgett et al. *2000*), we observed a remarkable decrease in the size of the spleen, mesenteric lymph nodes and Peyer's patches in prednisolone-treated gerbils, and had difficulty obtaining viable lymphocytes for further immunological analyses (unpublished data).

Therefore, in the present work, the humoral and lymphocyte immune responses to *E. multilocularis* infection and the effect of parasite antigens on lymphocyte responses were examined in the prednisolone-untreated Mongolian gerbils. This model may give us a clue to the mechanisms of parasite survival not only in the prednisolone-treated rodent alternative definitive hosts but also in the genuine definitive hosts of *E. multilocularis*.

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# Materials and methods

## Animals and parasites

The adult Mongolian gerbils (male and female) used in this study were produced and raised in our laboratory. Animals were kept in conventional conditions, fed commercial pellets (CE2, Clea Japan, Japan), and received water ad libitum.

The 'Noppro' strain of *E. multilocularis* used for infection was originally isolated from a red-backed vole (*Clethrionomys rufocanus bedfordiae*) at Ebetsu, Hokkaido, Japan in 1997 and maintained in cotton rats (*Sigmodon hispidus*) or Mongolian gerbils by serial intraperitoneal inoculation of metacestodes (secondary hydatidosis) in our laboratory. The

'Eiken' strain used for infection and antigen preparation was isolated from a red-backed vole at Nemuro, Hokkaido, Japan in 1986 and maintained by dog-cotton rat cycles in the Hokkaido Institute of Public Health; thereafter it was kept by secondary hydatidosis in our laboratory. From preliminary data, we considered that the infectivity and viability of these parasite strains were not different.

# Experimental design

## Time course of intestinal IgA secretion and worm burden

Parasite-specific intestinal IgA secretion of the following groups of gerbils was examined at 3, 5, 7, 14, and 21 days post-infection (DPI): gerbils untreated and orally infected with 20,000 live protoscoleces (untreated group), prednisolone-treated gerbils orally infected with 20,000 live protoscoleces (PA-treated group), untreated gerbils orally inoculated with 20,000 dead (frozen) protoscoleces, and untreated and uninfected gerbils (control group). Prednisolone treatment was performed according to Kamiya and Sato (*1990*a) with minor modifications: prednisolone acetate (Predonine, Shionogi, Japan) was used instead of prednisolone tertiary-butylacetate (PTBA). Worm burden was determined by counting parasites in the sediment of intestinal washings and mucus scraped from the intestine.

## Antibody and lymphocyte responses against *E. multilocularis*

Parasite-specific antibody and lymphocyte responses were examined in the gerbils orally infected with 20,000 live protoscoleces (infected group) and the gerbils subcutaneously immunized with protoscolex somatic antigen (PSO) (immunized group). Gerbils were immunized by subcutaneous administration of 150 $\mu$g of PSO in complete Freund's adjuvant (CFA, Sigma, USA) on 28, 21, and 14 days before autopsy. Untreated and uninfected gerbils were employed as controls (control group). At 14–15 days after infection or final immunization, the parasite-specific antibody and lymphocyte responses of each gerbil were evaluated.

# Antigen preparation

Metacestodes were recovered from gerbils and protoscoleces were isolated according to the methods of Smith and Davis (*1975*). To prepare the somatic antigens of protoscoleces (designated PSO), the protoscoleces were mixed with phosphate-buffered saline (PBS, pH 7.4) containing 1% deoxycholic acid (DOC), 5 mM iodoacetamide, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 $\mu$M pepstatin, disrupted first by homogenization using a glass homogenizer, then by sonication (30 W) and by repeated freeze-thaw lysis. Then, the suspension was spun at 10,000 *g* for 30 min at 4°C. The resulting supernatant was dialyzed against PBS and used as somatic antigens.

To prepare excretory-secretory (ES) antigens of protoscoleces (designated PES), live protoscoleces were incubated at 37°C in a 5% $CO_2$ incubator with Medium 199 (Gibco BRL, USA) supplemented with penicillin (100 IU/ml), streptomycin (1 $\mu$g/ml), glucose (4.5 $\mu$g/ml), and KCl (0.2 $\mu$g/ml) (pH 7.4). Culture supernatant was collected daily in the first 2 days, and thereafter every 2 days for a total of 14 days. Then, the culture supernatant was

filtrated through a 0.2 $\mu$m pore size filter (Advantec Toyo, Japan), placed in cellulose tubes (with a M.W. cutoff of 12,000–14,000, Sanko Junyaku, Japan), concentrated using polyvinylpirrolidone (PVP-360 Sigma, USA), and then dialyzed against PBS. After dialysis, the antigen was concentrated again with Centriprep YM-10 or Ultrafree-15 Centrifugal Filter Device BioMax 10 K (Millipore, USA). The protein concentration of antigen was determined by the Bio-Rad Protein Assay Kit I (Bio-Rad, USA).

## Parasite-specific antibody detection

Parasite-specific antibodies in sera and the intestine were detected by a standard enzyme-linked immunosorbent assay (ELISA). To prepare samples for the assay, sera were diluted 100-fold with PBS. The intestinal washings of the small intestine were prepared by flushing the intestine with 3 ml of cold PBS containing 0.02% $NaN_3$, then the intestinal fluid was spun at 700 *g* for 10 min and the supernatant was diluted again twofold with PBS containing 0.05% Tween 20 (PBS-T). Polystyrene ELISA plates (96-well) (Greiner, Germany) were coated with 50 $\mu$l/well of PSO (2 $\mu$g/ml) or PES (0.5 $\mu$g/ml) in carbonate bicarbonate buffer (pH 9.6) and left overnight at 4°C. The plates were then washed three times with PBS-T and blocked with 100 $\mu$l/well of 1% bovine serum albumin (BSA, fraction V, Sigma) in PBS and incubated for 1 h at room temperature (RT). After washing three times, 50 $\mu$l/well of sera or intestinal washings were added to the plates which were then incubated for 1 h at RT. After washing four times, 50 $\mu$l of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (diluted to 1:2,000) (American Qualex, USA) or HRP conjugated goat anti-mouse IgA (1:1,000) (Zymed Laboratories, USA) in 0.5% BSA-0.5% casein in PBS-T were added to each well and incubated for 1 h at RT. Then, the plates were washed five times and 100 $\mu$l/well of substrate solution containing 0.04% of *o*-phenylenediamine (Sigma) and 0.02% $H_2O_2$ in 0.1 M citric phosphate buffer was added and incubated for 30 min at 37°C. Finally, 50 $\mu$l/well of 4 N $H_2SO_4$ was added to stop the reaction and the optical density (OD) was measured at 490 nm.

## In vitro lymphocyte proliferation assay

After 14–15 days of infection or final immunization, spleens, mesenteric lymph nodes (MLN), and Peyer's patches (PP) were removed aseptically and the lymphocytes isolated from each organ. Single cell suspensions were prepared in RPMI 1640 (Gibco BRL, USA) supplemented with 10% inactivated fetal calf serum (FCS, Cosmo Bio, Japan), 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 50 $\mu$M 2-mercaptoethanol (Sigma). Cell viability was assessed using 0.5% trypan blue (Wako Junyaku, Japan) in PBS. Then, cells ($2\times10^5$ cells/100 $\mu$l/well) were cultured in 96-well culture plates (Corning, USA) with either PSO (50 $\mu$g/ml final concentration) or PES (5 $\mu$g/ml). Concanavalin A (ConA, type IV, Sigma) (5 $\mu$g/ml) and lipopolysaccharide (LPS, from *Escherichia coli* 0127: B8, Sigma) (25 $\mu$g/ml) stimulation were employed as positive controls for determining cell viability. Lymphocytes were also co-stimulated with PSO (50 $\mu$g/ml) and either ConA (5 $\mu$g/ml) or LPS (25

$\mu$g/ml). The degree of cell proliferation in 72-h cultures (37°C in 5% $CO_2$) was determined by a colorimetric MTT assay (Mosmann *1983*). Briefly, after incubation for 68-h, each well received 20 $\mu$l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) solution (5 mg/ml, dissolved in PBS). At 4 h after incubation, 100 $\mu$l of acid-isopropanol (0.04 N HCl in isopropanol) was added and mixed thoroughly. OD values were measured at 570 nm.

## Statistical analyses

Statistical analyses of antibody and lymphocyte proliferation data were performed with ANOVA and Scheffe's F test using StatView (version 5, SAS Institute, USA). *P* values <0.05 were considered significant.

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# Results

## Time course of intestinal IgA secretion and worm burden

In untreated gerbils, most of the protoscoleces were expelled within 3 DPI and a few parasites persisted in the intestine until 14 DPI. On the other hand, considerable numbers of worms (55–5,884) developed in the intestine of PA-treated gerbils, but the number of recovered worms varied (Table 1). Parasite-specific intestinal IgA levels were elevated from 7 DPI in the untreated group but were kept at a low level in the PA-treated group (Fig. 1). No elevation of specific IgA was observed in the gerbils inoculated with frozen (dead) protoscoleces.

**Table 1** *Echinococcus multilocularis* worm recovery in Mongolian gerbils. *PA* Prednisolone acetate

| Groups | Days post-infection | *n* | Worm recovery | |
|---|---|---|---|---|
| | | | Mean | (range) |
| Untreated | 3 | 3 | 0.7 | (0–2) |
| | 5 | 3 | 0.7 | (0–2) |
| | 7 | 3 | 0.7 | (0–2) |
| | 14 | 3 | 0.3 | (0–1) |
| | 21 | 3 | 0 | |
| PA-treated | 3 | 3 | 4,758.0 | (2,911–5,884) |
| | 5 | 3 | 2,077.0 | (534–4,050) |
| | 7 | 3 | 1,052.0 | (677–1,493) |
| | 14 | 3 | 2,315.7 | (55–3,493) |
| | 21 | 4 | 335.3 | (75–951) |

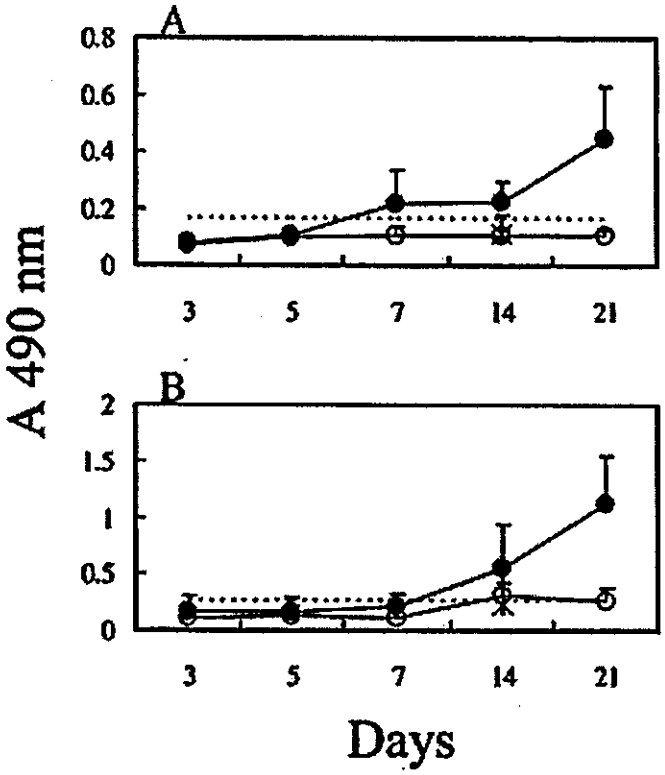


**Fig. 1** Time course of intestinal IgA levels to *Echinococcus multilocularis* in Mongolian gerbils. Untreated (*filled circles*) or prednisolone acetate (PA)-treated (*empty circles*) gerbils were orally inoculated with 20,000 live protoscoleces. Gerbils were killed 3, 5, 7, 14, and 21 days post-infection and the parasite-specific intestinal IgA levels were examined by ELISA. **A** Intestinal IgA levels to protoscolex somatic antigen (PSO). **B** Intestinal IgA levels to protoscolex excretory-secretory antigen (PES). The *cross* indicates intestinal IgA levels in untreated gerbils inoculated with frozen (dead) protoscoleces (14 days post-inoculation only). *Broken lines* indicate cut-off values (mean +3SD of the intestinal IgA levels in untreated and uninfected gerbils). Data are presented as mean+ SD of three/four gerbils. Each sample was tested in duplicate

## Antibody and lymphocyte responses against *E. multilocularis*

Parasite-specific serum IgG levels at 14–15 days post-infection or final immunization were significantly elevated in both immunized and infected groups (Fig. 2). The parasite-specific intestinal IgA level was markedly increased in the infected, but not in the immunized group. Higher responses to PES than those of PSO were observed in the immunized group (serum IgG responses) and in the infected group (serum IgG and intestinal IgA responses).

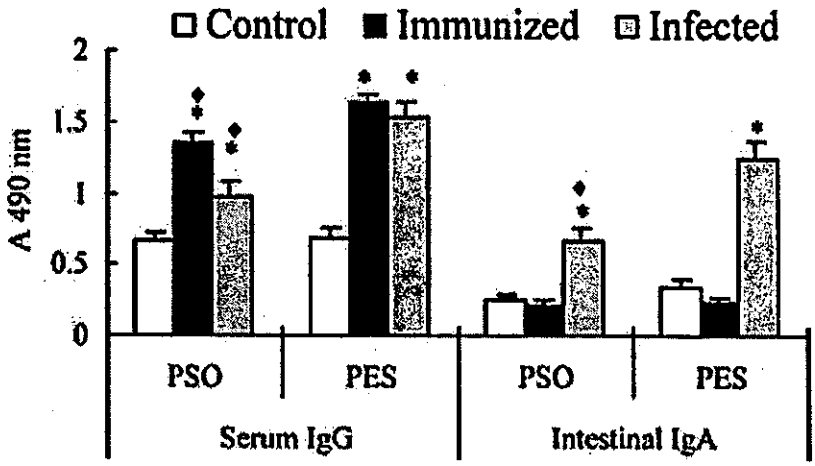


**Fig. 2** Specific serum IgG and intestinal IgA responses to *E. multilocularis* antigens in Mongolian gerbils. Gerbils were orally infected with live protoscoleces (20,000) once (infected group) or subcutaneously immunized with protoscolex somatic antigen (150 $\mu$g with CFA) three times (immunized group). Untreated and uninfected gerbils were used as a control group. After 14–15 days after infection or final immunization, serum and intestinal antibody levels were examined. *PSO* Protoscolex somatic antigen, *PES* protoscolex excretory-secretory antigen. Data are presented as mean + SD of seven gerbils per group. Each sample was tested in duplicate. Statistically significant differences were obtained between values of the treated group and the control group with the corresponding antigen (an *asterisk* indicates $P<0.05$). Significant differences were obtained between values of PES and PSO within groups (*filled diamond*: $P<0.05$)

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Parasite-specific lymphocyte proliferation was obtained only in PP cells of the infected group; however, the degree was relatively low: the maximum response was a threefold value of the non-stimulated cells (Fig. 3A). Parasite antigen-specific lymphocyte proliferative responses of other cells in the infected group were limited. In the immunized group, no lymphocyte response against parasite antigens was observed in any cells.

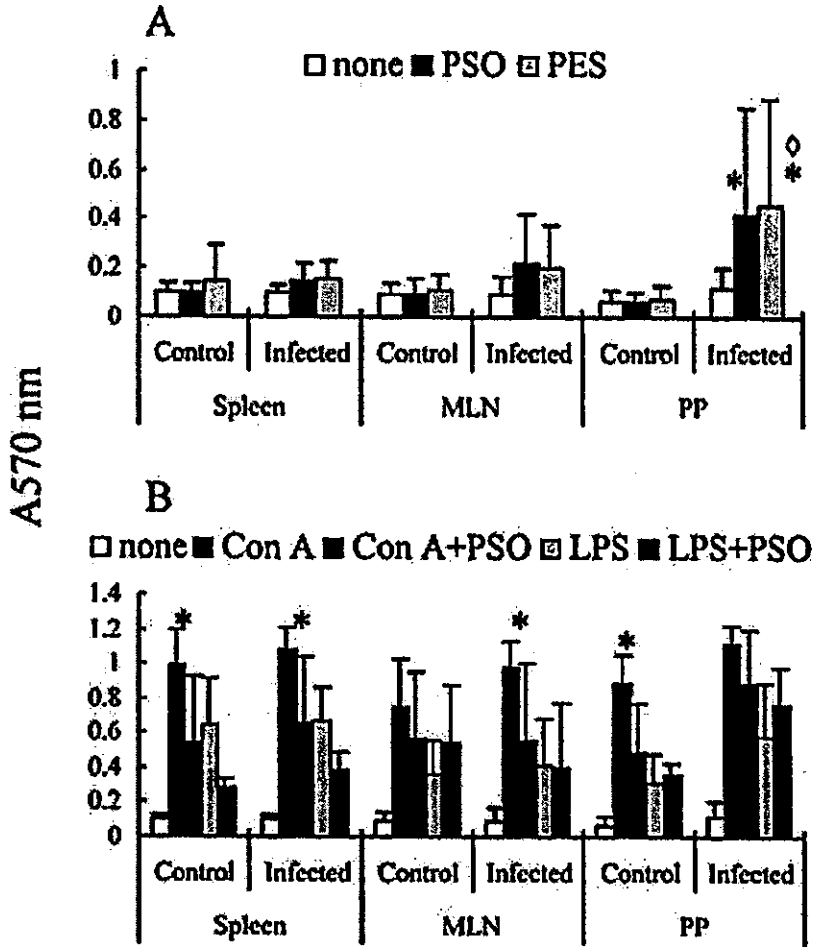


**Fig. 3a, b** Parasite specific blastogenesis and the effect of *E. multilocularis* antigen on mitogen-induced lymphocyte responses in Mongolian gerbils. At 14–15 days after infection, lymphocytes harvested from spleen, mesenteric lymph nodes (*MLN*) and Peyer's patches (*PP*) were stimulated with mitogens (ConA or LPS) or *E. multilocularis* antigens (*PSO* protoscolex somatic antigen or *PES* protoscolex excretory-secretory antigen), or co-stimulated with either mitogen plus PSO. Proliferative responses of 72-h cultures were assessed by MTT assay. A Parasite specific blastogenesis. B Lymphocyte responses stimulated by mitogens or mitogens plus PSO. Data presented are mean optical density+SD of seven animals per group. Each sample assay was performed in duplicate. Statistically significant differences were obtained between values of uninfected (control) and infected groups (A) or between values of ConA and ConA plus PSO-stimulated cells (B) (asterisk $P<0.05$). Differences was obtained between non-stimulated (none) and PES-stimulated cells in infected group (*empty diamond*; $P<0.05$)

## Effect of *E. multilocularis* antigen on mitogen-induced lymphocyte proliferation

PSO suppressed ConA-induced lymphocyte proliferative responses of naive cells from uninfected control gerbils and sensitized cells from infected gerbils (Fig. 3B). The reduction rates were 24–46% in the control group and 21–45% in the infected group. In contrast, PSO suppressed only LPS-induced splenocyte responses, but this was not statistically significant.

# Discussion

This paper focuses on the systemic and local immune responses to *E. multilocularis* infection in prednisolone-untreated gerbils, the definitive host model for *E. multilocularis* infection. In this model, although most worms were naturally expelled within a few days after infection, immune responses were induced. However, significant proliferative responses of lymphocytes against parasite antigens were obtained in only Peyer's patches cells in infected gerbils (Fig. 3A). In *E. granulosus* and *E. multilocularis* infected dogs, parasite-specific lymphocyte blastogenesis, especially of peripheral blood mononuclear cells (PBMC), was limited (Al-Khalidi and Barriga, *1986*; Deplazes et al. *1994*; Kato et al. 2005.

These observations indicate local stimulation (albeit of a low degree) of the parasite antigens on the intestinal immune system. A hypocellular response to parasite antigens could be considered due to oral tolerance (like phenomenon), induced by orally inoculating a large number of protoscoleces; however, this possibility could be negated because the immunized group also showed similar (or even lower) results. Accordingly, the potential effect(s) of immune modulation by parasite antigens is plausible.

PSO suppressed ConA-induced lymphocyte responses to a lesser extent than PES in dogs (Kato et al. *2005*); however, PSO effectively suppressed ConA-induced lymphocyte proliferative responses in both sensitized and naive gerbil (Fig. 3B). Although our results did not show the suppressive effect of PSO on LPS-induced lymphocyte responses, depressed LPS- and tuberculin purified protein derivative (PPD)-induced lymphocyte responses were reported in dogs infected with *E. granulosus* (Barriga and Al-Khalidi *1986*).

In hydatidosis, suppressed lymphocyte responses against ConA and parasite antigens occurred due to $CD8^{dull}$ suppressor cells (Kizaki et al. *1991*) or modified peritoneal macrophages (Rakha et al. *1991*). The macrophage modification was caused by an induced level of nitric oxide (Dai and Gottstein *1999*). Thus, *Echinococcus* infection/antigens may interfere with host immunity (e.g., antigen presenting function, cytokine production, and memory cell generation) by suppressing T-cell or macrophage function or by producing low avidity antibodies.

Our results indicating increased parasite-specific serum antibody levels in gerbils are consistent with those in dogs and prednisolone-untreated golden hamsters (Barriga and Al-Khalidi *1986*; Heath *1986*; Gasser et al. *1993*; Deplazes et al. *1994*; Inohara et al. *1996*; N. Kato et al. 2005 ). Increased parasite specific intestinal IgA in gerbils is similar to the results on fecal IgA levels reported in *E. granulosus*-infected dogs (Barriga and Al-Khalidi *1986*). Frozen (dead) protoscoleces did not stimulate intestinal IgA production (Fig. 1), suggesting that only continuous stimulation with ES product(s) or mechanical contact with living parasites results in antibody production. The most likely explanation for the antibody response, despite the suppressed cellular response by parasite antigens, may be antibody production by T-independent B-1 cells, but not by T-dependent B-2 cells. Several authors have reported antibody production to non-protein (carbohydrate-related) molecules in a T-independent manner (Dixon et al. *1982*; Al-Qaoud et al. *1998*; Dai et al. *2001*). Walker et al. (*2004*) demonstrated that the carbohydrate-rich secretory component of *E. multilocularis* metacestodes modulates the periparasitic cellular environment in alveolar echinococcosis. From preliminary analysis, it was also confirmed that PES has plenty of carbohydrate molecules. In the present study, significantly high levels of antibody were produced against PES (Fig. 2). This implies that most of the antibody is T-independent, mainly reacting with the carbohydrate-rich domain of the parasite antigens. Suppressed intestinal IgA in PA-treated gerbils indicates a contribution by intestinal IgA to worm expulsion. However, in the present study, the significant role of intestinal IgA in worm expulsion was not determined because prednisolone-untreated gerbils excreted worms within 3 days, but the intestinal IgA

level only increased from 7 DPI (Table 1, Fig. 1). Although T-independent antibody is generally considered non-protective, several papers describe the protective effects of mucosal T-independent antibodies (Franco and Greengerg *1997*; Al-Qaoud et al. *1998*; Szomolanyi-Tsuda and Welsh *1998*; Macpherson et al. *2000*). Further studies are necessary to reveal the role of parasite-specific mucosal IgA.

This paper describes the first attempt to evaluate the humoral and lymphocyte immune responses against *E. multilocularis* adult stage infection, using a prednisolone-untreated rodent definitive host model. In this model, similar observations to the responses in dogs infected with *Echinococcus*, especially local immune responses, were induced by oral inoculation with live protoscoleces. Moreover, it was found that parasite antigen could modulate the host immune system; therefore, infection induces both positive and negative stimulation of host immunity.

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# Modified Sugar Centrifugal Flotation Technique for Recovering *Echinococcus multilocularis* Eggs From Soil

**Kayoko Matsuo and Haruo Kamiya***, Department of Parasitology, Hirosaki University School of Medicine, Zaihu-cho, Hirosaki 036-8562, Japan

ABSTRACT: Among soil-transmitted parasitic diseases, alveolar hydatidosis due to the ingestion of *Echinococcus multilocularis* eggs is becoming a serious problem in Hokkaido, the northern most island of Japan. Dissemination of the infection far from the endemic areas can occur if motor vehicles transmit soil contaminated with eggs. No appropriate and validated method for recovering the taeniid eggs from soil is available. A modified sugar centrifugal flotation technique, using a sucrose solution of specific gravity 1.27 and 0.05% Tween-80, was evaluated as a method to successfully recover eggs from soil. Contamination levels as low as 10 eggs per gram could be detected. This method may be useful to determine the prevalence of *E. multilocularis*, its transmission, and the potential for by monitoring soil contamination with eggs.

Soil is contaminated with various kinds of helminth eggs, which can be transmitted to humans and other animals (Mizgajska, 1997; Chongsuvivatwong et al., 1999). *Echinococcus multilocularis* is the causative agent of alveolar hydatidosis and is distributed widely in the Northern Hemisphere. *Echinococcus multilocularis* eggs are deposited in the environment by defecation from the infected definitive hosts, mainly foxes. Human infection with *E. multilocularis* is more likely to occur by accidental ingestion of eggs in soil, contaminated vegetables, water etc., rather than through direct contact with definitive hosts, except dogs. Various techniques have been developed for ascarid egg detection from the soil (World Health Organization Chronicle, 1968; Uga et al., 1989; Ruiz de Ybanez et al., 2000). However, few reports for recovering taeniid eggs from soil are available.

A method for recovering ascarid eggs from sand (Uga et al., 1989) was adapted to a sugar centrifugal flotation technique (Ito, 1980) using a sucrose solution of specific gravity 1.27 (Nonaka et al., 1998). Soil was collected from the ground of Hirosaki University School of Medicine located in a nonendemic area for *E. multilocularis*. Twenty grams of soil mixed with 20 (equivalent to 1 egg per gram [EPG]), 200 (10 EPG), or 2,000 (100 EPG) *E. multilocularis* eggs was placed in 50-ml conical tube to which was added 40 ml of 0.05% Tween-80. The eggs were not infective, having been preserved in 70% ethanol since 1969. The mixture was stirred vigorously and sieved through 100-μm mesh. The suspension was centrifuged at 1,000 *g* for 5 min, and the supernatant was discarded. The sediment was resuspended in 7–8 ml sucrose solution (1.27 specific gravity). The suspension was transferred to 15-ml tubes and centrifuged again at 1,000 *g* for 15 min. Tubes were filled to the top, and a coverslip (24 × 24 mm) was placed on the tube. Coverslips were examined microscopically 2 hr later. A new coverslip was placed on the top of tube and left overnight to detect the remaining eggs. Statistical significance of the results was determined using Student's *t*-test. Data were expressed as mean ± SD, and a $P$ value of less than 0.05 was taken as the minimum level of significance.

Eggs were detected in all soil samples added with 100 EPG after 2 hr of incubation. More eggs were detected after overnight incubation compared with 2 hr of incubation. As shown in Table I, the egg recovery rate was 2.5, 4.8, and 10% in soil samples containing 1, 10, and 100 EPG, respectively. The egg detection rate significantly increased with increase in EPG. The present method could detect contamination of soil with taeniid eggs at a concentration of 10 EPG.

An average of 300 eggs per gravid segment was counted in *E. multilocularis* collected from the fox (Zeyhle and Bosch, 1982). Therefore, if only 1 gravid segment was mixed with 20 g of soil, the EPG in this locality will be more than 10. A total of 34,000 *E. multilocularis* adults were recorded from a heavily infected fox in endemic area of Japan (Morishima et al., 1999). *Echinococcus multilocularis* eggs deposited in feces and dispersed into soil could clearly contaminate a wide area. Furthermore, if fox heavily infected with *E. multilocularis* was involved in a motor accident, a huge number of eggs could be disseminated into the environment from injured viscera. According to Tsukada et al. (2000), infected foxes frequently encountered traffic accidents in Hokkaido, Japan. Therefore, motorcars contaminated with eggs could disperse the parasite to nonendemic areas.

It is somewhat difficult to evaluate the sensitivity of present techniques for application to field survey. However, the contamination of soil with *E. multilocularis* is not the same in different areas. The contamination of eggs in soil should be more intense around fox dens, showing that the wild rodents, its intermediate host, captured near the dens were heavily infected (Kamiya et al., 1977). Therefore, the sensitivity level of 1 EPG is still valuable for detection of eggs in certain instances such as in the soil of highly endemic areas or soil adhered to motorcars run over the injured fox viscera infected with *E. multilocularis*.

Recently, the present technique adapted for 2 kg of soil was used in a survey for detecting *E. multilocularis* eggs in soil left in the Hokkaido to Aomori ferryboat to monitor the transmission of eggs from endemic to nonendemic area. Although no helminth egg was detected, *Isospora* oocysts, mites, and eggs of mites were found (Matsuo et al., 2003). Of course, the soil collected from the endemic area must be incubated at 70 C for 12 hr (Nonaka et al., 1998) or frozen at −80 C for 3 days or more (Veit et al., 1995) to inactivate egg infectivity.

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* To whom correspondence should be addressed. *e-mail: hkamiya@cc.hirosaki-u.ac.jp*

TABLE I. Modified sugar centrifugal flotation technique for recovering *Echinococcus multilocularis* eggs from 20 g of soil.

| Time of incubation | Number of eggs detected (mean ± SD)† | | |
|---|---|---|---|
| | 20‡ (1 EPG) | 200‡ (10 EPG) | 2,000‡ (100 EPG) |
| 2 hr | 0.3 ± 0.5 (3/10)§ | 1.5 ± 0.7 (10/10)** | 64 ± 18.7 (10/10)** |
| Overnight | 0.2 ± 0.4 (2/10) | 8.1 ± 3.7 (10/10)*** | 137 ± 40.8 (10/10)*** |
| Total | 0.5 ± 0.5 (5/10) | 9.6 ± 4.0 (10/10)*** | 201 ± 54.5 (10/10)*** |

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
† Number of detected eggs were evaluated by Student's *t*-test and found significant, where $P < 0.05$, compared with that of sample containing 1 EPG.
‡ Number of eggs added.
§ Number of tubes detected eggs/examined (n = 10).

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