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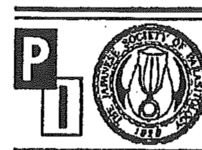

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Production and characterization of monoclonal antibodies against excretory/secretory products of adult *Echinococcus granulosus*, and their application to coproantigen detection

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Abstract

Two IgM murine monoclonal antibodies (MAbs), EgC1 and EgC3, were produced against the excretory/secretory (E/S) products of *Echinococcus granulosus* adult worms. Immunoblotting revealed that both predominantly recognized a 50 kDa antigen in the somatic extract and an 85 kDa component in the E/S products. Immunolocalization showed that both MAbs reacted with the tegument of the parasite, and additionally EgC3 reacted with parenchyma and the tegument lining the external surface of the reproductive organs. A coproantigen capture ELISA was developed using a rabbit polyclonal antibody against E/S products from adult tapeworms as catching antibodies, and each one of MAbs as detecting antibody. The assays detected seven out of eight (EgC1), and eight out of eight (EgC3) experimentally infected dogs (worm burdens ranging from 61 to 57,500), using heat-treated samples obtained at prepatent period, and none (n=8) of helminth-free samples. Time course analysis showed that, after a 12–25 days lag, coproantigen levels rose above cut off O.D. values and typically peaked around 30 days post-infection (DPI) at the end of the experiment. One dog experimentally infected with *Taenia hydatigena* metacestodes was slightly detected as positive at different time points after 30 DPI. Both MAbs showed a similar pattern of recognition, but *T. hydatigena* antigens were undetectable for a longer period, and reached lower O.D. values with EgC1. Interestingly, fecal samples from two experimentally infected dogs with *Echinococcus multilocularis* were not recognized by the EgC1 assay, suggesting a potential value as species-specific diagnostic tool.
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Keywords: Coproantigen; *Echinococcus granulosus*; Monoclonal antibodies

1. Introduction

Echinococcus granulosus, the dog/sheep tapeworm, is the causative agent of cystic echinococcosis, an important zoonosis widely distributed throughout the rural areas of the world. Many affected countries have established control programmes predominantly based on regular dosing of dogs, and in some cases a marked reduction in the transmission of the disease has been achieved [1–3]. Accordingly, accurate assessment of *E. granulosus* in dog populations is a critical requirement for evaluating the programme efficacy, and for estimating the potential infection risk for both human and ruminants. The purgation technique with arecoline hydrobromide has been widely used as the standard method for screening dog populations, but the examination of removed material is time-consuming, requires trained personnel, and it is not sensitive enough, as a single dose could detect less than 50% of *E. granulosus* infections [4].

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Detection of parasite antigens in feces has become an important alternative method for the diagnosis of intestinal infections caused either by protozoa or helminths [5,6]. It has the advantage of correlation with current parasitism, as parasite-derived antigens should not be present in the absence of infection. In this sense, different assays have been developed for the diagnosis of *E. granulosus* components in fecal samples using parasite specific polyclonal antibodies [7–10]. Although the sensitivity obtained with these assays has been reported higher than 90%, low parasite burdens with ≤100 worms were responsible for most false negative results [11]. Additionally, in most cases, false-positive reactions caused by infections with related canine tapeworms were observed.

In this context, we initially evaluated a sandwich ELISA system for *E. granulosus* coproantigen detection, using a monoclonal antibody produced against somatic extract of *Echinococcus multilocularis* [12,13]. Although the test showed a very high sensitivity (100%) in naturally and experimentally infected animals, cases of cross-reactivity with *Taenia hydatigena* were also observed.

In the present work, we produced and characterized, for the first time, MAbs against excretory/secretory (E/S) products from *E. granulosus* adult stage, and preliminarily studied their potential as diagnostic reagents for specific coproantigen detection.

2. Materials and methods

2.1. Experimental infections

The infections were performed as previously described by Malgor et al. [13]. Male and female crossbred dogs, aged 6 months to 2 years, were maintained under helminth-free conditions and fed commercial dog food and water ad libitum. One group of dogs was orally infected with 30,000–200,000 protoscoleces from bovine cysts (dogs 1–3), another group was infected with 25,000–65,000 protoscoleces obtained from ovine cysts (dogs 4–6), and a third one was infected with less than 1000 protoscoleces obtained from ovine cysts (dogs 7–8). They were euthanised before patency with an overdose of sodium pentobarbitone on 25 (dogs 7–8), 30 (dog 3), 31 (dogs 1–2), or 35 (dogs 4–6) days post-infection (DPI). The experiments were performed under the control of the Honorary Commission on Animal Experimentation (CHEA) of the University of the Republic in accordance with the Law on the Use of Animals in Experimentation, Teaching and Universitary Research (Ordenanza sobre uso de animales en experimentación, docencia e investigación Universitaria, Diario Oficial No. 25.467, Febrero 21 de 2000, 1440-C a 1444-C, carillas No. 64 a 68).

One dog was experimentally infected with seven *T. hydatigena* metacestodes, and the infection was maintained during the prepatent period (55 DPI), when the dog was treated with praziquantel (10 mg/kg).

2.2. Preparation of parasite extracts

2.2.1. E/S products

E. granulosus adults worms were recovered from the intestine of experimentally infected dogs at 35 DPI. Briefly, the small intestine was divided into three parts, opened and placed over a mesh in a Petri dish with the mucosae surface in Hank's balanced salt solution (HBSS), and incubated for various periods, during which adult worms were released. They were washed in HBSS (pH 7.2) containing gentamicin (200 μg/ml) and then maintained in Medium 199 (Gibco) pH 7.2 supplemented with glucose (4.0 g/l) and gentamicin (200 μg/ml), at 37 °C in a 5% CO_2 incubator. Approximately 7500 worms were cultivated in 150 ml of medium, which was replaced every 6 h during the first 24 h, then collected, and stored at −80 °C until processed. The medium containing the E/S components was concentrated using a YM-10 membrane (Amicon) followed by dialysis with PBS.

2.2.2. Somatic extracts

Adult *E. granulosus* worms obtained as above were washed in Tris–HCl buffer (pH 7.8) containing EDTA (25 μM) and PMSF (200 μM), homogenized, and ultrasonicated at 20 pulses/min (20% power). Sonicated material was centrifuged during 30 min at 10,000×*g* and supernatant was used as somatic extract.

2.3. Preparation of fecal samples

Feces from experimentally infected dogs were daily collected, mixed in a 1:4 ratio (w/w) with 1% formalin, heated at 70 °C for 12 h, centrifuged at 2200×*g* for 10 min, and the supernatant stored at −20 °C until used for coproantigen detection. Positive *E. multilocularis* fecal samples were collected at 45 DPI from two experimentally infected dogs as previously described [14]. Negative fecal samples were obtained on the day prior to the infection either with *E. granulosus* or *E. multilocularis*.

2.4. Monoclonal antibodies (MAbs) production

BALB/c mice were immunized with 100 μg of *E. granulosus* E/S antigen solution in Freund's complete adjuvant. Two weeks after priming, mice were boosted with the same amount of antigen in Freund's incomplete adjuvant. Three days before fusion, a second booster was given in saline. All the immunizations were done by intraperitoneal injection. After three days, mice were sacrificed and the spleen removed. Splenocytes were fused with X63 myeloma cells using a 50% polyethylene glycol 1500 solution in serum free Iscove's modified DMEM medium (IMDM), containing streptomycin sulfate (0.1 g/l)

and penicillin G (10^5 U/l). Fusion and cell-culture procedures were carried out essentially as described by De StGroth and Scheidegger [15]. Cell supernatants were screened for antibody activity using direct ELISA with *E. granulosus* E/S antigen. Hybridoma with suitable growth and higher secretion of antibodies against *E. granulosus* were repeatedly cloned by limited dilution in IMDM with 20% fetal bovine serum (Gibco) and cultured. Thymocytes from BALB/c mice were used as feeder cells. MAbs were recovered from cell culture supernatant.

2.5. ELISA assay

For MAbs screening and isotype determination, direct ELISA was performed as follows: flat-bottomed microtitre plates (Maxisorp, Nunc) were coated with 1 μg/ml antigen (50 μl/well) in 0.05 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.6) and left overnight at 4 °C. Blocking was done with 1% bovine serum albumin (BSA) in PBS (100 μl/well) for 2 h. Hybridoma culture supernatants (50 μl/well) were incubated for 1 h. Bound antibodies were detected after the addition of 50 μl of a horseradish peroxidase conjugated rabbit anti-mouse IgG+A+M (Sigma), diluted at 1/2000 in 0.5% BSA and 0.5% casein in PBS containing 0.05% Tween (PBS-T) for 1 h, and then 0.04% *o*-phenylenediamine, 0.07% H_2O_2 in citrate phosphate buffer (pH 5.5) were added for 10 min at 37 °C (100 μl/well). The reaction was stopped with 50 μl of 4 N H_2SO_4 and the plates were read at 492 nm. All the washes were done with PBS-T. Unless otherwise stated, all procedures were carried out at room temperature (RT). For isotype determination, the same protocol was followed, but horseradish peroxidase conjugated rabbit anti-mouse IgM and IgG (Sigma) were used.

2.6. Rabbit anti-E. granulosus E/S products polyclonal antibody

A New Zealand White rabbit was immunized three times subcutaneously with 100 μg of E/S products from adult *E. granulosus*, using Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for the first booster 2 weeks later. The third injection was in saline. IgG was purified from pooled sera using a Protein A affinity column (BioRad) according to manufacturer's instructions.

2.7. Immunoblotting

Somatic extract and E/S products of adult *E. granulosus* were separated in 10% SDS-PAGE under reducing conditions, and blotted onto a nitrocellulose membrane (Bio-Rad) using a semi-dry horizontal electro-transfer system. Blocking was done with 1% BSA in PBS at 4 °C overnight. The strips were then probed with hybridoma culture supernatant for 1 h at RT, and then incubated with horseradish peroxidase conjugated rabbit anti-mouse IgM (Sigma) diluted at 1/4000 in 0.1% BSA in PBS-T for 1 h at RT. Peroxidase reaction was visualized with 0.06% (w/v) diaminobenzidine tetrahydrochloride in 50 mM Tris–HCl (pH 7.6) and 0.03% (v/v) H_2O_2. The reaction was stopped after 5 min with distilled water.

2.8. Immunofluorescence on histological sections

Adult worms, fixed in 70% alcohol, were dehydrated, cleared, embedded in paraffin wax, and cut 4 μm thick. After dewaxing, sections were incubated with the MAbs (culture supernatant) for 1 h at 37 °C. After washing with PBS-T, a FITC conjugate anti-mouse IgM (Sigma) was added for 1 h at 37 °C. The sections were washed, then mounted and observed in a Zeiss fluorescence microscope.

2.9. Coproantigen detection

Sandwich ELISA for coproantigen detection was performed following the protocol described by Malgor et al. [13]. Flat-bottomed microtitre plates were coated with Protein A purified rabbit anti-*E. granulosus* E/S products antibody (5 μg/ml) in carbonate–bicarbonate buffer (pH 9.6), overnight at 4 °C. The plates were blocked with 1% BSA in PBS for 2 h at RT, and then incubated with fecal supernatant (50 μl/well) or different concentrations of parasite E/S products diluted in negative feces (500 to 10 ng/ml), for 2 h at RT. Then they were loaded with 50 μl of hybridoma culture medium, and after 1 h, 50 μl/well of HRP-conjugated anti-mouse IgM (1:4000) were added for another 1 h. Finally, 100 μl of *o*-phenylenediamine (0.04%) and H_2O_2 (0.07%) in citrate phosphate buffer (pH 5.0) were added for 10 min at 37 °C. The reaction was stopped by adding 50 μl of 4 N H_2SO_4, and plates were read at 492 nm. The cut off values for both MAbs were determined by

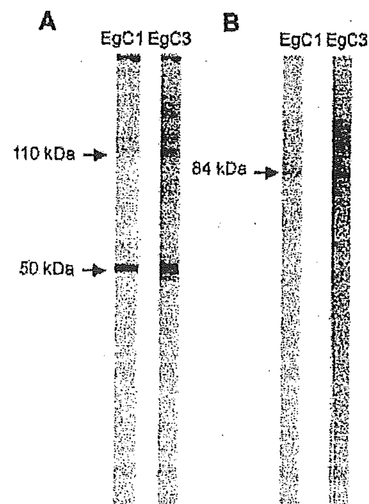

Fig. 1. Western blotting with EgC1 and EgC3 on *E. granulosus* somatic extract (A) and E/S products (B). Apparent molecular weights are shown on the left.

calculating the mean value+3S.D. of the samples collected immediately prior to infection (EgC1=0.088, EgC3=0.164).

3. Results

3.1. Monoclonal antibodies

Two clones of IgM MAbs were produced against the E/S products of adult *E. granulosus*, namely EgC1 and EgC3.

3.2. Immunoblotting

After SDS-PAGE, immunoblotting with both MAbs showed reactivity with a prominent single band of an apparent molecular weight of 50 kDa in the somatic extract, and with a band of 85 kDa in the E/S products (Fig. 1A and B). Additionally, EgC3 reacted with other minor slow migrating bands in the somatic extract (Fig. 1A).

3.3. Immunolocalization

Both MAbs exhibited high intensity staining, predominantly at the tegument of the parasite (Fig. 2A–C). Besides, EgC3 reacted with parenchyma and the tegument lining the external surface of the reproductive organs (Fig. 2A and B).

3.4. Coproantigen detection

Fig. 3 shows the detection of fecal antigens by EgC1 and EgC3 in experimentally infected dogs, harboring from 61 to 57,500 worms. At the last days of prepatent infection (25

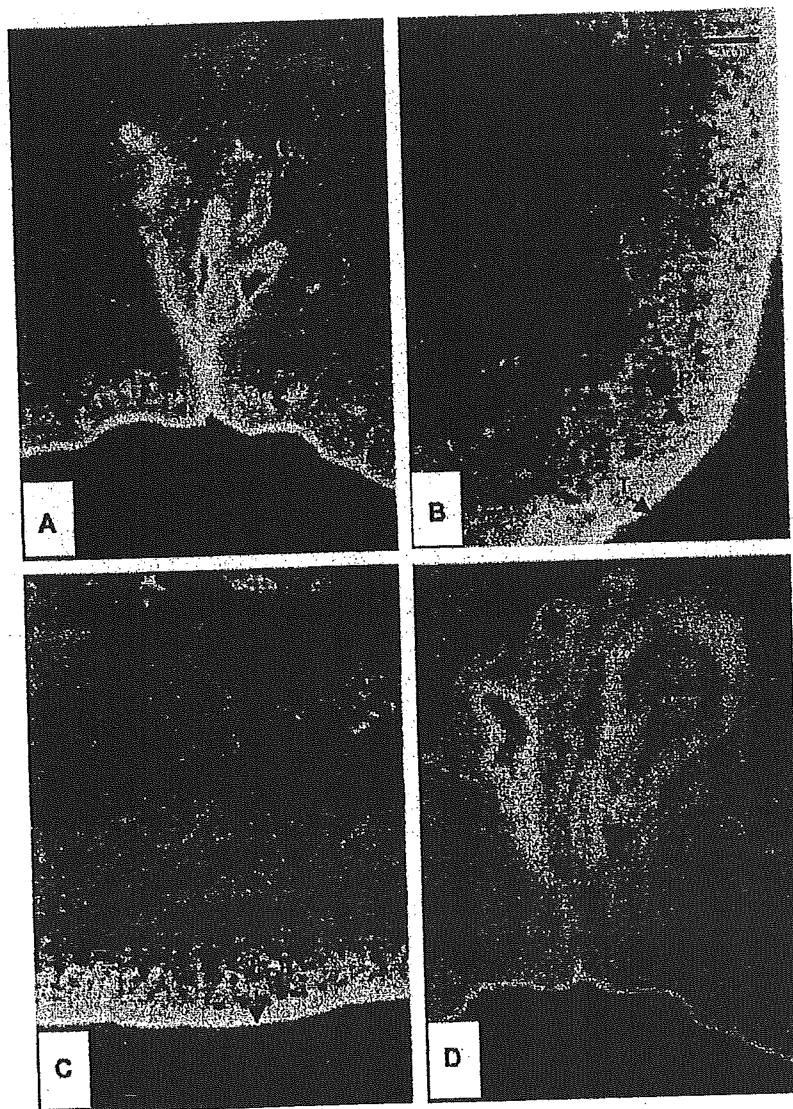

Fig. 2. Immunofluorescence with EgC1 and EgC3 on adult *E. granulosus* sections (posterior proglotid). (A) EgC3 recognition of tegument (T). (B) EgC3 recognition of parenchyma (P). (C) EgC1 recognition of tegument (T). (D) Control without MAb.

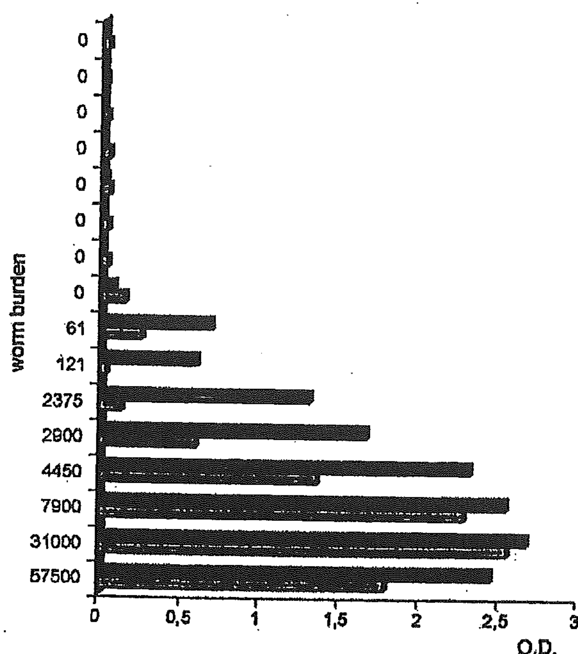

Fig. 3. Detection of *E. granulosus* coproantigens using a sandwich ELISA with a polyclonal antibody developed against *E. granulosus* E/S products as capture and EgC1 and Egc3 as detecting antibodies. Feces from dogs experimentally infected with different worm burdens were tested (samples from dogs harboring from 2375 to 57,500 worms were from 30 DPI, and samples from dogs harboring 61 and 121 worms were from 25 DPI). ▨, EgC1; ■, EgC3.

DPI for dogs harboring 61 and 121 worms, and 30 DPI for dogs harboring from 2375 to 57,500 worms), all infected dogs were detected as positive for EgC3, and seven out eight for EgC1. None of the helminth-free controls showed false positive reactivity.

The sensitivity of both systems was preliminarily determined evaluating the detection limit for serial dilutions of parasite E/S products in negative feces. The detection limit for EgC1 was below 30 ng/ml and for EgC3 was below 7 ng/ml, equivalent to 120 and 28 ng of parasite components/g of feces, respectively. Fig. 4 expose the individual time course of coproantigen detection in each of the eight dogs experimentally infected with *E. granulosus*. Both MAbs detected released fecal antigens during the prepatent period studied. Using EgC3, fecal samples became positive at 12 DPI in the dogs harboring higher worm burdens (dogs 1 and 2), or later at 15–24 DPI in dogs with less than 10,000 worms (dogs 3 to 6), followed by a rise in O.D. values that remained positive until the end of the experiment. In dogs carrying worm burdens between 61 and 121 (dogs 7 and 8), the coproantigens were detected at the end of the experimental infection (25 DPI), with O.D. values in the same order as those from higher worm burden on 25 DPI. The ELISA assay with EgC1 showed a similar pattern, but antigens were undetectable for a longer period, and reached lower O.D. values.

However, when feces from a *T. hydatigena*-infected dog were assayed, both MAbs showed positive O.D. values in

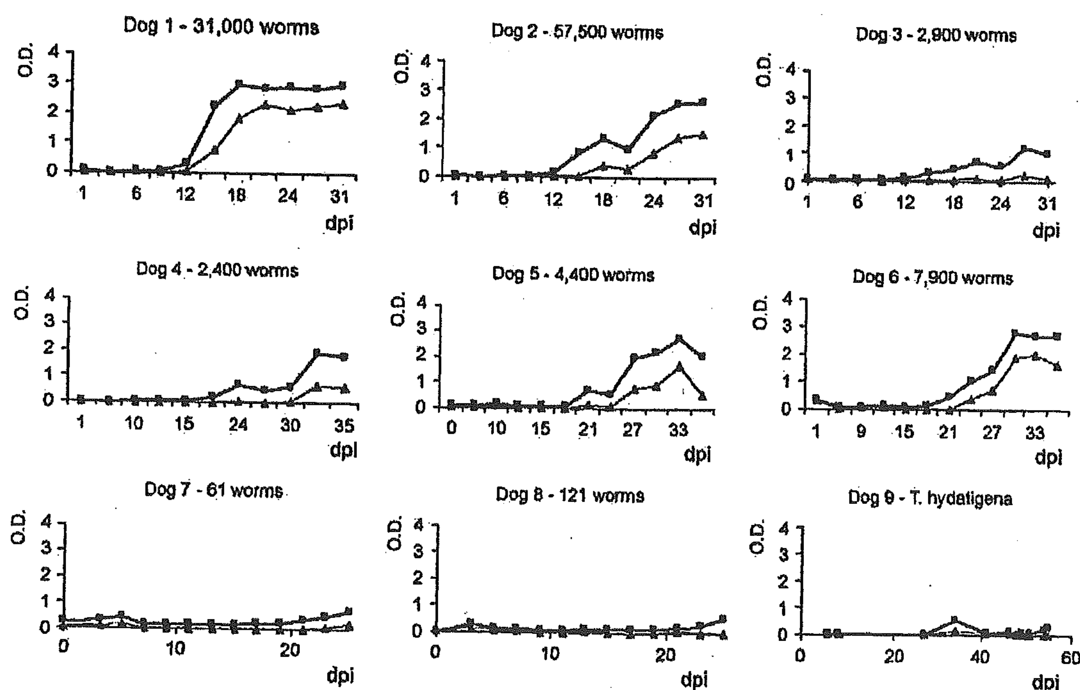

Fig. 4. Time course coproantigen detection in the prepatent period of infection of dogs experimentally infected with different worm burdens of *E. granulosus* or *T. hydatigena*. DPI: days after infection; ▲, EgC1; ■, EgC3.

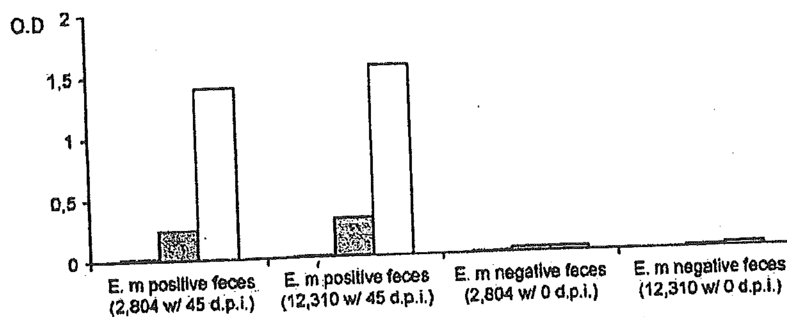

Fig. 5. Coproantigen detection by EgC1 ■, EgC3 ▨ and EmA9 □ in feces from dogs experimentally infected with *E. multilocularis*.

samples at different time points from 30 DPI until the end of the experiment on 55 DPI.

An indication of specie-specificity of EgC1 is shown in Fig. 5. EgC1 did not recognize either *E. multilocularis* somatic extract (not shown) or positive feces from infected dogs. The results were compared with EmA9, an MAb prepared against somatic extract of *E. multilocularis* [16].

4. Discussion

It is increasingly recognized that an accurate measurement of the prevalence of canine infection is a critical requirement in order to establish the epidemiological status of cystic echinococcosis in a given situation. However, the use of the standard purgation method with arecoline hydrobromide is highly problematic mainly due to its low sensitivity and operational difficulties, making it unsuitable for the screening of large dog populations. In this context, the immunodetection of soluble released antigens in fecal supernatants has gained increasing support as an alternative method capable of overcoming these difficulties [10]. Aimed at developing a highly specific assay, we produced two MAbs, EgC1 and EgC3.

The accuracy of coproantigen detection in canine echinococcosis is critically dependent on the parasite burden, as initially observed by Deplazes et al. [8], who detected only one in eight dogs infected with less than 100 worms, using polyclonal antibodies produced against E/S products from adult tapeworms. Another study using the same assay showed that 92% of dogs harboring more than 100 worms were positive, while detection capacity dropped to 30% in those animals with less than 100 parasites [11]. Similarly, a burden-related effect was observed in another field study that employed a coproantigen capture ELISA with affinity-purified polyclonal IgG anti-*E. granulosus* somatic homogenate. In this case, false negative coproantigen samples were from dogs with less than 20 worms detected at purge. It has been shown that the average worm burden of *E. granulosus* is about 200/dog in endemic areas for cystic echinococcosis [17].

For the coproantigen detection ELISA utilizing EgC1 and EgC3, time course profiles of coproantigen detection during experimental infections were very similar. Fecal samples became positive during the prepatent period after a lag phase of 12 to 25 DPI, being later for dogs with lower worm counts. After detection, coproantigen levels showed a steady rise that peaked at about 30 DPI. Within the range of parasite counts, EgC3 showed higher values than EgC1, and also a trend of positive correlation between OD values and worm burden (not observed with EgC1). Dogs infected with less than 121 worms were detected by EgC3 near day 25 (EgC1 only detected dog 7), when the experimental infection was finished. The O.D. values for these samples (25 DPI) were similar to those of dogs harboring higher worm burdens.

These findings suggest that the detection limit of the coproantigen assay is related to the biomass and the antigen production capacity of growing parasites. Alternatively, it is possible that some *E. granulosus* antigens released to the intestinal lumen during the early phase of development were stage-specific and hence, not recognized by EgC1 and EgC3, produced against E/S products from older prepatent worms.

Unlike previous reports that utilized either polyclonal or monoclonal-based assays, none of the studied dogs exhibited strong fluctuations in coproantigen excretion levels [7,13,14] indicating an even distribution of released parasite antigens in the feces.

Cross-reactivity with *Taenia* spp. constitutes another major hurdle for the development of a highly specific coproantigen detection method for echinococcosis. The cross-reaction has been reported for all the developed coproantigen tests [7,8,13] and it can be undoubtedly a problem in countries like Uruguay, where *T. hydatigena* is in hyperendemic steady state [18]. Recently, a major field study conducted by Christofi et al. [10] in Cyprus revealed that ECHINOTEST, a commercial coproantigen kit based in the polyclonal-based assay developed by Allan et al. [7], had a sensitivity of 83% and a specificity that ranged from 80% to 98%, depending on the presence of *Taenia* spp. infection in the group under evaluation.

Our results showed that MAbs reacted with fecal supernatants from a dog infected with seven worms of *T. hydatigena* at different prepatent time points starting on 35 DPI until the end of the experiment on 55DPI. However,

extensive field studies in naturally infected animals with *Taenia* are necessary to assess assay specificity, particularly using EgC3.

This false positive reaction with *Taenia* positive samples contrasts with the lack of reactivity showed by EgC1 with patent feces from *E. multilocularis* experimentally infected dogs, harboring >1000 worms. Such species-specificity might be useful in epidemiological settings where both *Echinococcus* species coexist, as in parts of Central Europe and China [19].

The capacity of both MAbs for spotting animals for treatment before eggs can contaminate the environment is a valuable feature for control campaigns where rates of reinfection in dogs are being monitored. Additionally, both MAbs detect heat-resistant epitopes, possibly carbohydrate moieties, allowing the sterilization of fecal samples by heating, thus rendering them safe for the personnel involved.

Acknowledgements

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ORIGINAL PAPER

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Modified cellular immune responses in dogs infected with *Echinococcus multilocularis*

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Abstract Parasite-specific antigen responses and lymphocyte blastogenesis in dogs orally inoculated with *Echinococcus multilocurālis* metacestodes were examined. Serum IgG1 (Th2-oriented) and IgG2 (Th 1-oriented) levels against somatic and excretory-secretory (ES) antigens of protoscoleces and adult worms increased from 7 days post-infection (DPI), with the highest responses against protoscolex excretory-secretory antigen (PES). Specific blastogenesis of peripheral blood mononuclear cells (PBMC) against the parasite antigens was not observed during the 21-day infection period, but Peyer's patches cells from one out of two dogs at 21 DPI showed blastogenesis against PES (stimulation index: 4.65). Interestingly, only at 7 DPI were concanavalin A (ConA)-induce proliferative responses of PBMC reduced. Moreover, ConA-induced proliferative responses of lymphocytes from various origins were suppressed by the addition of parasite antigens, especially with PES. These data suggest that although both Th1- and Th2-oriented humoral immune responses were observed in *E. multilocularis* infected dogs, the parasite antigens, especially PES, may have incompletely suppressed lymphocyte responses in these dogs.

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Introduction

Echinococcus multilocularis is the causative agent of a parasitic zoonosis, alveolar hydatidosis. The life cycle exploits a prey–predator relationship (voles as the intermediate hosts and carnivores as the definitive hosts), and human cases are caused by accidental ingestion of parasite eggs. A countermeasure against the source of infection (parasite eggs) has been successfully developed by baiting wild foxes with the anthelmintic praziquantel (Schelling et al. 1997; Tackmann et al. 2001; Tsukada et al. 2002; Hegglin et al. 2003). Compared to deworming, immunoprophylaxis of the definitive hosts would have more advantages for a long-lasting preventive effect.

Although some evidence suggests the existence of protective immunity to *Echinococcus* in the definitive hosts both in experimental infections (Herd et al. 1975; Gemmell et al. 1986; Heath 1986) and field studies (Tackmann et al. 1998; Morishima et al. 1999; Yimam et al. 2002), the mechanisms have not yet been fully elucidated. Despite the need, little information is available on immunity to *Echinococcus* infections in definitive hosts because of its biohazardous nature and animal welfare problems. Although there are some works on the humoral and cellular immunity to *E. granulosus* infection, little work has been done on *E. multilocuralis* infection. In dogs infected with *E. granulosus*, at 35 days post-infection (DPI), parasite specific blastogenesis of local lymph node cells (mesenteric, popliteal, and Peyer's patches) were obtained from some dogs, and those of peripheral blood mononuclear cells (PBMC) resulted in low levels in a subset of dogs (Deplazes et al. 1994). According to Al-Khalidi and Barriga (1986), *E. granulosus* infection at 29 DPI depressed the ability of non-stimulated PBMC to proliferate, but enhanced concanavalin A (ConA)-stimulated proliferative responses. In contrast, many workers reported suppressed ConA- and antigen-induced splenocyte proliferation in the intermediate

hosts of *Echinococcus* (Rakha et al. 1991; Kizaki et al. 1991; Dai and Gottstein 1999; Dematteis et al 2001).

Thus, it is hypothesized that the immune system, especially the intestinal immune system of the definitive host, may be stimulated by *E. multilocuralis* infection. To clarify this, antibody and lymphocyte responses to primary infection of *E. multilocularis* in dogs were examined. In addition, the effect of parasite antigens on ConA-induced lymphocyte proliferative responses was examined to assess immune modulation by parasite antigens.

Materials and methods

Parasites

The parasite used in this study was the 'Eiken' strain of *E. multilocularis*, originally obtained from a red backed vole (*Clethrionomys rufocanus bedfordiae*) in Nemuro, Hokkaido, Japan in 1986 and kept by dog–cotton rat (*Sigmodon hispidus*) cycles in the Hokkaido Institute of Public Health. Thereafter, this strain was maintained in Mongolian gerbils (*Meriones unguiculatus*) by serial passages of metacestodes (i.e., secondary hydatidosis) in our laboratory.

Animals and experimental infections

Two beagle dogs (male, 16 months old), kindly provided by the New Drug Development Research Center (Iwamizawa, Hokkaido, Japan), were kept in our laboratory and supplied with commercial dog food (CD-5, CLEA Japan, Tokyo, Japan) and water ad libitum. For initiating experimental infections in dogs, the *E. multilocuralis* metacestodes of 8 months post-infection were recovered from a gerbil, and the dogs were orally given about 230,000 protoscoleces. Then the dogs were bled on 0, 3, 7, 10, 14, 17 and 21 DPI. Peripheral blood was utilized for lymphocyte proliferation tests. Sera were also separated from the blood and stored at −40°C until used for antibody detection. Feces were collected everyday from 7 days before infection and stored at −40°C until needed.

E. multilocularis coproantigen detection

Excretion of *E. multilocularis* coproantigen was monitored by the previously reported sandwich enzyme-linked immunosorbent assay (ELISA). The assay using a murine monoclonal antibody directed against somatic antigens of adult *E. multilocularis*, EmA9 (Kohno et al. 1995), was performed according to the method described by Morishima et al. (1999).

Antigen preparation

Metacestodes were recovered from gerbils and protoscoleces were isolated according to the method of Smith and Davis (1975). Adult worms (21 days old) were obtained from the rodent alternative definitive hosts described by Kamiya and Sato (1990) with a minor modification: prednisolone acetate (Predonine, Shionogi, Japan) was used in place of prednisolone tertiary butylacetate (PTBA).

To prepare somatic antigens of protoscoleces and adult worms (called PSO and ASO, respectively), isolated protoscoleces or adult worms 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 μM pepstatin, disrupted first by homogenization using a glass homogenizer, then by sonication (30 W), and finally by repeated freeze-thaw lysis. Then, the suspension was subjected to centrifugation at 10,000 *g* for 30 min at 4°C. The resulting supernatant was dialyzed against PBS and used as a somatic antigen.

To prepare excretory-secretory (ES) antigens of protoscoleces and adult worms (called PES and AES, respectively), live protoscoleces or adult worms were incubated at 37°C in a 5% CO_2 incubator with Medium 199 (Gibco BRL, USA) supplemented with penicillin (100 IU/ml) and streptomycin (1 mg/ml), glucose (4.5 mg/ml), and KCl (0.2 mg/ml) (pH 7.4). Then, culture supernatant was filtrated through a 0.2 μm pore size filter (Advantec Toyo, Japan), placed in cellulose tubes with a cutoff molecular weight of 12,000–14,000 (Sanko Junyaku, Japan), concentrated using polyvinylpirrolidone (PVP-360 Sigma, USA), and then dialyzed against PBS. After dialysis, antigen solution was concentrated again with Centriprep Ym-10 or Ultrafree-15 Centrifugal Filter Device BioMax 10 K (Millipore, USA). Protein concentration of each antigen was determined by using the Bio-Rad Protein Assay Kit I (Bio-Rad, USA).

Parasite-specific antibody detection

E. multilocularis specific antibodies (serum IgG1 and IgG2, IgA, and fecal IgA) were detected by ELISA. Antigens and their concentrations utilized for plate coating were as follows: PSO (5 μg/ml), PES (0.5 μg/ml for IgG antibodies, 1 μg/ml for IgA antibodies), ASO (5 μg/ml), and AES (0.5 μg/ml for IgG antibodies, 1 μg/ml for IgA antibodies). After coating the plate with 50 μl of antigens in 0.05 M $NaHCO_3$-Na_2CO_3 buffer (pH 9.6) overnight at 4°C, the plate was washed with PBS containing 0.05% Tween 20 (PBS-T) three times and blocked for 1 h with 100 μl/well of 1% bovine serum albumin (BSA, fraction V, Sigma, USA) in PBS. Then, the plate was washed with PBS-T three times and incubated either with 50 μl/well

of sample sera or fecal supernatant for 1 h, all of which were assayed in duplicate. Sera were diluted 100-fold with PBS. Feces (10 g) were mixed with three times the volume (v/w) of PBS-T containing 0.02% sodium azide. The mixture was spun at 20,000 *g* for 15 min at 4°C and the resulting supernatant was diluted again twofold with PBS-T and used for antibody detection. After incubation of the plate with samples, the plate was washed again and incubated either with 50 µl/well of horseradish peroxidase (HRP)-conjugated goat anti-dog IgG1 (γ1) (diluted 1:10,000 in 0.5% BSA-0.5% casein in PBS-T), HRP-conjugated sheep anti-dog IgG2 (γ2) (1:10,000) or HRP-conjugated goat anti-dog IgA (α) (1:1,000) (all products from Bethyl Laboratories, USA) for 1 h. Then the plate was washed again and incubated with 100 µl/well of 0.04% *o*-phenylenediamine (Sigma) and 0.02% H_2O_2 in 100 mM citric phosphate buffer (pH 5.0) for 30 min at 37°C. Finally, 4 N H_2SO_4 was added to stop the reaction and the optical density (OD) at 490 nm of each well was read using an ELISA plate reader (Microplatereader, Model 450, Bio-Rad). The cut-off value of the ELISA for specific antibody detection was determined by calculating the mean + 3SD of the samples collected before infection.

Lymphocyte proliferation tests

Peripheral blood mononuclear cells (PBMC) were isolated using Histopaque-1077 (Sigma Diagnostics, USA) according to the manufacturer's instructions. At 21 DPI, dogs were sacrificed by injecting an overdose of sodium pentobarbital (Nembutal, Dainippon Pharmaceutical, Japan), the spleen, mesenteric lymph nodes (MLN), and Peyer's patches (PP) were removed and lymphocytes were 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, Tokyo, Japan), 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol (Sigma, USA). Cell viability was assessed using 0.5% trypan blue (Wako Junyaku, Japan) in PBS. Then, cells (2×10^5 cells/100 µl/well) were cultured either with PSO (50 µg/ml final concentration) or PES (5 µg/ml) in 96-well culture plates (Corning, USA). Cells were also cultured with ConA (type IV, Sigma, USA) (5 µg/ml) for a positive control of cell reactivity or without stimuli. In addition, cells were co-stimulated with ConA and one of the parasite antigens. The cells were cultured at 37°C in a 5% CO_2 incubator for 72 h. The degree of cell proliferation was determined by a colorimetric MTT assay (Mosmann 1983) and the color development was measured at 570 nm. Stimulation indices (SI) were expressed as the ratio of the proliferation of stimulated cells to that of non-stimulated cells. An SI≥2 was considered as significant (Gottstein et al. 1990).

Results

E. multilocularis coproantigen detection

During the course of experimental infection, excretion levels (OD values) of coproantigen from both dogs increased at 7 DPI and remained high until the end of the experiment (21 DPI) (Fig. 1). This indicates that *E. multilocularis* was successfully established in the intestine of the dogs during the experiment.

Parasite-specific antibody detection

Serum antibody levels to *E. multilocularis* antigens are shown in Fig. 2. Serum IgG1 levels specific to four *E. multilocularis* antigens increased from 10 to 14 DPI, with the highest response observed with PES. In contrast, serum IgG2 levels increased earlier from 7 DPI, with the highest response observed with PES. An increase in the serum IgA level was observed in dog 2, with the highest response obtained with PES, while the increase was not obvious in dog 1. Parasite-specific fecal IgA titers showed high OD values before infection and high variation of daily OD values after infection.

Lymphocyte proliferation tests of PBMC

During the course of infection, responses of PBMC against ConA at 7 DPI decreased remarkably, but were restored thereafter (Fig. 3). Responses of PBMC against PSO and PES were not remarkably different from those of non-stimulated cells throughout the infection. Co-stimulation of PBMC with ConA and the protoscolex antigens, particularly with PES, resulted in lower responses compared with the responses with ConA mono-stimulation (maximum 60.7% reduction in dog 1 at 7 DPI).

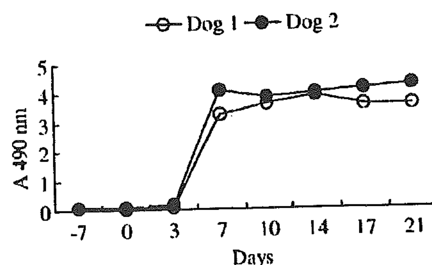

Fig. 1 Time course of *Echinococcus multilocularis* coproantigen detection in dogs. Dogs were orally infected with *E. multilocularis* metacestodes (containing 230,000 protoscoleces). *E. multilocularis* coproantigen was measured by sandwich ELISA using monoclonal antibody EmA9

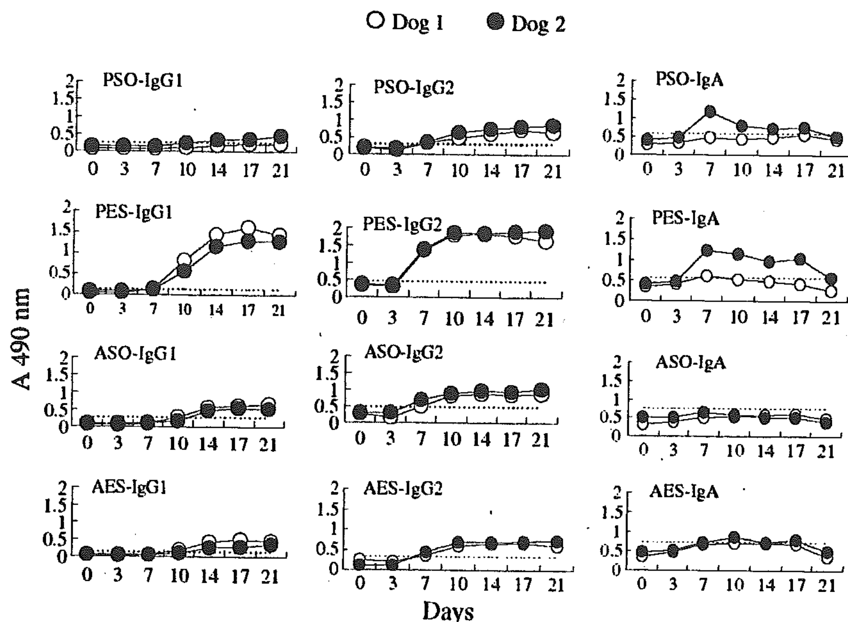

Fig. 2 Time course of serum antibody levels in dogs infected with *E. multilocularis*. *E. multilocularis* antigen specific IgG1, IgG2, and IgA levels were measured by ELISA. Data are presented as individual samples. Each sample assay was performed in duplicate. *PSO* Protoscolex somatic antigen, *PES* protoscolex excretory-secretory (ES) antigen, *ASO* adult worm somatic antigen, *AES* adult worm ES antigen. *Broken lines* indicate the cut-off values (the mean OD values +3SD of sera before infection)

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.

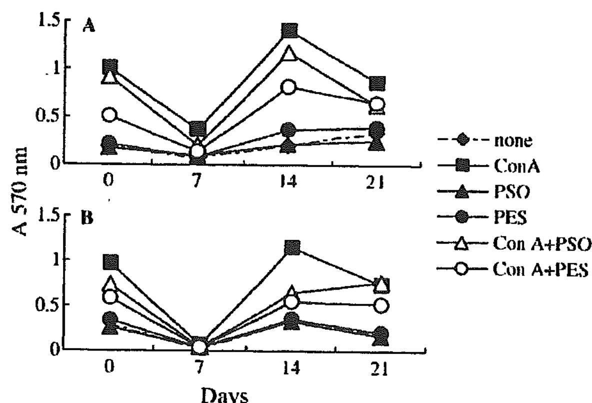

Fig. 3 Time course of *E. multilocularis* specific lymphocyte responses and the effects of parasite antigens on dog peripheral blood mononuclear cells (PBMC). Dog PBMC were stimulated with concanavalin A (*ConA*) or parasite antigens, or co-stimulated with ConA and either of the parasite antigens. Cellular 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

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)

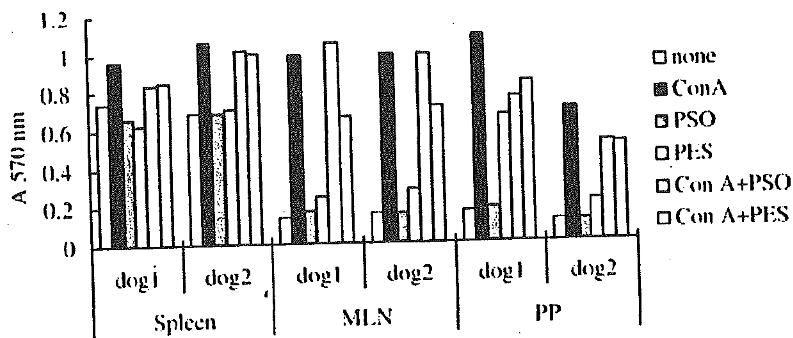

Fig. 4 *E. multilocularis* specific lymphocyte responses and the effects of parasite antigens on dog lymphocytes at 21 days post-infection. Lymphocytes from spleen, mesenteric lymph node (*MLN*) and Peyer's patches (*PP*) at 21 days post-infection were stimulated with concanavalin A (*ConA*) or parasite 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

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

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≥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

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

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

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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.

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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 1990a) and golden hamsters (*Mesocricetus auratus*) (Kamiya and Sato 1990b). 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*.

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 (1990a) 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 μg 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 μM 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 μg/ml), glucose (4.5 μg/ml), and KCl (0.2 μg/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 μm 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 µl/well of PSO (2 µg/ml) or PES (0.5 µg/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 µl/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 µl/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 µl 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 µl/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 µl/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 µg/ml streptomycin and 50 µM 2-mercaptoethanol (Sigma). Cell viability was assessed using 0.5% trypan blue (Wako Junyaku, Japan) in PBS. Then, cells (2×10^5 cells/100 µl/well) were cultured in 96-well culture plates (Corning, USA) with either PSO (50 µg/ml final concentration) or PES (5 µg/ml). Concanavalin A (ConA, type IV, Sigma) (5 µg/ml) and lipopolysaccharide (LPS, from *Escherichia coli* 0127: B8, Sigma) (25 µg/ml) stimulation were employed as positive controls for determining cell viability. Lymphocytes were also co-stimulated with PSO (50 µg/ml) and either ConA (5 µg/ml) or LPS (25 µg/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 µl 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 µl 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.

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.

Antibody and lymphocyte responses against *E. multilocularis*

Parasite-specific serum IgG levels at 14–15 days post-infection or final immunization were significantly ele-

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)

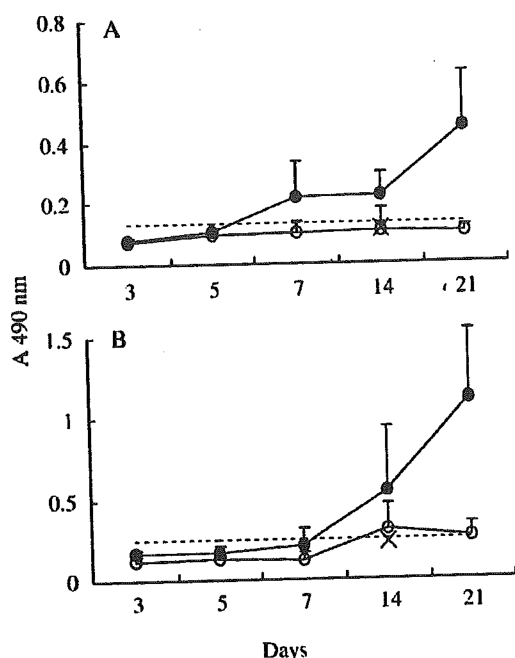

Fig. 1 Time course of intestinal IgA levels to *Echinococcus multilocularis* in Mongolian gerbils. Untreated (*filled circles*) or prednisolone acetate (PA)-treated (*empty circles*) gerbils were orally inoculated with 20,000 live protoscoleces. Gerbils were killed 3, 5, 7, 14, and 21 days post-infection 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

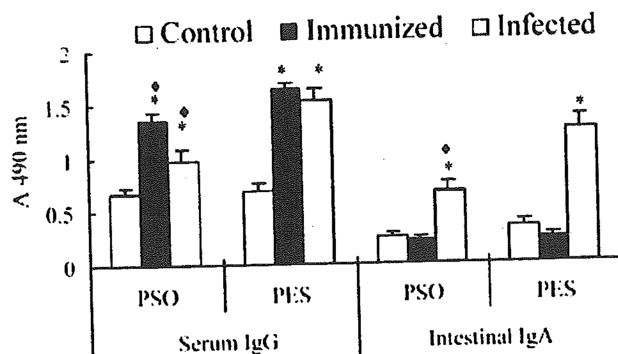

Fig. 2 Specific serum IgG and intestinal IgA responses to *E. multilocularis* antigens in Mongolian gerbils. Gerbils were orally infected with live protoscoleces (20,000) once (infected group) or subcutaneously immunized with protoscolex somatic antigen (150 μg with CFA) three times (immunized group). 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*: $P < 0.05$)

vated 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).

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.

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 (although it was 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.

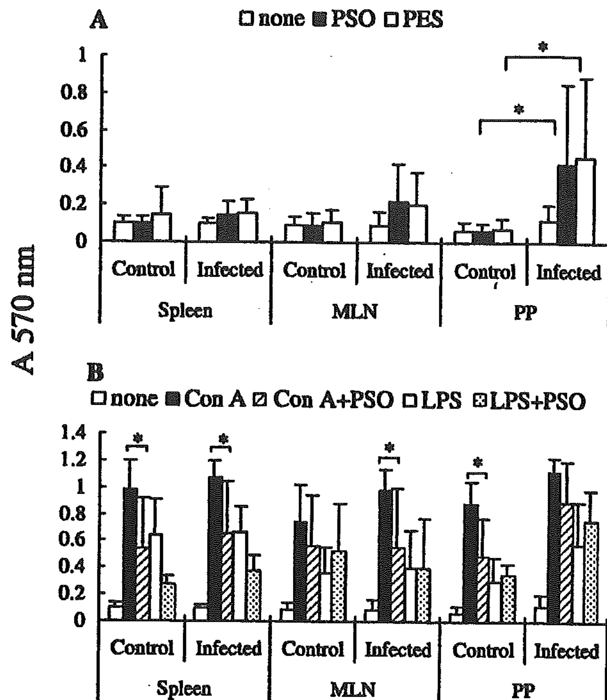

Fig. 3A, B Parasite specific blastogenesis and the effect of *E. multilocularis* antigen on mitogen-induced lymphocyte responses in Mongolian gerbils. At 14–15 days after infection, lymphocytes harvested from spleen, mesenteric lymph nodes (*MLN*) and Peyer's patches (*PP*) were stimulated with mitogens (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 + PSO-stimulated cells (**B**) (*asterisk* $P < 0.05$). Difference was obtained between non-stimulated (none) and PES-stimulated cells in infected group (*empty diamond*; $P < 0.05$)

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; 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 antibody.

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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