Local and systemic immune responses to *Echinococcus granulosus* in experimentally infected dogs


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**Abstract**

Local and systemic immune responses were studied in six dogs experimentally infected with the dog/sheep tapeworm *Echinococcus granulosus*. All dogs developed similar IgG antibody response to parasite antigens. In contrast, IgE and IgA responses differed widely. No relationship between IgA responses and parasite burden at the end of the infection were observed. Further, clear differences in the anti-parasite IgA response in serum as compared with specific IgA forming cells in mesenteric lymph nodes were observed within the same dog. An inverse association of anti-parasite IgE and parasite load seemed to be present, with the strongest IgE response in the one dog that had no worms in the intestine at the end of the experiment. No differences were observed in the numbers of intestinal mast cells and goblet cells among all infected dogs. However, the dog with no detectable parasite load had a marked reduction of detected mast cells in the submuscular and muscular layer of the mucosa. Our data give new insight into the immune response of dogs during *E. granulosus* infection and provide information that may be useful for the rational design of vaccines for the control of hydatid disease.

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1. Introduction

Cystic echinococcosis, caused by *Echinococcus granulosus*, is an important zoonosis affecting agriculturally-based countries. Natural intermediate hosts, particularly cattle and sheep, become infected after ingestion of eggs released in the faeces of infected dogs. Humans are accidental hosts of this parasite, usually becoming infected through contact with infected dogs (Thompson, 1995). *E. granulosus* has worldwide geographical distribution, and in areas where the infection is endemic it constitutes a major public health problem and is a cause of important economical losses (Eckert et al., 2000; Torgerson et al., 2000). New areas of infection are being reported, giving to Echinococcosis the status of an emerging zoonosis (Eckert et al., 2000).

Although considerable effort has been directed towards the understanding of some critical aspects of the immunobiology of the parasite in the intermediate hosts, including humans (Heath, 1995), very little is known about the immunology of the infection in dogs, as hosts for the adult parasite. A vaccine candidate for the intermediate host has been tested in sheep, showing a high degree of protection (Lightowlers et al., 1996, 1999, 2000; Woollard et al., 1998). However, due to the particular characteristics of the cycle of the parasite, an effective vaccine for dogs could be very useful, not least by reducing the production of infectious eggs which are the source of infection in humans (Wakelin, 1997). Vaccination of dogs would be also the most cost-effective control measure, since hydatidosis is mainly endemic in cattle-rearing countries, which would make vaccination of livestock very costly, particularly for farmers in developing countries. Further, although local control programs based on the treatment of dogs with anti-helminth drugs may reduce the infection, they are very expensive to maintain, and recent surveys have shown that if those measures are not maintained for long periods (at least 30 years) dogs are soon reinfected, suggesting that additional measures are required for permanent control (Economides et al., 1998; Eckert et al., 2000).

Attempts at vaccinating dogs against *E. granulosus* with various parasite-derived immunogens have given conflicting results, and there are doubts as to whether dogs can develop protective immunity (Gemmell, 1962; Movsesijan et al., 1968; Heath, 1995; Lightowlers et al., 2000). This lack of progress in the development of effective vaccines is most likely due to: (a) an almost total lack of knowledge of the immunology of *E. granulosus* infection in dogs; (b) a poor understanding of the immune mechanisms involved in the expulsion of intestinal parasites particularly in dogs, since almost everything known so far in the field comes from studies in rodents infected with intestinal nematodes or with the cestode *Hymenolepis diminuta*; and (c) the particular difficulties of working with dogs, including the lack of data and tools for immunological studies, which is unfortunately necessary as there are no experimental models that could reproduce the development of the larval stage to adult worm in the intestine of the definitive host.

In this context, there are scarce reports on the immune responses in dogs infected with *E. granulosus*, and those have mainly concentrated in the study of the systemic antibody response, searching for improved diagnostic methods (Jenkins and Rickard, 1986; Gasser et al., 1989, 1992; Spinelli et al., 1996). A few works have also shown that parasite-specific humoral and cellular responses can be elicited in dogs upon infection (Al-Khalidi and Barriga, 1986; Barriga and Al-Khalidi, 1986; Deplazes et al., 1994).
In this study, we experimentally infected six dogs with *E. granulosus* to measure the local and systemic humoral and cellular immune responses elicited in them, and discussed the results in terms of the observed differences in parasite burden at the end of the infection.

2. Materials and methods

2.1. Dogs

Worm-free dogs were purchased from local breeders in Uruguay at three months of age and kept in our facilities for another three months before being used. They were fed with commercial food and water ad libitum. For the experiment, littermates of unspecified lineage weighing approximately 7 kg were used.

Coprological examination using sugar centrifugal-flotation technique (Ito et al., 1989) to screen for intestinal parasites was routinely conducted over the 3 months period before the start of the experiment. At days 60 and 30 before infection all dogs were treated with a mixture of praziquantel and abendazole (Basken Plus, König, Germany) and examined afterwards to confirm that they were free from parasites before the experiment.

2.2. Experimental infections

*E. granulosus* protoscoleces (PE) were aseptically obtained from a single cyst from an infected sheep. PE were washed in PBS, their viability assessed by 5% eosin exclusion and flame cell activity (Smyth and Zena, 1974), and counted under a low magnification binocular microscope. To perform the experimental infection, aliquots containing 20,000 viable PE in 1 ml of PBS were included in a raw minced meat pellet and given to each dog after a day of fasting.

2.3. Sample collection

Before the end of the prepatent period (4 weeks after experimental infection) all dogs were euthanased with an overdose of pentobarbitone sodium, the peritoneal cavity was rapidly opened and the entire small intestine removed. The intestine was divided into three segments of equal longitude (anterior, middle and posterior) (Lymbery et al., 1989), and from each segment, sections of 1 cm were cut for histological analysis. To assess parasite burden, the rest of the segments were opened longitudinally along the line of mesenteric attachment and incubated into saline solution at 37°C for 1 h. After that the mucosa was scraped into saline, fixed with 4% formalin and heated at 70°C for 12 h. The number of worms were counted under a low magnification binocular microscope.

In addition, Peyer’s patches (PP), mesenteric lymph nodes (MLN) and spleen were removed for analysis of local and systemic immune responses.

2.4. ELISA determination of antibody responses

A crude antigen preparation from the whole parasite was obtained by sonication of PE in PBS, followed by sterilizing filtration and quantitation of protein content using the BCA
method (Sigma, St. Louis, USA) as previously described (Carol et al., 1997). The preparation (SPE) was aliquoted and stored at $-20^\circ$C until use.

Antibody responses were assessed by ELISA in sera obtained from the dogs at different time points, as previously described (Chabalgoity et al., 2000). Briefly, 96-well microtitre plates (Nalge Nunc International, Denmark) were coated overnight at room temperature with 0.1 $\mu$g SPE per well. Individual sera were diluted in PBS–1% BSA, added to the wells and incubated for 90 min at $37^\circ$C. Peroxidase conjugated secondary antibodies specific to dog immunoglobulins IgG, IgA or IgE (Bethyl Laboratories, Montgomery, USA) were added at appropriate dilution and the plates were incubated for 1 h at $37^\circ$C. Plates were developed with orthophenylene diamine (OPD, Sigma, St. Louis, USA) prepared as per the manufacturer’s instructions for 15 min at $37^\circ$C. The reaction was stopped with 2 M $\text{H}_2\text{SO}_4$ before readings were taken at 490 nm.

2.5. **ELISPOT**

Antibody secreting cells (ASC) were enumerated in mesenteric lymph nodes (MLN). ELISPOT plates (MAHA, Millipore, France) were sensitized by overnight incubation with 5 $\mu$g SPE per well diluted in PBS with gentamicin sulphate (40 $\mu$g/ml) and blocked with BSA 5 mg/ml at $37^\circ$C for 1 h. Cells (2 $\times$ $10^5$ per well and 4 $\times$ $10^4$ per well) were then dispensed in duplicate in the sensitized wells. Untreated wells served as controls. Cells were cultured in WMB 752/1 medium (Sigma, St. Louis, USA), 10% foetal calf serum, 25 mM glutamine, 40 mg/ml gentamicin sulphate for 18 h at $37^\circ$C in a 5% CO$_2$ atmosphere, and then washed with PBS 0.1% Tween 20 (Sigma, St. Louis, USA). Incubations with unlabelled goat anti-dog IgA (Nordic, The Netherlands) antibody, was followed by alkaline phosphatase conjugated rabbit anti goat IgG. The development of spots was performed with nitro blue tetrazolium/bromo-chloro-indolyl phosphate. The reaction was stopped by washing the plates thoroughly in running tap water. Spots were counted under a low magnification binocular microscope and arithmetic means were calculated. Spots recorded in the control wells were subtracted from each corresponding mean value. The final result is expressed as specific ASC/10$^6$ cells.

2.6. **Western blotting assays**

Antibody responses were also assessed by Western blotting, performed as previously described (Chabalgoity et al., 1996). Samples of SPE were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and probed with sera obtained from each dog either immediately before the experimental infection or 28 days after it. Peroxidase conjugated secondary antibodies specific to dog immunoglobulins IgA or IgE (Bethyl Laboratories, USA) were added at appropriate dilutions, and developed using LumiGLO chemiluminescent substrate as per the manufacturer instructions (New England Biolabs, USA).

2.7. **Proliferation assays**

Proliferative responses were assessed in cultures of peripheral blood mononuclear cells (PBMC), MLN, or Peyer’s Patches (PPs) as previously described (Chabalgoity et al., 2000).
For preparation of PBL, blood samples were collected in heparin tubes at the time of euthanasia. After centrifugation the buffy coat was collected, diluted in medium and overlaid on Histopaque 1077 (Sigma). Samples were centrifuged and the interface containing the PBMC was harvested, and diluted in RPMI 1640 (Sigma, HEPES modification). Cells were washed twice before being resuspended in complete RPMI (CRPMI: 1640 with 10% heat inactivated new born calf serum (NBCS), 0.1% of 0.05 M 2-mercaptoethanol, 2% 0.2 M glutamine, 10 mg/ml streptomycin, 100 U/ml penicillin) and counted. Cell cultures were set up in round bottomed 96-well plates (Corning, NY) at 5 × 10^5 cells/culture in 200 μL and SPE antigen or Concanavalin A were added in triplicate in volumes ranging between 5 and 20 μL. Cultures were incubated at 37 °C in 95% humidity, 5% CO₂. After 3 or 6 days in culture 1 μCi of [³H]thymidine was added to each well and the plates were incubated for a further 18 h before being harvested on filters and incorporation of radioactive label determined. Values are expressed as the mean of the stimulation index obtained for each of the triplicates (value of a specific antigen divided by mean value of the three negative control) ± standard error of the mean (S.E.M.).

2.8. Histology

For histopathological evaluation sections of 1 cm in length from the three different segments of the intestine were obtained from each dog and fixed in either 4% buffered formalin or in Carnoy’s fluid. After embedding in paraffin wax, serial sections of 4 μm were cut from each tissue block. Formalin fixed sections were stained with periodic acid Schiff (PAS) for the demonstration of goblet cells. Sections of tissue fixed in Carnoy’s solution were stained with toluidine blue to demonstrate the sulphated acid glycosaminoglycans in the mast cells (MC) granules (Kube et al., 1998). Villous goblet cells were counted at the tip and base of the villi using a 40× ocular grid, and are expressed as cells per five grid areas (Lloyd et al., 1991). Mast cells in the muscular layer were counted using the same methodology. The quantification of positively stained mucosal MC was performed by using a square eyepiece graticule (objective × 25, eyepiece × 10, 10 × 10 squares with a total area of 0.0169 mm²). Mucosa and submucosa were evaluated separately. The total area of each section was evaluated. Results are expressed as cells/mm².

3. Results

3.1. E. granulosus challenge and parasite recovery

The number of parasite recovery 28 days after experimental infection with 20,000 protoscoleces varied between the different dogs. Whereas in one dog no parasite could be recovered, the rest of the animals harboured 1.0–2.5% of the infective dose (range of recovery 195–484 worms) found almost exclusively in the anterior and middle sections of the intestine (Table 1).
Table 1
Parasite load at the time of sacrifice of dogs experimentally infected with 20,000 protoescoleces

<table>
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<tr>
<th>Dogs</th>
<th>Anterior intestine</th>
<th>Middle intestine</th>
<th>Posterior intestine</th>
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<th>Percentage of recovery</th>
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<td>126</td>
<td>0</td>
<td>484</td>
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<td>0</td>
<td>208</td>
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</table>

3.2. Antibody response

Dogs were bled immediately before the experimental infection and at days 14 and 28 after infection. Anti-parasite antibody responses were evaluated by ELISA, Western blot and ELISPOT, and screened against a parasite antigenic preparation (SPE).

3.2.1. ELISA and Western blot analysis

ELISA results from sera tested for anti-SPE antibody responses, showed that all infected dogs developed a similar IgG response after infection (Fig. 1). In contrast, the anti-parasite IgA and IgE responses varied between individual dogs. Only two dogs (dog numbers 3 and 6) developed a strong serum IgA response at both time points after infection as detected by ELISA (Fig. 2). However, western blot analysis using serum prior to infection and serum obtained 28 days after infection, showed specific recognition of several bands in the range of 97–37 kDa only in dog number 3 (result not shown). Sera from the other dogs weakly
recognized some bands, but without differences between sera obtained before and after challenge. Anti-parasite IgE responses showed a different pattern. Dog number 2, which had no detectable worms at necropsy, showed the strongest anti-SPE IgE response (Fig. 3). Furthermore, an inverse association between the IgE response and the number of worms recovered was observed (Fig. 4). Western blot analysis for IgE antibodies gave unclear results, most likely due to low sensitivity of the assay.

Fig. 2. Anti-SPE IgA antibody response as detected by ELISA on sera taken immediately before the experimental infection and at days 14 and 28 after infection. Results are expressed as OD values from individual dogs experimentally infected with 20,000 protoescoleces.

Fig. 3. Anti-SPE IgE antibody response as detected by ELISA on sera taken immediately before the experimental infection and at days 14 and 28 after infection. Results are expressed as OD values from individual dogs experimentally infected with 20,000 protoescoleces.
3.2.2. In vitro antibody production
ELISPOT analysis of antibody secreting cells in MLN, showed a different pattern of IgA antibodies as compared with IgA responses in sera. In MLN there were significantly more IgA+ ASC cells in dog number 2 (Fig. 5).

3.3. Cellular responses

Systemic and local proliferative responses to parasite antigen stimulation, were evaluated in vitro either in cultures of PBL, or in cultures of MLN and PPs. Cultures of PPs cells from
Fig. 6. Proliferative responses of Peyer’s patches upon in vitro stimulation with SPE. Results are expressed as stimulation index (SI) of cell cultures from individual dogs stimulated with 0.1 mg per well (solid bars) or 1 mg per well (open bars) of SPE. Each value represents the mean for three different cultures ± S.E.M.

all dogs responded to Con A stimulation, albeit with clear differences in the magnitude of the response between individual dogs (result not shown). In contrast, only the culture of one dog proliferated after stimulation with SPE, and only at one of the two different concentrations of SPE used (Fig. 6). Similar results were obtained in PBL and MLN (result not shown).

3.4. Analysis of cell populations in the gut

Gut sections were stained for enumeration of mast cells and goblet cells. Overall, the number of goblet and mast cells were similar to those found in uninfected dogs (our own unpublished results). No differences in the total number of goblet cells (Fig. 7A), or mucosal MC (Fig. 7B) were found between the different dogs. However, the dog with no detectable worms had fewer mast cells in the muscular layer (Fig. 7C).

4. Discussion

We experimentally infected six dogs with a low challenge dose of *E. granulosus*, and 4 weeks after the challenge we found variations of parasite load in the dog’s intestine ranging from no parasites in one dog to 2.5% of recovery in two others (Table 1). The variability in parasite burden between dogs is a common finding during experimental infections with *E. granulosus* (Turner et al., 1936; Gemmell et al., 1986; Barriga and Al-Khalidi, 1991). Further, it has been postulated a bimodal distribution in the number of worms recovered from dogs challenged with different infective doses, with a few dogs been heavily infected and others developing very few or no worms at all (Turner et al., 1936; Barriga and Al-Khalidi, 1991). In a dose-related experiment it was shown that in dogs infected with less than 17,500 protoscoleces there were always some dogs that developed no worms, but in dogs infected
Fig. 7. Histological analysis of gut sections showing number of goblet cells (A) and mast cells (B and C) from individual dogs. Results are expressed as number of goblet cells in the crypt (GCC) and the villi (GCV) per five grid areas (A); number of mast cells/mm² in mucosa and submucosa, after counting the total area of each section (B); and number of mast cells in the muscularis mucosae (MMMC), submuscular layer (SMMC) and the muscular layer (MMC) per five grid areas (C).
with higher doses (175,000 protoscoleces), all dogs had at least some worms (Gemmell et al., 1986). Our results using a challenge dose of 20,000 PE are consistent with those findings. We have also found a clear site specificity in the localization of worms, with the majority being found in the anterior third of the intestine. A similar uneven distribution of *E. granulosus* in the intestine of the dogs has been previously reported (Lymbery et al., 1989).

However, to the best of our knowledge, there are no previous works assessing in a comparative fashion the immune responses among dogs with such differences in parasite load. Here, we analysed local and systemic immune responses among the dogs, looking for immunological parameters that could explain those differences.

All dogs developed similar IgG antibody responses to protoscolex somatic antigens (Fig. 1) suggesting that all animals have been similarly infected, making infectivity variations in the challenge a less likely explanation for the observed differences in worm numbers. Comparison of the local vs. systemic IgA responses was particularly intriguing: whereas the strongest systemic response was observed in the dog carrying the largest number of worms (Fig. 2), the larger number of parasite-specific IgA+ ASC was found in MLN from the dog that had rejected the challenge (Fig. 5). Given the localization of the worm, it may be that a preferential induction of local over systemic responses is present, which would make the local concentration of IgA producing plasma cells higher. Also, although some authors have suggested that neutralizing-secretory IgA specific for *E. granulosus* would have no effect on the scolex (Heath, 1995), others speculated that antibodies to the rostellar surface of the worm may inhibit development by interfering with uptake of essential nutrients (Smyth, 1972). Further, some authors reported from results of one experiment that higher titres of total IgA and IgG in serum and faeces were associated with less mature parasites (Barriga and Al-Khalidi, 1986). A role for IgA in protective responses has been proposed for other helminths with an intestinal phase. In rats that rapidly expel an infection with the intestinal nematode *Heligmosomoides polygyrus*, a negative correlation between IgA and worm burden has been found (Ben-Smith et al., 1999).

The anti-SPE IgE responses showed the most clear differences between individual dogs. The worm-free dog had a much stronger parasite-specific IgE response as compared to the others (Fig. 3). Furthermore, an inverse association of anti-SPE IgE and parasite load seem to be present. Although the small number of animals used precluded the use of statistical analysis, a clear trend could be seen, the dogs with the lower load showing the strongest IgE responses (Fig. 4). IgE responses have been involved in protection in many tissue-dwelling parasites (Behnke, 1990), and also in some intestinal nematodes (Nawa et al., 1994; Else and Finkelman, 1997; Finkelman, 1997; Maizels and Holland, 1998), and particularly in *H. diminuta* which, similarly to *E. granulosus* in dogs, is another “exclusively” intestinal cestode (Ishih and Uchikawa, 2000). Also, the protective response against intestinal parasites usually involves intestinal mast cells together with the IgE response (Maizels and Holland, 1998). However, in *H. diminuta* infection, it has been suggested that whereas a reduction in worm biomass was related with an increase in serum IgE and in mucosal mast cell numbers, those mechanisms are not solely responsible for worm expulsion in rats, suggesting that other cells like goblet cells may also play an important role on cestode expulsion (Ishih and Uchikawa, 2000).

We found no differences in the amount of goblet cells or mucosal mast cells between dogs (Fig. 7). However, it is noteworthy that cells were counted in tissue samples obtained
at day 28 after infection, and therefore we cannot rule out that differences in those cell populations could be apparent earlier in the infection, having a direct relevance on anti-parasite immunity. Interestingly enough, the worm-free dog had lower numbers of mast cells at the muscular layer of the intestine (Fig. 7C). Since the method used for mast cell detection relies on staining of intracellular granules, that result could suggest that mast cells are degranulated in that dog. Degranulation of mast cells in the muscular layer would provoke muscular contraction altering the anatomy of the intestine, and thus may result in an unsuitable environment for installation of the parasite.

Proliferative responses could not be detected in vitro against SPE, although a wide range of experimental conditions and antigen concentration were used. One explanation could be that other antigens, i.e. antigens from the membrane of the cyst or developmentally regulated antigens associated with *E. granulosus* adult tissues are involved in proliferative responses. Other authors have shown in a previous report that proliferative responses in dogs experimentally infected with *E. granulosus* could only been observed when dogs were infected with a mixture of PE plus cyst membranes and hydatid fluid (Deplazes et al., 1994). In preliminary studies, we have detected the production of higher concentrations of IL-2 in cells from MLN stimulated with adult-worm secretory antigens as compared with cells stimulated with SPE (our unpublished results), suggesting that proliferative responses may still be obtained in dogs infected solely with PE when other worm-derived antigens are used for the assay.

Overall, our results could be taken to suggest that increased parasite-specific IgE and local IgA may be related with protection of dogs against a challenge with the parasite. Nevertheless it is noteworthy that complete rejection was observed in only one dog, and therefore we cannot exclude that, in that particular dog, expulsion of the challenge dose arose as the result of several factors including others than the specific immune-mediated mechanism. However, the results presented here also suggest that immune effector mechanisms may be operative against the parasite, thus reinforcing the need for further immunological studies in dogs.

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**References**


