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Echinococcus granulosus: Different cytokine profiles are induced by single versus multiple experimental infections in dogs

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ABSTRACT

Modulation of host responses is an important strategy by which parasites ensure successful establishment and persistence. Host counteraction against this modulation may be required for the host to develop resistance to infection. In this pilot study, experimental infection of dogs with *Echinococcus granulosus* induced a strong polarization of the cytokine response towards a Th2 phenotype. Consecutive rounds of infection and cure induced resistance to infection resulting in a dramatically lower parasite burden. Repeatedly-infected resistant dogs also lost immune polarization and developed a balanced Th1/Th2 response. No major differences were observed in the production of regulatory cytokines (IL-10, TGF-β) between dogs with high parasite load and dogs with only few intestinal parasites. These results suggest that *E. granulosus*-driven immunomodulation contributes to successful infection in the definitive host. This information might be relevant for the development of more effective vaccines against this stage of the parasite.

1. Introduction

Echinococcus granulosus, the causative agent of cystic echinococcosis, is still endemic in many countries on all continents and continues to be an important cause of morbidity and mortality. Echinococcosis is an emerging zoonotic disease (new areas of infection are frequently reported), and also a re-emerging problem in some regions. (Eckert and Deplazes, 2004; Moro and Schantz, 2006; Schantz, 2006). The life cycle of *E. granulosus* involves two mammalian hosts. The definitive hosts are primarily dogs which harbor adult worms in their small intestines. Natural intermediate hosts, particularly sheep and cattle, become infected after ingestion of eggs released in the faeces of infected dogs. Humans are accidental intermediate hosts of this parasite (Thompson, 1995).

E. granulosus presents different immunological relationships with its hosts. Great effort has been invested to understand the immunobiology of the parasite in the intermediate host (Baz et al., 2006; Siracusano et al., 2008; Zhang et al., 2003). However, there is scarce information about the immunologic response in the definitive host, likely because of the difficulties of working with dogs, the scarcity of tools to evaluate immune responses in them, and the lack of an appropriate alternative experimental model for the establishment of infections with the tapeworm stage.

The immune system of dogs responds to a primary infection with *E. granulosus*, but the responses appear weak and ineffective suggesting immunomodulation by the parasite. The scoleces of worms successfully installed in dogs' intestines adhere strongly to crypts and establish intimate contact with the gut mucosae (Howell and Smyth, 1995; Thompson, 1995). Dogs develop serum IgG and local IgA responses to infection and show parasite-specific proliferation of Peyer's patch cells. There is also preliminary evidence for a role of IgE in the protection against infection (Deplazes et al., 1994; Jenkins and Rickard, 1986; Moreno et al., 2004). Moreover, peripheral lymphocytes from dogs experimentally infected with *E. granulosus* display an enhanced proliferative response to the mitogen Concanavalin A (ConA), and dogs with the highest responses had significantly fewer parasites than less reactive dogs (Al-Khalidi and Barriga, 1986). Notwithstanding, the dynamics of cellular recruitment and the profile of the inflammatory response in the gut of dogs upon infection have not yet been characterized.

Here we report that *E. granulosus* primary infection modifies the cytokine profile of dog cells in response to a mitogenic (ConA)

Abbreviations: mRNA, messenger RNA; Dpi, day post-infection; cDNA, complementary DNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ct, threshold cycle; SC, stimulated cells; NSC, non-stimulated cells; Bp, base pair.

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stimulation. Dogs experimentally infected with *E. granulosus* showed a strong polarization towards a Th2 phenotype as analyzed by mRNA (messenger RNA) levels of several cytokines in cells isolated from Peyer's patches and spleen. We also show that the cytokine responses in dogs that undergo successive rounds of infection and cure, resulting in dramatically decreased tapeworm establishment, are similar to the cytokine profile of non-infected dogs.

2. Materials and methods

2.1. Dogs

Eleven dogs of mixed breeds, aged between 3 and 6 months, were purchased from the Animal Control Center in Lima, Peru The dogs were maintained under helminth-free conditions at the facilities of the School of Veterinary Medicine of the Universidad Nacional Mayor de San Marcos, also in Lima, where they were fed with commercial dog food and water *ad libitum* for at least 3 months before the beginning of experiments. Coprological examinations to screen for intestinal parasites were routinely conducted during the quarantine period. Thirty days before infection all dogs were treated with Drontal Plus (Bayer) at standard doses (praziquantel 5 mg/k, pyrantel 5 mg/k and febantel 50 mg/k), and had a further coproparasitological examination to confirm that they were free from parasites before the experiment (Eckert et al., 2001). The protocol was approved by the Animal Ethic Committee of the School of Veterinary Medicine of the Universidad Nacional Mayor de San Marcos.

2.2. Experimental infections

E. granulosus protoscoleces were aseptically obtained from ovine cysts. For experimental infection purposes, aliquots of a single pool containing 80,000 viable protoscoleces in 1 ml of PBS were given orally to each dog after a day of fasting as previously described (Casaravilla et al., 2005; Moreno et al., 2004; Petavy et al., 2008). Briefly, protoscoleces were allowed to decant, washed with PBS, and counted under light microscope after confirming their viability by eosin exclusion and flame cell activity (Smyth and Davies, 1974). Protoscolex suspensions were then diluted to the desired concentration and given to the dogs immediately after. Dogs were restrained and the veterinarian on charge verified that the animal swallowed the entire dose.

Nine dogs were used for experimental infections, divided in three groups of three dogs each. Group 1 received a single experimental infection. Groups 2 and 3 received three and six rounds of infection and cure, respectively. All infections were interrupted by treating the dogs with arecoline (4 mg/k, 1 or 2 doses) and praziquantel 5 mg/kg afterwards, before the end of the pre-patent period, by day 35 post infection. The results of each round of experimental infections were assessed by evaluating worms after arecoline purgation. Fifteen days later, dogs were re-infected with identical doses of protoscoleces. The control group consisted of two uninfected dogs that received only PBS.

2.3. Sample collection and worm burden

Following the last infection but before the end of the prepatent period (35 dpi after infection) all dogs in each group were euthanized with an intravenous overdose of sodium pentobarbital. The peritoneal cavity was rapidly opened and the Peyer's patches of the small intestine and spleen were removed for *in vitro* analysis. To assess parasite burden the rest of the small intestine was opened longitudinally and incubated in saline at 37 °C until no more worms were liberated. Most worms were released naturally and the remainders were carefully scraped from intestine. The worms were counted under a magnifying glass.

2.4. Cell cultures

Whole cell suspensions from Peyer's patches and spleens were obtained by mechanical disruption of the organ (followed by red cell lysis in the case of spleen), and used directly for *in vitro* stimulation assays as previously reported (Moreno et al., 2004). Briefly, cells were suspended in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Gibco), 2.5 mM L-glutamine (Sigma), 0.05 mM 2-mercaptoethanol, 5000 units/ml penicillin, 5 mg/ml streptomycin and 10 mg/ml neomycin. The number of cells was adjusted to 5×10^6 cells per ml. Cell suspension was stimulated with 5 μg/ml of ConA (Sigma). Stimulated and non-stimulated cultures were incubated in duplicate for 16 h at 37 °C in 5% CO_2-humidified atmosphere Finally cells were lysed in TRIzol (Invitrogen), shipped to Uruguay and stored at −80 °C until RNA isolation was done.

2.5. Total RNA extraction and reverse transcription

RNA was extracted from cells in TRIzol according to the manufacturer's protocol. Total RNA was dissolved in nuclease free water and quantified by UV-spectrophotometry. Genomic DNA was removed from RNA before reverse transcription by incubation of 1 μg of total RNA with 0.35 IU DNase I (Invitrogen). First strand complementary DNA (cDNA) synthesis was carried out immediately by adding 0.5 μg oligo dT primer (Invitrogen) and 200 U M-MLV reverse transcriptase (Invitrogen) in a final volume of 20 μl. cDNA generated was diluted to 200 μl in nuclease free-water and stored at −20 °C until use.

2.6. Quantification of relative cytokine mRNA levels

Quantification of canine IL-4, IL-13, IFN-γ, IL-10, and TGF-β mRNA was conducted by real time PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene. Primers were purchased from Operon (Cologne, Germany) and are all described in Table 1. Real-time PCRs were performed using QuantiTect SYBR Green PCR Kit (QIAGEN), and 2.5 μl of diluted cDNA in the presence of 0.5 μM of each specific primer. Quantitative PCR was carried out using the Rotor-Gene 6000 (Corbett Life Science). Samples were initially denatured for 15 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min during which the fluorescence data were collected (Manna et al., 2006).

We first compared the average GAPDH expression in stimulated and non-stimulated spleen and Peyer's patch cells and found that the groups were not statistically different ($P = 0.0862$). We therefore concluded that the GAPDH gene could be used as a single normalizing gene.

The relative mRNA amount in each sample was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) were $\Delta Ct = Ct_{Cytokine} - Ct_{GAPDH}$, and expressed as the ratio between relative amount to GAPDH of stimulated (SC) and relative amount to GAPDH of non-stimulated (NSC) cells.

2.7. Statistical analysis

One-tailed Mann–Whitney test was used for non-parametric comparisons with a significance level set at $P < 0.05$. All calculations were performed with GraphPad Prism (GraphPad Software, San Diego, California, USA).