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.

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].
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. multilocularis 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 post-infection with an overdose of sodium pentobarbital on 25 days (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 University 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% CO2 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 NaHCO₃/Na₂CO₃ 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+λ+M (Sigma), diluted at 1/2000 in 0.5% BSA (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. They were then loaded with 50 µl of HRP-conjugated anti-mouse IgM (1:4000) were added for another 1 h. Finally, 100 µl of o-phenylenediamine (0.04%) and H₂O₂ (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 H₂SO₄, and plates were read at 492 nm. The cut off values for both MAbs were determined by 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) H₂O₂. The reaction was stopped after 5 min with distilled water.
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 days prepatent infection).
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...
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.

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