Toxocara canis 30-35 kDa excretory-secretory antigen is an important marker in mice challenged by inocula containing different parasite load levels

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ABSTRACT

The Western-blotting technique was applied to identify antigenic fractions of excretory-secretory *Toxocara canis* antigen recognized by IgG antibodies throughout an experimental infection in mice challenged by different inocula. Mice were inoculated with 5, 50 and 500 embryonated eggs and serum samples were collected 15, 30, 60, 90 and 120 days post-infection. Serum samples were analyzed using an excretory-secretory *Toxocara* antigen. Antibodies recognized antigenic fractions from 30 to 90 kDa. The protein fraction of 30-35 kDa was the most frequently recognized regardless of the size of inoculum and the stage of infection represented by the different collection times, but the antigenic recognition was more evident in groups infected with 50 and 500 eggs. This study presents an antigenic panel of the excretory-secretory antigen of *T. canis* and suggests that the 30-35 kDa antigenic fraction is a promising marker of the infection and should be further explored in future studies on experimental toxocariasis.

KEYWORDS: BALB/c mice. *Toxocara canis*. Toxocariasis. Western blotting. Immunodiagnosis. Antigenic fractions.

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INTRODUCTION

Toxocariasis, a zoonosis caused by *Toxocara canis* and *Toxocara cati* nematode larvae, causes a variety of syndromes, including visceral larva migrans, ocular larva migrans, common or covert toxocariasis and neurotoxocariasis[1-4]. Rats and mice are paratenic hosts, widely used in research due to the similarities of their infections with those of humans with respect to the host-parasite relationship, larvae migration patterns, in addition to being easily maintained in the laboratory[5-8].

In the absence of direct evidence of the parasite infection in paratenic hosts, indirect detection, especially of antibodies using immunological methods has a relevant role in the diagnosis of toxocariasis[9]. The literature has highlighted the use of Western-blotting (WB) techniques for the investigation of many immunological aspects[1,2,8-12]. The recognition of antigenic fractions of the parasite by IgG antibodies throughout experimental infections induced by different inocula has not yet been evaluated. This study aimed to describe an antigen recognition panel by IgG anti-*Toxocara* antibodies in mice infected with different *T. canis* inocula throughout the infection.

MATERIALS AND METHODS

Twenty-five BALB/c specific pathogen-free male mice, 6-8 weeks old,

acquired from Centro de Bioterismo of the Faculdade de Medicina of the Universidade de Sao Paulo, were divided into three groups (I, II and III). Six animals in each group were infected via intragastric intubation with 5, 50 or 500 embryonated *T. canis* eggs (L3), suspended in 200 µL of saline solution, respectively. Seven non-infected mice received 200 µL of saline solution (negative controls). All the animals received water and diet ad libitum and were treated in accordance with the ethical guidelines of the Animal Ethics Committee of the Instituto de Medicina Tropical de Sao Paulo (protocol CPE-IMT/254) and Ethics Committee on Animal Studies of the Faculdade de Medicina of the Universidade de Sao Paulo (protocol 162/15). Animals in all groups were anesthetized with xylazine and ketamine at concentrations of 10 mg/kg and 100 mg/kg, respectively, and serum samples were obtained 15, 30, 60, 90 and 120 days post-infection (dpi) by the retro orbital plexus, divided into aliquots and stored at -20 °C. Animals were euthanized by intravenous administration of a thiopental overdose of 100 mg/kg. Confirmation on the loss of consciousness and death were made by cervical dislocation at the end of the experiment.

T. canis excretory-secretory antigen (TES-Ag) was prepared following the procedure described by Savigny[13] with modifications made by Elefant *et al.*[14]. Briefly, *T. canis* eggs were collected from the uterus of adult females, embryonated in 2% formalin at room temperature and observed weekly for embryonation. When embryonated, eggs were collected in a conic tube and centrifuged at 170 X g for 5 min with saline solution until the formalin was completely removed. Then, the precipitate was re-suspended in 5% sodium hypochlorite for 5 min for the removal of the proteic outer layer. Next, the eggs were centrifuged with saline solution as described before. Then, they were transferred into an Erlenmeyer containing glass pearls and RPMI medium (RPMI 1649 Sigma Aldrich, a subsidiary of Merck Inc., Burlington, MA, USA) with 80 µg/mL of gentamicin. This content was gently shaken for up to 30 min to release larvae, and then transferred into a modified Baermann apparatus for 2 h. The medium was transferred into 5 mL tubes and incubated at 37 ºC, and was afterwards removed and replaced with fresh medium at weekly intervals. A protease inhibitor (5 mL/mL of 200 mM phenyl- methyl-sulfonyl-fluoride) was added to the supernatant removed from the medium. Later, the antigen in the supernatant was concentrated with the aid of an Amiconfilter (Millipore Inc., a subsidiary of Merck Inc., Burlington, MA, USA) , and its protein concentration was determined by the Lowry assay[15].

One hundred and forty micrograms of the TES antigenic fraction (88 µg/µL) were subjected to polyacrylamide gel electrophoresis (SDS-PAGE), using a 12% gel. The samples were suspended in buffer [0.5 M Tris-HCl (pH 6.8), 10% sodium dodecylsulfate (SDS), 20% glycerol, 0.1 M dithiothreitol, 0.2% bromophenol blue] and heated at 100 °C for 5 min. After the addition of buffer (0.3% Tris, 1.5% glycine), electrophoresis was performed at 40 mA and 100 V together with a molecular weight standard (10-260 kDa; Thermo Fischer Scientific, Waltham, USA). Then, the proteins on the gel were transferred to polyvinylidene fluoride (PVDF) membranes (0.2 µm) (Bio-Rad Laboratories, Hercules, CA, USA) in a Mini Trans-Blot system with buffer (10 mM Tris-glycine, 95 mM 20% ethanol, 10% SDS) at 200 mA and 50 V. The membranes were cut into strips of approximately 3 mm and stored until use[16].

The WB technique was carried out as described previously[14,16]. Membranes were blocked for 1 h at room temperature with phosphate-saline buffer (PBS, pH 7.2), Tween 20, and 5% skimmed milk powder (PBSTSM) under constant agitation. The membranes were washed three times with PBS-Tween solution (PBS-T). Serum samples from each animal of all groups were diluted 1: 50 in 1% PBSTSM and added to the membranes, followed by an incubation at 4 °C for 18 h under constant agitation. The membranes were washed six times for 5 min each with PBS-T, and incubated for two h with anti-mouse IgG conjugated with horseradish peroxidase (γ-chain specific; Sigma-Aldrich, USA) diluted 1: 200 in 1% PBSTSM. Binding was detected using DAB substrate (Sigma-Aldrich, USA) in the dark until bands appeared. The antigenic components were analyzed by the VisionWorks LS Analysis software (Analytik Jena GmbH, Germany). Data were expressed as absence or presence of recognizable bands.

RESULTS

Immunogenic fractions were recognized by IgG anti-*Toxocara* antibodies in serum samples of the three groups of infected mice throughout the experiment (Figure 1). Serum samples from negative control animals did not show any recognition of TES-Ag proteins. Serum antibodies sequentially recognized different TES components. While group I mice began to show reactivity 30 dpi, groups II and III animals showed recognition at the beginning of the infection (15 dpi). It was observed that the reactivity of antibodies was strongly increased by the end of the infection (120 dpi).

Immunogenic fractions of TES-Ag were detected at 60-65, 40-45 and 30-35 kDa, in group I. In groups II and III, antigenic fractions of 90-95, 80-85, 70-75, 60-65, 50-55, 40-45 and 30-35 kDa were recognized. The 30-35 kDa
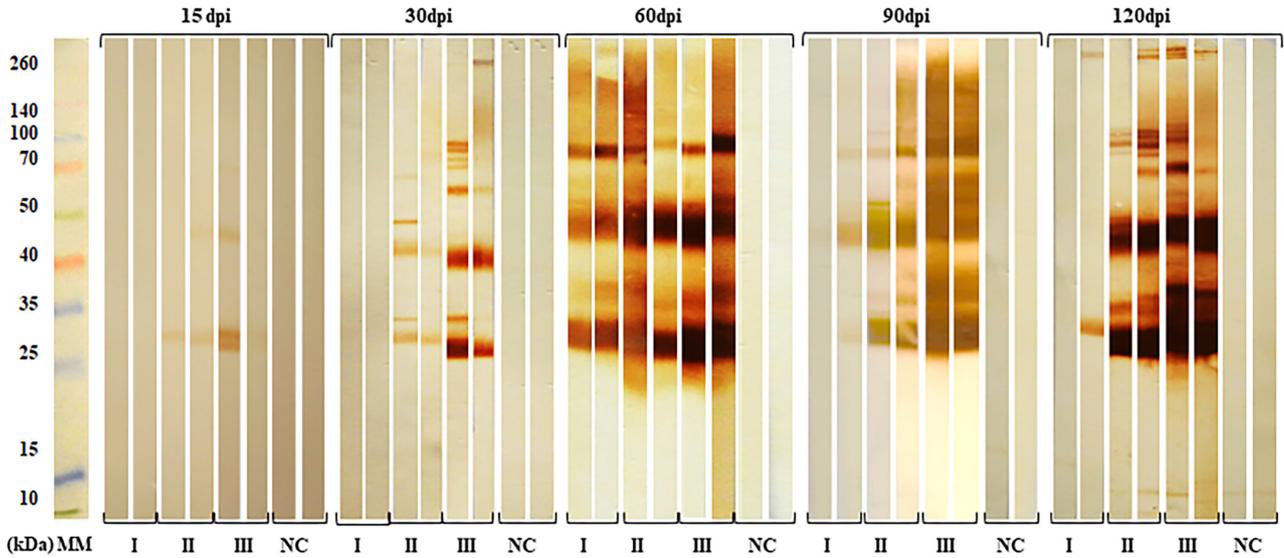
Figure 1 - Dynamics of the recognition of protein fractions by anti-*Toxocara* IgG antibodies in serum samples of mice from groups I, II and III, at collection times corresponding to 15, 30, 60, 90 and 120 dpi. MW = molecular weight marker; NC = negative controls.

Table 1 - Frequency of antigenic fractions recognized by IgG anti-*Toxocara* antibodies from animals of groups I, II and III, according to the days post-infection (dpi). Numbers represent percentages of detection.

kDa	15 dpi			30 dpi			60 dpi			90 dpi			120 dpi		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
90-95	0	0	0	0	0	16.7	0	16.7	0	0	0	0	0	0	0
80-85	0	0	0	0	0	16.7	0	33.3	0	0	66.7	33.3	0	100	83.3
70-75	0	0	0	0	0	16.7	0	33.3	0	0	66.7	33.3	0	100	83.3
60-65	0	0	0	0	0	0	83.3	100	83.3	33.3	66.7	100	0	83.3	100
50-55	0	0	0	0	33.3	100	0	0	33.3	0	0	0	0	66.7	66.7
40-45	0	33.3	33.3	16.7	100	66.7	83.3	100	100	66.7	100	100	0	100	100
30-35	0	66.7	66.7	16.7	100	100	100	100	100	66.7	100	100	33.3	100	100

bands was the most frequently recognized antigenic fraction amongst infected animals, followed by the 40-45 kDa fraction (Table 1).

DISCUSSION

Few studies have been performed using WB in experimental toxocariasis[11,17,18], but none of them assessed the recognition pattern of antigenic fractions related to the inoculum size. The present study observed sequential antigen recognition throughout the experimental infection in all groups. This fact might be related to antigenic components secreted by *T. canis* larvae during their migration to various tissues, or even to metabolites released during larval elimination[17]. Recognition of a greater number of antigenic fractions by IgG antibodies from mice infected with 1,000 *T. canis* larvae from 30 dpi onwards has been demonstrated[18], and it was also observed in this study. Therefore, this study suggests that the number of TES-Ag

fractions recognized by IgG antibodies can estimate whether an infection is in its initial or advanced stage[17].

The panel of immunogenic fractions identified in the present study showed distinct and variable recognition. The number of antigenic fractions recognized in all groups was directly related to the parasite load (inoculum size). This fact might explain the recognition of smaller numbers or less intense antigenic fractions in group I. The immune response of mice infected with *T. canis* has been related to the inoculum[6,19]. We can infer that the remaining larvae from group I might not have stimulated the immune response as observed in animals from groups II and III that were challenged by higher parasite load levels.

Throughout the experimental infection, an increase in the number of protein fractions recognized by IgG in the animals was observed. Similar results were revealed in experimental infections of gerbils, rabbits and other mice[11,17,18]. However, it is important to emphasize that all these studies evaluated only inoculum sizes containing

higher doses (≥ 1,000) *T. canis* larvae or eggs.

Interestingly, the 30-35 kDa fractions were recognized by groups II and III at the initial phase of the infection. Other authors observed protein fractions of 32 kDa 20 dpi in gerbils and of 35 kDa 14 dpi in rabbits inoculated with 1,000 and 5,000 *T. canis* eggs, respectively[11,17]. In contrast, in group I, this fraction was only recognized after 30 dpi. These results may indicate that this protein fraction is not immunogenic enough at the initial phase of the infection, especially in the presence of low inocula sizes. In addition, Nguyen *et al.*[18] demonstrated that 33.1 kDa E/S protein of *T. canis* was the most reactive fraction recognized by specific IgG in animals and humans. Unlike the present study, serum samples from rabbits were evaluated only after 30 dpi. In previous studies, this fraction has been applied to the diagnosis of human toxocariasis[9,18,20-22].

CONCLUSION

As far as we know, this is the first study that describes an antigenic recognition panel by antibodies present in serum of animals inoculated with small size inocula (low parasite load) as well as intermediate and high parasite load inocula, at different times post infection[23]. In addition, the recognition of the 30-35 kDa fraction in experimental animals infected with low parasite load (5 eggs) can mimic the course of infection in occult human toxocariasis[19].

The migration pattern of *Toxocara* larvae in mice starts with the visceral stage andsubsequently evolves to the myotropic-neurotropic stage, corresponding to the collection times 10 dpi and after 10 dpi, respectively[5]. A possible re-migration to the tissues via the bloodstream may occur[19,24,25]. Therefore, the increase and decrease of protein fractions might be related to the larval migration pattern at that time or the elimination of parasites by the immune system, or the momentary parasite load. Thus, our TES-Ag antigenic panel highlights the 30-35 kDa fraction as an important marker of murine infection caused by *T. canis*, especially in the advanced stages of infection, even in the occurrence of infection with very low larval load. These results support further research for the improvement of WB for diagnosis of murine and human toxocariasis.

AUTHORS' CONTRIBUTIONS

GRF and MAC: investigation, validation, writing, visualization; DMCLM: investigation; FMP: conceptualization, methodology, writing, supervision; RCBG: writing, review, editing, resources; SAZL: conceptualization, methodology, resources, writing, supervision, project administration, funding acquisition.

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