SANDWICH ELISA DETECTION OF EXCRETORY-SECRETORY ANTIGENS OF *TOXOCARA CANIS* LARVAE USING A SPECIFIC MONOCLONAL ANTIBODY

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Abstract. We produced a new monoclonal antibody (mAb) to the excretory-secretory (ES) antigens of *Toxocara canis* larvae. The mAb (IgG1) reacts specifically with the 120 kDa protein of many ES molecules and does not have any cross-reactivity with adult *T. canis* antigens. Sandwich ELISA to detect the ES antigens was performed using the mAb and rabbit polyclonal antiserum. The lower limit for the detection of ES antigen was 4 ng/ml; assay was proportional within a concentration range of 4 ng/ml to 1 μg/ml of ES antigen. This assay system may prove valuable when seeking to quantify parasite burden early in infection and when determining the efficacy of anthelmintic treatment.

INTRODUCTION

Toxocariasis is an important zoonotic disease caused by the second-stage larvae of *Toxocara canis* and *Toxocara cati*. The infection is acquired through the ingestion of embryonated eggs that hatch in the host's intestine. A definitive diagnosis of toxocariasis can be confirmed by finding an pathohistological examination, *Toxocara* larvae in biopsy material. Because of the difficulty of detecting the fine larvae in tissue, the disease is diagnosed on the basis of an elevated specific antibody titer determined by enzyme-linked immunosorbent assay (ELISA) using larval excretory-secretory (ES) antigens. Treatment is based on the administration of systemic corticosteroids. Some authors have reported that anthelmintics decrease eosinophilia and improve clinical scores, but their indications are still under discussion. (Lobovska *et al*, 1988; Sturchler *et al*, 1989; Magnaval *et al*, 1992). The antibody titer is not reliable for the estimation of the effect of anthelmintic administration because it remains elevated after initial infection with *T. canis* larvae, as Fenoy *et al* (1992) have reported. The measurement of ES antigens may be more useful than antibody assay when estimating the duration of disease because antigens are secreted from active larvae; furthermore, the variation of ES antigens in serum or in intraocular fluid, accompanied by data regarding the clinical course of illness, may be helpful when determining the indications for drug administration.

To enable the design of a sensitive method for the detection of ES antigens, we produced a monoclonal antibody (mAb) that reacts specifically with ES antigens and that does not have any cross-reactivity with other parasitic helminths; the mAb recognizes the 120 kDa soluble antigen, one of the major components of ES antigens. We report on a sandwich ELISA method for the detection of ES antigens that have reacted with mAb.

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MATERIALS AND METHODS

Preparation of ES antigen

Ova of *T. canis* were collected and hatched as previously described (De Savigny, 1975;

Kondo *et al*, 1984). The *in vitro* maintenance of larvae in serum-free medium followed earlier methods: 1,000 larvae/ml RPMI 1640 containing penicillin, streptomycin, HEPES and glucose supplemented to 1%, were incubated at 37ºC in an atmosphere of 5% CO_2. Weekly changes of culture supernatant were pooled, filtrated on 22 μm Millipore and stored at -80ºC until use.

Hybridoma cells producing monoclonal antibodies

BALB/c mice were immunized with 3 doses of whole ES antigens subcutaneously at 3-week interval. Each immunizing dose consisted of 100 μl of soluble antigens (100 μg protein) emulsified in 100 μl of either Freund's complete adjuvant (first dose) or Freund's incomplete adjuvant (second and third doses). Six days after the final (third) injection, spleen cells were collected and fused with one-third of their number of P3-X63-Ag8-U1 (P3U1) cells in polyethylene glycol and seeded into microtiter plates in hypoxantine-aminopterin-thymidine (HAT)-supplemented RPMI-10S culture medium. Growing wells were screened by ELISA. After initial screening, several representative hybridoma cell lines were cloned by two rounds of limited dilution on feeder layers of BALB/c thymocytes. Five cell lines remained positive for and specific to ES antigens throughout the cloning procedure and were named 14D3, 15F1, 15F4, 15H4, and 18H4. Four cell lines were classified into IgM. Only one cell line (14D3) produced IgG1: 14D3 was grown in ascitic fluid in pristane-primed BALB/c mice and then partially purified from the ascitic fluid by two precipitation steps carried out in 45% saturated ammonium sulfate solution at pH 7.4. IgG concentration was determined by absorbance at 280 nm.

ELISA

To detect anti-ES specific antibodies, ELISA was performed using the following method: ES antigen at 10 μg/ml in 0.1M carbonate-bicarbonate buffer, pH 9.6, was used to coat the ELISA plates (MS8896-F; SUMILON). Other antigens (*Dirofilaria immitis, Ascaris suum* and adult *T. canis*) were also used in 10 μg/ml as soluble antigens. Mouse sera were diluted to 1:1,000 (v/v) and peroxidase-conjugated anti-mouse IgG and IgM were diluted to 1:2,000 in 3% BSA, 0.05% Tween-20 in PBS (3% BSA-T-PBS). Monoclonal antibody was also diluted in 3% BSA-T-PBS. One mg/ml 2,2′-Azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) and 1:1,000 (v/v) H_2O_2 in citrate buffer, pH 4.2, were used as a substrate. Antibody titer was estimated by determining the optical densities at 415 nm (OD_{415}) of the products: two wells per serum were tested.

Sandwich ELISA

Plates for sandwich ELISA were coated with 30 μg/ml of mAb overnight at 4ºC. The plates were then incubated with ES antigens diluted in 3% BSA-T-PBS for 1 hour, with rabbit anti-ES polyclonal serum at a dilution of 1:2,000 (v/v) for 1 hour and with peroxidase-conjugated anti-rabbit IgG at a dilution of 1:4,000 (v/v).

Western blotting

Western blot analysis was performed on ES antigens. Ten μg of ES/tracks was electrophoresed on 10% polyacrylamide gel, and the electrophoresis was monitored using bromophenol blue tracking buffer. The current was set at 50 mA. The relative molecular weights were calculated using prestained protein molecular weight standards. The grafite electrodes were covered with nitrocellulose paper impregnated with TRIS/glycine/SDS/methanol transfer buffer, pH 9.4. The transfer lasted 30 minutes at 20 V. The nitrocellulose was washed for 3 hours in a rotating bath with 1% skimmed milk in PBS in order to block the remaining free sites of the matrix, and then incubated for 1 hour with mAb diluted at 10 μg/ml in 3% BSA-T-PBS or mouse sera diluted at 1:200. Blots were then probed with anti-mouse IgG diluted at 1:200 and finally incubated in substrate, 3,3′- diaminobenzidine (DAB) 40 mg in 100 ml-50 mM Tris-HCl, H_2O_2 40 μl.

Mouse and rabbit serum

Anti-ES serum was collected from BALB/c

mice that had produced hybridomas after immunization with ES antigens subcutaneously for 3 weeks. Normal mouse serum (NMS) was collected from age-matched BALB/c mice. Rabbit anti-ES serum was prepared by the same immunizing method.

RESULTS

No cross-reactivity of mAb with other helmintic antigens

The species-specificity of mAb 14D3 and hyper-immunized mouse serum (HIS) was investigated by ELISA. MAb 14D3 diluted at 10 μg/ml in 3% BSA-T-PBS and mouse sera diluted at 1:1,000 (v/v) were used to detect ES and other antigens (*D. immitis*, *A. suum* and adult *T. canis*). HIS reacted strongly with *D. immitis* and adult *T. canis* and weakly with *A. suum*; mAb 14D3 reacted specifically with ES antigens. No cross-reactivity with adult *T. canis* demonstrated that the target antigen of the mAb was specific for the larval stage. NMS did not react with any antigens (Table 1).

Target antigen of the monoclonal antibody

SDS-polyacrylamide gel electrophoresis (PAGE; 10% gels) revealed that crude ES antigens were composed of 5-6 major and 3-4 minor bands ranging in molecular weight from 15 kDa to 200 kDa (data not shown).

Tabel 1
The species-specificity of the monoclonal antibody.

	OD_{415}		
Antigen	14D3	HIS	NMS
Dirofilaria immitis	0.08	2.17	0.09
Ascaris suum	0.04	0.29	0.09
Adult *T. canis*	0.04	2.80	0.08
ES	1.56	2.14	0.07

HIS: hyper immunized serum
NMS: normal mouse serum

Western blot analysis was performed to show which component was the target antigen of the mAb. The mAb reacted with only one band, while HIS reacted with at least 8 bands, most of which were major proteins (Fig 1). NMS did not bind with any components of ES antigens. The molecular weight of the target antigen recognized by mAb 14D3 was estimated to be 120 kDa.

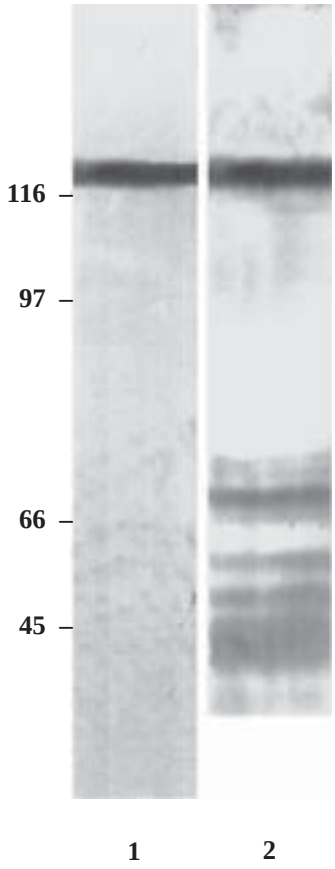

Fig 1–Western blot analysis. Ten μg of ES antigens were electrophoresed on each track of 10% polyacrylamide gel: track 1 is incubated with hyper-immunized sera and track 2 is incubated with monoclonal antibody. The molecular weights indicated in are kDa.

Sensitivity of mAb 14D3

To determine the proper concentration of mAb 14D3 for sandwich ELISA, the sensitivity of the mAb was evaluated by ELISA. Plates for ELISA were coated with 10 μg/ml of ES antigens. OD_{415} was proportional to monoclonal antibody concentration within the range 0.01 μg/ml to 1 μg/ml and showed the maximum value over 30 μg/ml; the optimal concentration of mAb 14D3 to coat the plates for sandwich ELISA was therfore considered to be 30 μg/ml (Fig 2).

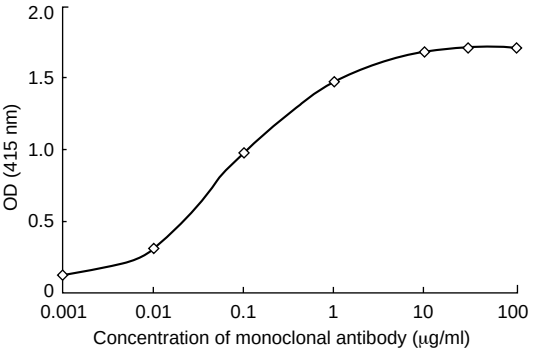

Fig 2–Sensitivity of mAb 14D3. ES antigen at 10 μg/ml was used to coat ELISA plates. Mouse sera were diluted at 1:1,000 (v/v) and peroxidase-conjugated anti-mouse IgG and IgM were diluted at 1:2,000.

Sandwich ELISA to detect ES antigens

To establish sandwich ELISA limits for detecting ES antigens, a standard curve was made with a mAb 14D3 (30 μg/ml)-coated ELISA plate by diluting ES antigens in 3% BSA-T-PBS at concentrations from 0.01 to 10,000 ng/ml. The anti-ES antigen rabbit serum (1:2,000) was used as the detecting antibody. As shown in Fig 3, OD_{415} of 0.1 and 1 ng/ml ES antigens were 0.052 and 0.069 respectively; the OD_{415} of 4 ng/ml was 0.160, which was twice as high as those of the lower concentrations. A positive indication of the presence of ES antigens was therefore obtainable at levels as low as 4 ng/ml. The values of OD_{415} were proportional within the range of 4 ng/ml to 1 μg/ml of ES antigen concentrations. The OD_{415} of 1 μg/ml ES antigens was 2.549. Over 1μg/ml, OD_{415} reached a plateau (Fig 3).

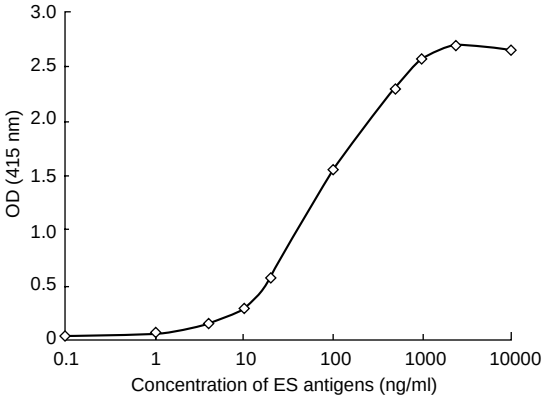

Fig 3–Sandwich ELISA to detect ES antigens. Plates coated with 30 μg/ml of mAb were incubated with ES antigens diluted in 3% BSA-T-PBS and with rabbit anti-ES polyclonal serum at a dilution of 1:2,000 (v/v).

DISCUSSION

In this paper, we intended to produce a mAb with high specificity for ES antigens and we succeeded in obtaining an anti-ES specific IgG1 mAb, 14D3, that has no cross-reactivity with adult *T. canis* antigens. SDS-PAGE showed that whole ES antigens consisted of a range of proteins with molecular weights from 15 kDa to 200 kDa. Western blot analysis revealed that mAb 14D3 reacted with one of the major bands, which was estimated to be the 120 kDa molecule of ES antigens. Maizels *et al* (1984; 1987) also reported the SDS-PAGE profiles of ES antigens and produced some mAbs against the antigens. Their Tcn-2 (IgM) reacted with 55, 70, and 120 kDa molecules, and their Tcn-3 (IgG1) consistently precipitated the 32 kDa molecule and showed some binding to the 120 kDa molecule. Our mAb 14D3 reacted with the 120 kDa molecule only. This result suggested that the epitope recognized by mAb 14D3 was different to those recognized by mAbs, Tcn-2 and Tcn-3.

ES antigens-detecting assays have been described by some authors. Aguila *et al* (1987) detected circulating immune complexes in se-

rum using a mAb specific for 177-77 kDa molecules of ES antigens. Robertson *et al* (1988) reported that the mAb-based two-site sandwich assay discriminates between *T. canis* and the related feline ascarid *T. cati*; they found the antigens in sera from UK patients diagnosed with visceral or ocular toxocariasis, and in four asymptomatic Papua New Guinean children as well as in experimental animals; their assay system, using Tcn-2 and Tcn-3, was able to detect the target antigens in concentrations as low as 20 ng/ml, comparable to the threshold by Bowman *et al* (1986), who employed a polyclonal antiserum directed at all components of ES. Luo *et al* (1999) reported that their sandwich ELISA was able to detect the circulating antigens in some sera with positive antibodies to *T. canis*: they conducted their study using guinea pig anti-TES-IgG and rabbit anti-TES-IgG; their positive limit for detection was as low as 78 ng/ml. In the present paper, we designed the assay using mAb 14D3 and rabbit anti-ES polyclonal serum. The sensitivity of sandwich ELISA using the anti-120 kDa soluble protein of ES monoclonal antibody was very high: the assay could detect traces of ES antigens at concentrations as low as 4 ng/ml.

The application of monoclonal antibody in immunodiagnosis confers many advantages, including decreased false-positive reactions and cross-reactivity, increased reproducibility, and standardization of testing. Monoclonal antibody in the present study reacts with the 120 kDa soluble protein of ES and has high specificity without any cross-reactivity to the soluble antigen of adult *T. canis*, *A. suum* and *D. immitis*. While this study was not designed to examine improved methods for the immunodiagnosis of visceral larva migrans, the remarkable specificity and sensitivity of the monoclonal antibody produced in our laboratory suggest that further studies employing this and similar antibodies may lead to more specific immunodiagnostic tests.

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