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Immunodiagnostic Value of an α-Stichocyte-derived Antigen Isolated by Affinity Chromatography for Trichinosis

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Abstract

We isolated 160-kDa molecule (Ts-α 160) from α-stichocytes of *Trichinella spiralis* muscle larvae with affinity chromatography using a mouse monoclonal antibody and examined reactivities of Ts-α 160 and crude extracts of *T. spiralis* larvae (Ts-crude) by the enzyme-linked immunosorbent assay (ELISA) against serum samples which had been obtained from 13 patients 33 to 109 days after infection. Antibodies against Ts-α 160 were detected with higher titers than those against Ts-crude in every case. The application of Ts-α 160 to the ELISA eliminated false-positive reactions which were observed against Ts-crude in serum samples of patients with trichuriasis, gnathostomiasis, paragonimiasis westermani and paragonimiasis miyazakii, although weak positive reactions against Ts-α 160 were observed in cases with dirofilariasis or fascioliasis. The present results suggest that Ts-α160 can be utilized as a unique antigen for the immunodiagnosis of human trichinosis from about 5–6 wk after infection. In addition, Ts-α 160 was found to be a component of excretory-secretory products derived from the infective muscle stage larvae.

Key words: *Trichinella spiralis,* affinity chromatography, α-stichosome, antigen, diagnosis

Introduction

In order to develop reliable diagnostic tests for parasite infections, it is essential to employ reagents which are sensitive and highly specific to the parasite concerned. In most laboratories, however, extracts of whole organisms have been employed as conventional antigens. This can lead to a false diagnosis if the possibility of cross-reaction is overlooked.

Many immunological methods for diagnosis of trichinosis have been employed to resolve these disadvantages (Roth, 1941; Bozicevice *et al.*, 1951; Price and Weiner, 1956; Kozar *et al.*, 1964; Kagan and Norman, 1970; Despommier *et al.*, 1974; Barret-Conner, 1976; Gamble and Graham, 1984; Despommier, 1986). Based on experimental results, Ruitenberg and his colleagues (Ruitenberg *et al.*, 1977a, b; van Knapen *et al.*, 1982) have suggested that the enzyme-linked immunosorbent assay (ELISA) is highly sensitive and specific to diagnose trichinosis, and that the assay has advantages when compared with other immunological methods.

We have prepared a partially purified antigen ($Ts\text{-}S_3$) from stichocytes of *Trichinella spiralis* muscle larvae according to the method of Despommier and Lacetti (1981a, b), and compared its reactivity with that of crude antigens of *T. spiralis* against serum samples from chronic patients with trichinosis by the complement fixation test (CFT), double diffusion test in agar gel (DDT) and ELISA (Kobayashi *et al.*, 1987). The results indicated that $Ts\text{-}S_3$ was superior to Ts-crude in specificity in the ELISA.

We have also obtained a monoclonal antibody (TS32D12) of IgG_1 class specific to a 160-kDa

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molecule protein in the α-stichosome of *T. spiralis* infective muscle larvae (Niimura *et al.*, 1988). Results obtained from *T. spiralis* infected mice have suggested that the 160kDa molecule, if it is separated by immunoaffinity chromatography coupled with TS32D12, is unique in the diagnosis of trichinosis (Niimura and Kojima, 1991).

The aim of the present study is to examine the specificity and sensitivity of the ELISA for the diagnosis of human trichinosis by using this particular antigen.

Materials and Methods

Parasites

Trichinella spiralis (originally derived from N.I.H., USA) was maintained by serial passage in male ddy mice. Muscle larvae were recovered by digestion of infected muscles with a solution of 1% pepsin/HCl as described previously (Niimura and Kojima, 1991).

Purification of a monoclonal IgG_1 antibody

A monoclonal IgG_1 antibody was purified from ascites of mice bearing a hybridoma (TS32D12) (Niimura *et al.*, 1988) by using a Mono Q HR5/5 prepacked anion exchange column (Pharmacia) connected to a fast protein liquid chromatography (FPLC) system.

Antigens

A crude homogenate (Ts-crude) and a partially purified antigen (Ts-S_3) were prepared as described (Niimura *et al.*, 1988). Another antigen of 160-kDa molecule in α-stichosome was separated by immunoaffinity chromatography using a slightly modified method of Niimura and Kojima (1991). In brief, purified TS32D12 (7.9 mg) was coupled to Formyl-Cellulofine matrix (1 g/wet weight) according to a technical manual (Seikagaku Kogyo, Tokyo) with a coupling agent, sodium cyanoborohydride (Nacalai Tesque, Inc. Kyoto). Ts-S_3 was incubated with Formyl-Cellulofine matrix in 0.2 M Tris-HCl, pH 7.2, at 4°C, overnight. After washing the matrix, the gel was embedded into a column (1.0×5 cm). Ts-α160 was eluted from the column with 0.3 M acetate buffer at pH 3.0, and the eluent was neutralized with Tris-base.

Protein concentration was determined by the Coomassi dye-binding method using bovine serum albumin as a standard (Bradford, 1976).

Serum samples

Serum samples were collected from patients who had eaten uncooked bear meat, which had been imported from China at a restaurant in Mie Prefecture in Japan, 1982. They were diagnosed as trichinosis by immunological methods such as latex agglutination test (LA), circum-larval precipitin test (CLP) and countercurrent immunoelectrophoresis (CIE) (Yamaguchi *et al.*, 1982). Reference serum was obtained from a patient, from whom larvae were detected by muscle biopsy (Ozawa *et al.*, 1981). Additional serum samples were obtained from patients with other kinds of helminthiasis. Trichuriasis and paragonimiasis westermani were diagnosed by stool examinations, and gnathostomiasis was determined by identifying larvae by muscle biopsy. Anisakiasis was determined by removing larvae from the gastric mucosa with biopsy forceps. Dirofilariasis, toxocariasis, angiostrongyliasis, paragonimiasis miyazakii and fascioliasis were diagnosed by several immunological techniques in our laboratory. Normal sera were collected from medical students of Chiba University, who were proved to be parasite free by stool examinations and none of whom had ever consumed raw bear meat.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed by a modified method of Voller (1976) and Tanaka *et al.* (1979). Microassay plates (Nunc immuno-plate 1) were coated with 50 μl of antigen (2 μg protein/ml) in 0.05 M carbonate buffer (pH 9.6) at 4°C overnight. After blocking the plates, 50 μl of diluted sera were added to wells. Peroxidase-labeled goat anti-human IgG/IgM (Miles-Yeda) and 2-2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) (Nacalai Tesque, Inc. Kyoto) were used as the conjugate and substrate, respectively. The rate of reaction was measured against optical density of normal

sera by microplate photometer at 405 nm.

Western blot analysis

Western blot analysis was carried out as previously described (Niimura *et al.*, 1988).

Excretory-secretory (E-S) products

E-S products were recovered from culture supernatant of *T. spiralis* mature muscle larvae (5,000 worms/ml) which were maintained in Dulbeco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine and antibiotics (penicillin 100 units/ml and streptomycin 100 μg/ml) for a period of 38 hr at 37°C in a humid atmosphere of 5% CO_2. Culture fluid was harvested by filtration, dialyzed with PBS and concentrated under pressure by a collodion-bag (Sarutorius Membranfilter). Western blot analysis and protein determination were performed as described above.

Results

Separation of Ts-α160

Ascites of mice bearing hybridoma TS32D12 (Niimura *et al.*, 1988) were applied on an anion-exchange Mono Q column to separate TS32D12. The antibody was eluted in peak 3 which was obtained by a stepwise gradient with 0.14 M sodium chloride. The purified TS32D12 was coupled to Formyl-Cellulofine matrix and Ts-S_3 was incubated with the matrix. Ts-α160 was eluted from the affinity column. Recovery rates of the antigen ranged from 8 to 11% of original protein of Ts-S_3. Specific activity of Ts-α160 increased 1.5–1.6 fold than Ts-S_3 against serum samples of 6–8 week after infection of *T. spiralis*. Purity of Ts-α160 was confirmed by the Western blot (Fig. 1). Heat treatment of Ts-α160 at 100°C for 5 min completely destroyed its antigenicity (Fig. 1, lane 3).

Evaluation of Ts-α160

Reactivities of Ts-crude and Ts-α160 were examined against serum samples from 13 patients with trichinosis (Fig. 2). Sample No. 1 was obtained 109 days after infection from a patient with positive muscle biopsy (Ozawa *et al.*, 1981).

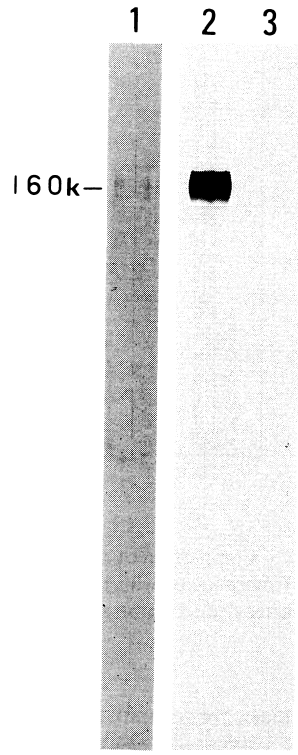

Fig. 1 Western blot analysis of the fraction of Ts-α160 eluted by Formyl-Cellulofine column chromatography. SDS-PAGE was carried out under reduced condition. Lane 1, stained with Coomasi brilliant blue. Lanes 2 and 3, incubated with TS32D12. Lane 3: treated at 100°C.

Serum samples of No. 2–5 and No. 6–13 were obtained 43 days and 33–50 days after infection, respectively, from patients with positive LA, CLP and CIE (Yamaguchi *et al.*, 1982). As shown in Fig. 2, antibody reactions against Ts-α160 were detected at a higher level than those against Ts-crude in every case by the ELISA.

Cross-reactions were observed when Ts-crude was used for several serum samples from patients with either trichuriasis, dirofilariasis, gnathostomiasis, paragonimiasis westermani, paragonimiasis miyazakii or fascioliasis. Ts-α160 was able to eliminate false-positive reactions observed in serum of trichuriasis, gnathostomia-

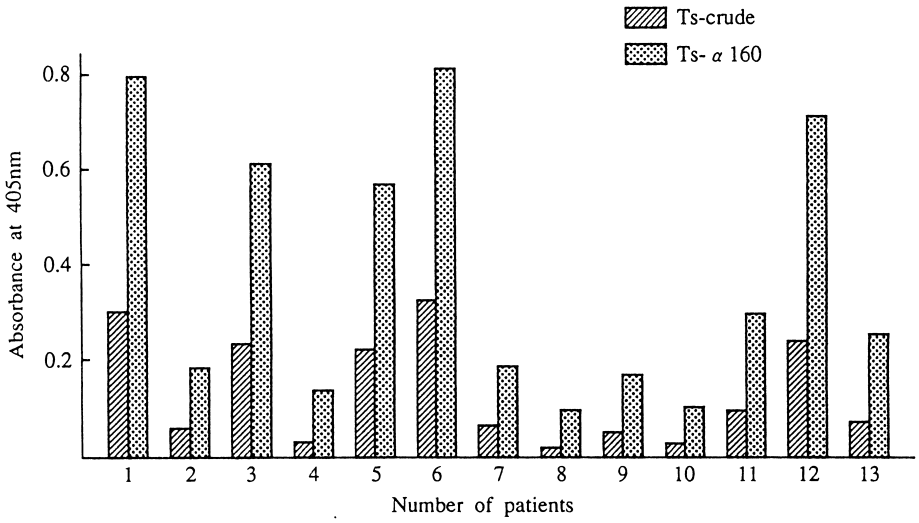

Fig. 2 Comparison of reactivity of Ts-α 160 and Ts-crude against serum samples (1:80 dilution) from patients with proven trichinosis. Absorbance at 405 nm represents net optical density after deduction of control values.

sis, paragonimiasis westermani and paragonimiasis miyazakii, although weak reactions were observed even with this antigen in cases of dirofilariasis or fascioliasis (Fig. 3).

E-S products

The molecular size of E-S products was analyzed by Western blotting. The presence of epitopes of 160-kDa molecule recognized by TS32D12 was demonstrated under reduced condition (Fig. 4).

Discussions

The detection of antibodies in the host serum against antigens derived from developmental stages of *T. spiralis* is an important aspect of immunodiagnosis of trichinosis. The present study demonstrated that Ts-α 160, if isolated by affinity chromatography in which TS32D12 was coupled to Formyl-Cellulofine matrix, could be utilized as a specific antigen for the ELISA in the diagnosis of trichinosis.

In our previous studies, only a glycoprotein with molecular weight of 160-kDa (Niimura *et al.*, 1988) was found to be useful for immunodiagnosis, and this molecule has been isolated from the secretory granules of stichocytes as a stage-specific antigen by immunoaffinity chromatography. In mice infected with *T. spiralis,* antibodies against the 160-kDa molecule (Ts-α 160) were detected in the serum at 6 wk post infection (PI) and thereafter (Niimura and Kojima, 1991). On the other hand, studies on localization of Ts-α 160 revealed that the antigen disappeared soon after infection (23 hr PI) from the stichocytes of *T. spiralis* in the enteral stage, and that immature larvae in the muscle stage (14 days PI) did not possess this antigen. However, a marked increase of the antigen was observed in mature muscle larvae on day 21 PI and thereafter. These results suggest that Ts-α 160 is formed in the granules of α-stichocytes of muscle larvae from 3 to 4 wk PI, and that the molecule is excreted or secreted with the granules, inducing production of antibodies from 5 to 6 wk PI (Niimura and Kojima, 1991).

Takahashi *et al.* (1990) have also investigated the time course of antibody responses against *T. spiralis* muscle larvae in rats. IgG-class antibodies against stichocyte granules and esophagus occupying substances were detected at 6 wk PI,

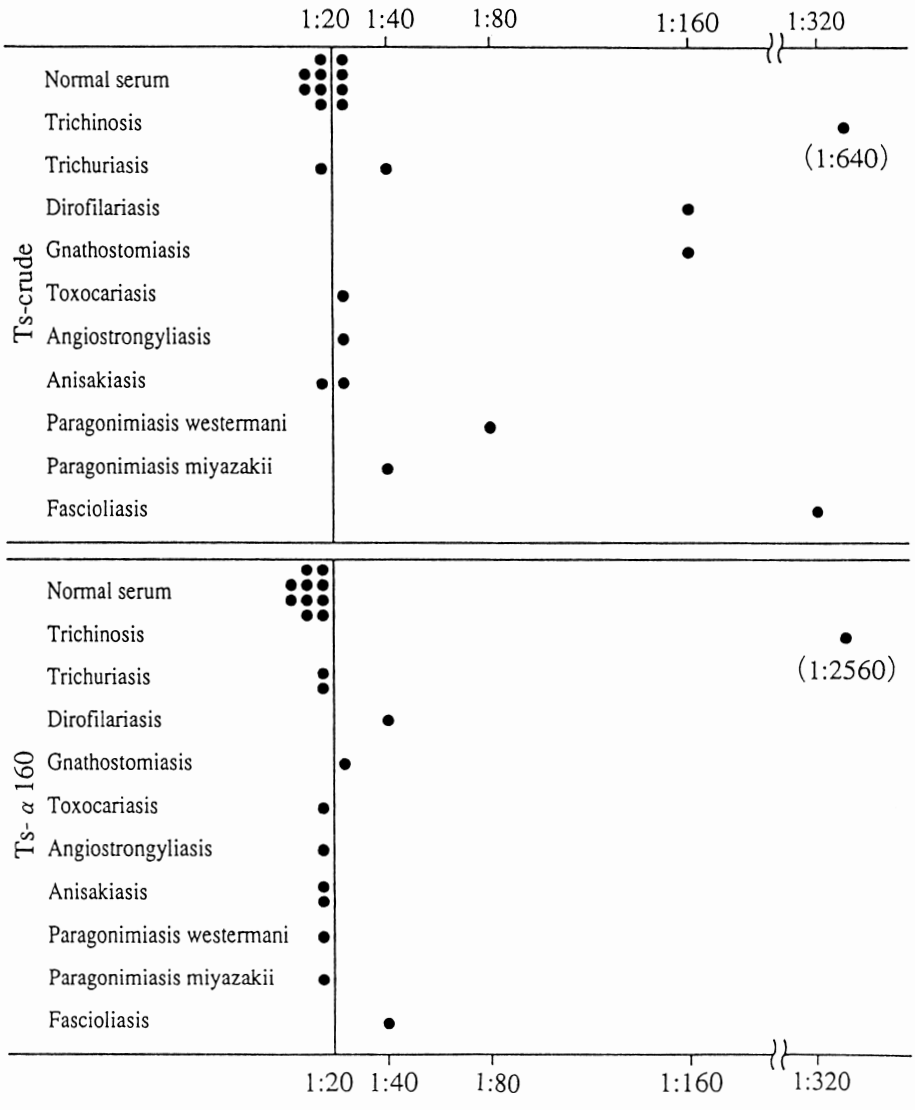

Fig. 3 Comparison of results of the ELISA by using Ts-α160 and Ts-crude against serum samples from patients with proven helminthiases. Titers were determined by the endpoint of serum dilution which showed 3-fold higher absorbance at 405 nm than mean absorbance (1:20 dilution) of normal sera.

whereas antibodies of the same isotype bound to other tissues including the cuticle inner layer(s), hypodermis and hemolymph became detectable at 2 wk PI.

To examine whether Ts-α160 antigen might be useful for detection of antibodies in human infections, ELISA was carried out for serum samples obtained from 13 cases 33–109 days after infection including the proven case by muscle biopsy. Responses against Ts-α160 were always higher than those against Ts-crude in every case (Fig. 2). Cross-reactions were observed in serum samples from cases with dirofilariasis or fascioliasis, but there would be no misleading to a false diagnosis if controls were appropriately provided (Fig. 3). In addition, Ts-α160 showed

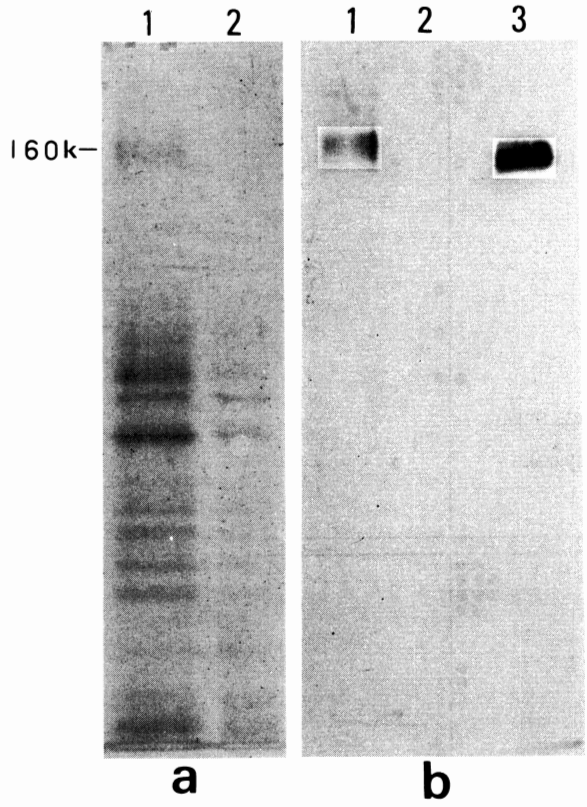

Fig. 4 Analysis of E-S products. E-S products were subjected to 10% SDS-PAGE under reduced condition and they were transferred to nitro-cellulose membrane. a. The membrane was stained with Coomassi brilliant blue. Lane 1, reduced form. Lane 2, treated at 100°C, for 5 min. b. Westen blot. Lanes 1 and 2, correspond to a. Lane 3, Ts-α160.

a higher ELISA value even if compared to that of Ts-S_3.

Thus, Ts-α160 can be utilized as a unique antigen for the immunodiagnosis of human trichinosis 5–6 wk after infection, although it was not clear if antibodies were also detectable in earlier stages of the infection because serum samples from patients in early stages of infection were not obtained in the present study. Several investigators have attempted to identify and purify various *T. spiralis* antigens to increase the diagnostic value. The E-S antigen and other stichocyte-derived antigens were shown to be superior to crude antigens (Graham, 1984; Homan *et al.*, 1992). Antigens of 45-, 48- and 53-kDa molecules were identified as major components in the E-S fraction of *T. spiralis* muscle larvae (Gamble and Graham, 1984). Among them, the 54-kDa antigen seemed to be promising for the development of a rapid large-scale screening assay (Homan *et al.*, 1992).

In our hands, it is likely that Ts-α160 is excreted or secreted from stichocytes of mature muscle larvae of *T. spiralis* (Fig. 4), and epitopes of this molecule seemed to be destroyed by heat treatment (Figs. 1 and 4), which was in accordance with our previous observations (Niimura *et al.*, 1988). In addition, this antigen molecule may be used as a tool for elucidation of the physiological function of the stichosome,

because it seems to have an enzymatic activity in degenerating casein (data to be published). The functional role of this molecule is currently under investigation.

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