[Jpn. J. Parasitol., Vol. 40, No. 3, 253–259, June, 1991]

Immunocytolocalization and Properties of a Target Antigen Recognized by a Monoclonal Antibody to *Trichinella spiralis*

MUNETOSHI NIIMURA AND SOMEI KOJIMA[1)]

(Accepted for publication; May 8, 1991)

Abstract

Immunocytolocalization of the 160kDa molecule that was recognized by a mouse monoclonal antibody to *Trichinella spiralis* was examined by the indirect immunofluorescent technique and by using a scanning confocal imaging system throughout the developmental stages of the parasite. The 160kDa molecule present in the infective larval stage diminished from α-stichosome during the enteral development and almost completely disappeared at 23 hr post infection. This molecule was not detected in adult worms at day 6, embryos at day 7, nor in muscle larvae at day 14. However, it was demonstrated in α-stichosome of the late muscle stage-larvae from day 21 at least up to day 180. Moreover, the scanning confocal imaging system revealed that the molecule was present in the granules of α-stichocytes of the late muscle stage-larvae. Antibody responses of infected BALB/c mice against the affinity-purified 160kDa antigen (Ts-α160) became detectable with low titers at week 4, followed by a rapid increase in titers at week 8. The responses remained with high titers thereafter. On the other hand, the antibody responses against crude antigens became positive even at day 7 and remained higher in titers up to week 6 than those against the purified antigen. Analysis of glycoproteins revealed that asparagin-linked polysaccharide chain was present in the molecule. Passive transfer experiments with the monoclonal antibody failed to induce protection in mice against a challenge infection with *T. spiralis*.

Key words: *Trichinella spiralis*, monoclonal antibody, α-stichocyte, antigen, property

Introduction

A number of research groups have now raised monoclonal antibodies against different life-stages of *Trichinella spiralis* (Silberstein and Despommier, 1984; Gamble and Graham, 1984; Ortega-Pierres *et al.*, 1984; McLaren *et al.*, 1987; Niimura *et al.*, 1988). The muscle-stage larva of *T. spiralis* has been the focus of most of these investigations.

Despommier *et al.* (1976, 1981a) demonstrated that the principal secretory organ, the stichosome of the infective L_1 larvae, contained a full complement of antigens specific for that stage, polypeptides of 50/55kDa and 48kDa molecules which were localized in α- and β-stichocytes by an immune-labelling technique (Silberstein and Despommier, 1984). Such antigens induce high levels of protection in mice (Silberstein and Despommier, 1984, 1985; Gamble 1985), and the anatomical fate of these proteins has been analyzed in the enteral stage of larvae and adults according to each developmental stage (Capo *et al.*, 1986). By using a monoclonal antibody of the IgG_1 class (MoAb-IgG_1) which binds only to epitopes present on a 160kDa molecule in α-stichosome of *T. spiralis* muscle larvae (Ts-α 160), we also have suggested that the 160kDa molecule is to be of great value in immunodiagnosis because of its specificity (Niimura *et al.*, 1988).

Department of Parasitology, Chiba University School of Medicine, Chiba 280, Japan.

[1)]*Present address: Department of Parasitology, Institute of Medical Science, the University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108.*

新村宗敏（千葉大学医学部寄生虫学教室）
小島莊明（東京大学医科学研究所寄生虫研究部）

This study was supported by a Grant-in-Aid for Scientific Research (No. 02670165) from the Ministry of Education, Science and Culture of the Japanese Government.

The aim of the present study is to determine whether or not the 160kDa molecule is present throughout the developmental stages of the worms from the enteral to muscle stage. Antibody titers of the sera from mice infected with each stage of *T. spiralis* were determined against Ts-α160 by the enzyme-linked immunosorbent assay (ELISA). Carbohydrate moiety of Ts-α160 and protective effects of MoAb-IgG_1 to a challenge infection were also examined.

Materials and Methods

Parasites and antigens

The strain of *T. spiralis* used for the present experiment was originally derived from the NIH, USA, and has been maintained by passage through Wistar rats in our laboratory for 15 years. Infective L_1 larvae were obtained from stock-infected mice by peptic digestion of minced carcasses in 1% pepsin/HCl for 1 hr at 37°C under constant agitation. After washing with distilled water, the larvae were lyophilized and kept at –20°C.

A crude homogenate was prepared by grinding lyophilized worms in saline using a Potter-Elvehjem tissue grinder on ice. The homogenate was centrifuged at 12,000 × g for 60 min at 4°C, and the supernatant was used as the crude antigens (Ts-crude). A 160kDa molecule in α-stichosome was separated by immunoaffinity chromatography as follows; briefly, the MoAb-IgG_1 separated from ascites using an anion exchange column (Niimura *et al.*, 1988) was coupled to Formyle-Cellulofine (Seikagaku-kogyo, Tokyo) with a coupling agent, sodium cyanoborohydride (Nakarai Chemicals, Tokyo). A partially purified antigen (Ts-S_3), isolated from the stichosome of *T. spiralis* infective larvae according to Despommier and Laccetti (1981a, b), was incubated with Formyl-Cellulofine matrix in 0.2M Tris-HCl, pH 7.2. After washing the matrix, Ts-α160 was eluted from the column with 0.3M acetate buffer at pH 3.0, and the eluat was neutralized with Tris-buffer.

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

Chronic serum samples

A group of five male BALB/c mice (6 wk old) received per os 30 infective *T. spiralis* larvae each. Serum samples were collected from each mouse at 1, 2, 4, 6, 8, 14 and 22 wk post-infection (PI).

Immunocytolocalization of the target antigen

Each of naive male mice received per os 1,000 infective *T. spiralis* larvae. The worms were recovered from the intestine with a Baermann apparatus at 6, 14, 23 and 30 hr, respectively. Newborn larvae were collected by incubating adult female worms in Dulbecco's modification of Eagle's medium (DMEM) containing 15% fetal calf serum and antibiotics. Muscle larvae were recovered with a Baermann apparatus on days 14 and 21, or with peptic digestion on day 28, 35 and 180, respectively. The worms collected at each time-point were fixed in 95% ethanol and they were embedded in paraffin. Microscopic sections were cut at 5μm. To determine the localization of the antigen, indirect immunofluorescent technique was performed with FITC-conjugated goat anti-mouse IgG (used at a dilution of 1:60) according to Hamashima and Yasuda (1971). Confocal analysis was carried out on cross sections of α-stichosome of muscle larvae at 35 days PI with a Bio-Rad MRC-500 scanning confocal imaging system. Morphological observation was performed on sections stained with HE and PAS-reagent.

Enzyme-linked immunosorbent assay

ELISA was performed by a modified method of Voller (1976) and Tanaka et al. (1979). Microassay plates (Falcon, 3912) were coated with 50μl of antigen (2μg protein/ml) in 0.05M carbonate buffer (pH 9.6) at 4°C overnight. After blocking the plates, 50μl of diluted sera (1:80) was added to wells. Peroxidase labeled goat anti-mouse IgG/IgM (Miles-Yeda) and 2-2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) (Nakarai Chemicals, Tokyo) were used as the conjugate and substrate, respectively.

Western blot analysis

Western blot analysis was carried out according to Towbin *et al.* (1979). Glycoprotein

analysis was performed on nitrocellulose sheets with peroxidase-conjugated lectin kit-A (Seikagaku-kogyo, Tokyo) (Kijimoto-Ochiai *et al.*, 1985).

Passive immunization

Eight wk old female ICR mice were infected orally with 150 infective larvae of *T. spiralis*. Group 1 received an i.p. injection of 50μg MoAb-IgG$_1$ with a volume of 0.25ml in saline one day before and on the day of the infection. Mice of group 2 were injected with the antibody on days 6 and 7 at the time point corresponding to the adult and embryo stage. Group 3 was treated similarly on days 13 and 14 when immature larvae were found in the muscle. Control mice received an i.p. injection of 0.25ml saline as in mice of group 1. Mice were sacrificed on day 35, and muscle larvae were recovered by pepsin-HCl digestion. Statistical analysis for difference of recovery of the worms between experimental groups and a control group treated with MoAb-IgG$_1$ was performed using Student's *t*-test.

Results

Immunocytolocalization of the target antigen

Localization of the target antigen in worms of the enteral stage was studied at 6, 14, 23 and 30 hr, and on day 6 PI, time points corresponding to the development from L_1 larvae to adults. As shown in Table 1, MoAb-IgG$_1$ was found to bind to the α-stichocytes of the worms within the enteral stage until 23 hr from the beginning of the infection, while the binding was not demonstrated in the stichocytes of the worms of the pre-adult (30 hr) or adult (6 day) stage. Newborn larvae (day 7) did not possess the target antigen recognized by this monoclonal antibody. As for the worms recovered from the muscle, immature larvae (day 14) did not possess the antigen, while a marked increase of the antigen was noted in the α-stichocytes of mature muscle larvae (day 21, 28 and 35). The antigen was also detected on day 180 PI.

It was further clarified by a confocal laser fluoro-microscope that Ts-α160 was present on the granule in α-stichocyte of *T. spiralis* infective muscle larvae (Fig. 1).

Table 1 Immunocytolocalization of the target antigen in *Trichinella spiralis* recognized by a monoclonal antibody

Time after infection	Developmental stage	Antigen recognized by MoAb*
Enteral phase		
6 hrs	$L_1 - L_2$	+
14 hrs	$L_2 - L_3$	+
23 hrs	$L_3 - L_4$	+
30 hrs	$L_4 - L_5$	−
6 days	Mature adult	−
Muscle phase		
7 days	Newborn larvae	−
14 days	Immature muscle larvae	−
21 days	Mature muscle larvae	+
28 days	Mature muscle larvae	+ +
35 days	Mature muscle larvae	+ +
180 days	Mature muscle larvae	+

*: Monoclonal antibody of IgG$_1$ class.

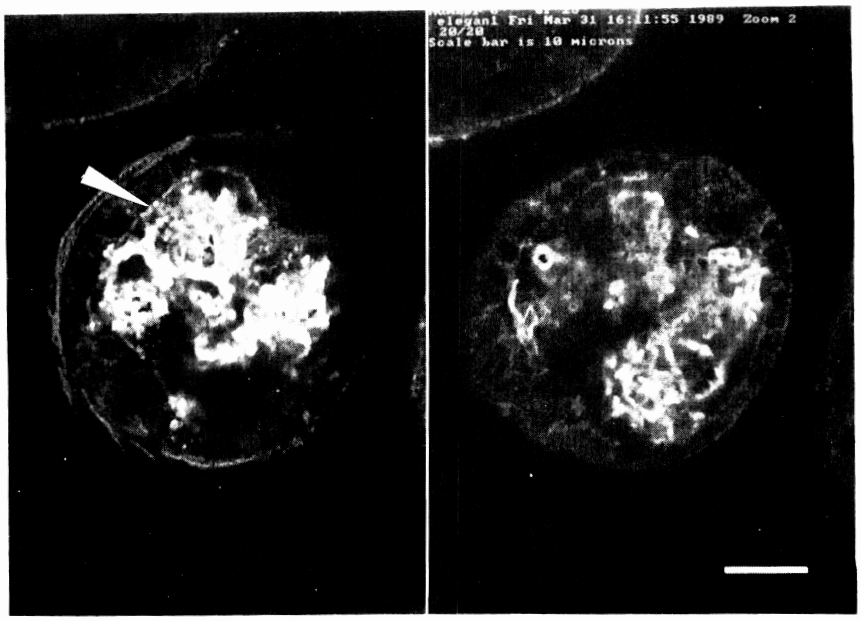


Fig. 1 Immunolocalization of Ts-α160 in a α-stichocytes of *T. spiralis* muscle larvae. Cross sections of a worm. A series of scanning confocal images were taken at 0.5μm after treatment with MoAb-IgG$_1$. The arrow shows the stichocyte wall of the worm, scale bar, 8.5μm.

Antibody detection in chronic serum

As shown in Fig. 2, antibody responses against Ts-α160 became detectable at 6 wk PI and titers rapidly increased at 8 wk, reaching a peak at 14 wk. On the other hand, antibody responses against Ts-crude were detected as early as 1 wk PI, and the responses were significantly higher in titers than those against Ts-α160 up to 6 wk PI. However, almost similar responses were observed until 14 wk as observed against Ts-α160, although they showed a tendency to decrease thereafter.

Lectin binding assay and passive transfer of immunity

The presence of N-linked carbohydrate chain on Ts-α160 was estimated, because the antigen had asparagin-linked polysaccharide chain which reacted with Con A and LCA lectins (Fig. 3). Nonspecific peroxidase reaction was not observed on Ts-α160. There was no significant decrease in recovery of worms among experimental groups of mice passively transferred MoAb-IgG$_1$ when compared with the worm recovery in control mice (Table 2).

Discussion

An important aspect in immunodiagnosis of trichinosis, and of parasitic diseases in general, is identification of the specific antigen which is unique to the parasite species concerned. In our previous report (Kobayashi *et al.*, 1987), we used a crude antigen for three immunological tests, i.e., the complement fixation test (CFT), double diffusion test in agar gel (DDT) and ELISA, for the diagnosis of 474 suspected cases with trichinosis. However, the results of these tests did not always correspond with each other. Moreover, we observed that antibody titers to the crude antigen gradually decreased from about 7 wk PI (Kobayashi *et al.*, 1987), despite the fact that at least in cases with paragonimiasis, the CFT with a crude antigen remained positive with high antibody titers for years if chemotherapy was not performed (Yokogawa *et al.*, 1961).

In the mouse model with *T. spiralis* infection, antibody responses against Ts-α160 were compared with those against Ts-crude (Fig. 2). When Ts-α160 was used for the ELISA, antibodies were detected at 4 wk PI. Titers rapidly

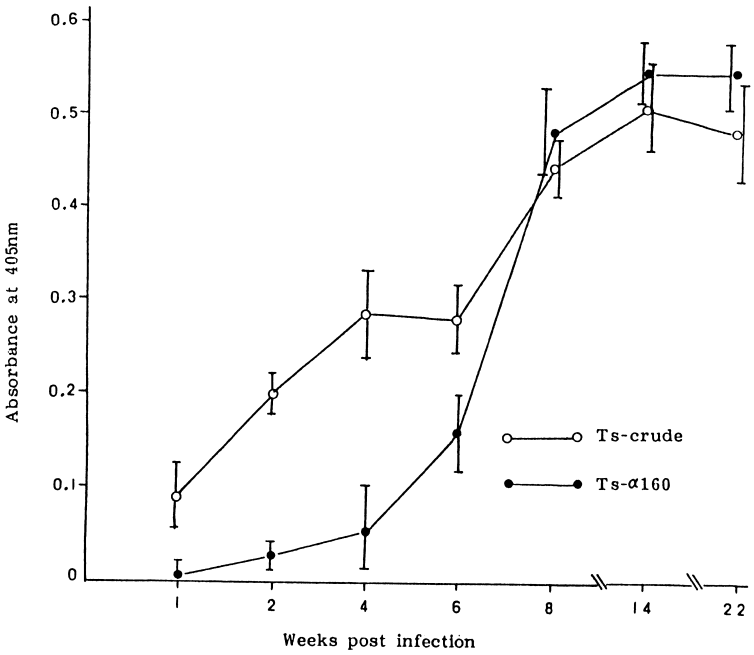
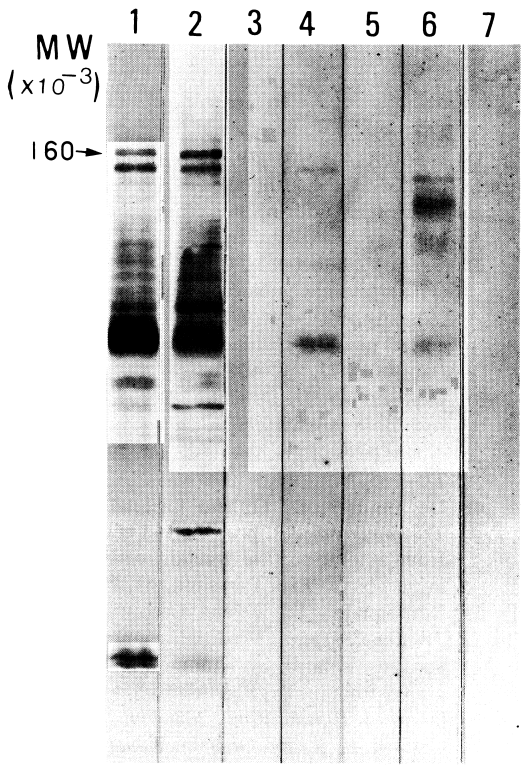


Fig. 2 Reactivity of Ts-crude and Ts-α160 against serum samples from mice infected with *T. spiralis*.



increased at 6–8 wk and responses remained with high titers until 22 wk PI. On the other hand, responses against Ts-crude became detectable as early as at 1 wk PI, and the increase in titers was observed from 6–8 wk as in the responses to Ts-α160. However, antibody responses to crude antigen tended to decrease gradually after 14 wk.

In addition, studies on immunocytolocalization of Ts-α160 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 (14 days PI) did not possess

Fig. 3 Detection of polysaccharide in Ts-α160 with lectin-peroxidase reagents.

Ts-S_3 was subjected to electrophoresis under reduced condition on a 10% SDS-polyacrylamid gel, and transferred to nitrocellulose membrane. The membrane was cut apart to each lane. Pieces of the membranes were stained by lectins, respectively. Lane 1, Concanavalin A (Con A)-peroxidase. Lane 2, Lentil lectin (LCA)-peroxidase. Lane 3, Peanut lectin (PNA)-peroxidase. Lane 4, *Phaseolus vulgaris* lectin (PHA-E4)-peroxidase. Lane 5, Caster bean lectin (RCA120)-peroxidase. Lane 6, Wheat germ lectin (WGA)-peroxidase. Lane 7, Peroxidase.

Table 2 Effects of passive transfer of a monoclonal antibody* against a 160kDa molecule of α-stichosome of *T. spiralis* muscle larvae on *T. spiralis* infection

	Control	Group 1	Group 2	Group 3
No. of worms recovered	18,007 ± 3,326	19,357 ± 3,176	17,990 ± 2,290	17,409 ± 2,159

*: Monoclonal antibody of IgG_1 class.

Passive immunization and recovery of worms from the muscle were performed as described in the text. Values are means ± standard error for a group of 5 mice.

this antigen. However, a marked increase of the antigen was observed in mature muscle larvae (21–35 days PI). The antigen was detected also in 180 days PI.

These results suggest that the 160kDa molecule is formed in the granules of the α-stichocyte of muscle larvae from 3 to 4 wk PI, and that the molecule is excreted or secreted from the granule, inducing production of antibodies from 5 to 6 wk PI. The titers of antibodies against Ts-α160 in human serum samples, which had been collected from 5 to 15 wk after infection, seemed to be higher than those of antibodies against Ts-crude in every case (data to be published).

The chlonology of class-specific antibody responses against *T. spiralis* muscle larvae has also been investigated by Takahashi *et al.* (1990a). They reported that IgG-class antibodies reacting to the cuticle surface, stichocyte granules and on esophagus-occupying substances were detected at 6 wk PI, while early responses were demonstrated against other anatomical structures such as the cuticle inner layer(s), hypodermis, hemolymph, intestinal gland cell granules, cytoplasmic granules in the cords and so on. They also reported that the cuticle surface of the muscle larvae shared antigenicity with stichocyte α-granules and the esophagus-occupying substance (Takahashi *et al.*, 1990b).

In our experiments, it was not clear whether MoAb-IgG_1 might react to the esophagus-occupying substance. However, it was evident by means of immunofluorescent staining, Western blot analysis (Niimura *et al.*, 1988) and analysis with a confocal laser fluoro-microscope (Fig. 2) that this antibody reacted only to the 160kDa antigen in α-stichocyte granules.

Ts-α160 consists of asparagin-linked polysaccharide chain and this antigen is a unique molecule for *T. spiralis,* because it is not detected in any other helminth (Niimura *et al.*, 1988). Consequently, the target molecule (160kDa) of the MoAb-IgG_1 exhibits the excellent antigenic properties in terms of immunodiagnosis of trichinosis.

Acknowledgement

The authors wish to thank Dr. Masashi Kobayashi for his technical help.

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