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Monoclonal Antibodies to *Trichinella spiralis* Muscle Larvae that Mimic Serum Antibody from Trichinellosis Mice and Discriminates Various Isolates of the Worm

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Abstract

Two *Trichinella spiralis* (Ts) specific monoclonal antibodies (MoAbs), designated TY10 and TY64, were established by fusing P3X63-Ag8.653 myeloma cells with spleen cells from BALB/c mice immunized with Ts muscle larval crude antigens. Based on the reaction patterns of Western immunoblotting analysis using TY10 as a probe, obtained with various isolates of Ts and *Trichinella pseudospiralis* (Tps) were classified into four groups. Group 1 consisted of the isolates from USA (U), Poland (P), Thailand (T), and China (C) and gave 4 major bands (42kD, 47kD, 65kD and 67kD) when specimens were prepared under reducing conditions using 2-mercaptoethanol; group 2 consisted of two Ts isolates from Japan ((Yamagata (Y) and Iwasaki (I)) with 3 major bands of 42kD, 47kD and 67kD; group 3 was the Scandinavian isolate (S) of Ts with 3 major bands of 42kD, 47kD and 66kD; and group 4 was Tps with 3 major bands of 39kD, 60kD and 70kD. Western immunoblotting patterns did not differ irrespective of the date of worm isolation or of the species of the worm-infected host. TY 64 mainly reacted with a 32kD antigen of all Ts isolates and Tps. The antigens recognized by TY10 were detected in whole stichocytes, on the surface and in the anterior portion of the gut. The Western immunoblotting pattern obtained with TY10 was quite similar to that obtained with the sera collected from mice 2 months or longer after Ts infection.

Key words: *Trichinella spiralis*, *Trichinella pseudospiralis*, isolates, monoclonal antibody, Western immunoblotting, immunohistochemistry

Introduction

Trichinellosis is difficult to diagnose by fecal examination because of the specific developmental history of the worm. At present, muscle biopsy appears to be the most reliable method. However, the amount of specimen that can be obtained is limited, and the procedure is painful. Development of a reliable immunoserological method would be of great benefit in the diagnosis of this infectious disease. However, cross-reactivity among heterologous parasites is one problem that must be first overcome (Kobayashi *et al.*, 1987). To this end, purification of Ts specific antigens has been attempted using MoAbs (Gamble and Graham, 1984; Niimura *et al.*, 1988). We also established Ts specific MoAbs to Ts muscle larvae (ML) that mimic Western immunoblotting patterns obtained with the sera of trichinellosis mice.

Materials and Methods

Parasites: ML, 5–12 weeks old, of 7 isolates of Ts and *T. pseudospiralis* (Tps) used for this series of experiments were as follows: ML of a Yamagata isolate (Y) from a racoon dog, *Nyctereutus procyonoides viverrinus* from Yamagata City, Japan (Saito and Yamaguchi, 1985); an Iwasaki isolate (I), from a Japanese black bear in Iwasaki Village, Aomori Prefecture, Japan; a USA isolate (U), from a pig; a Polish isolate (P), from a wild pig; a Thai isolate, from a human; a Polar isolate (S), from a

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polar bear imported from Scandinavia (through the courtesy of Emeritus Professor T. Yamaguchi, Hirosaki University School of Medicine, Fukumoto *et al.*, 1987); a Changchun isolate (C), from a dog in China (through the courtesy of Associate Professor Liu Zao-Min, Norman Bethune University of Medical Sciences, Changchun, China); and Tps, from a racoon in the Northern Caucasus region of USSR (through the courtesy of Emeritus Professor T. Yamaguchi mentioned above). All ML obtained from these isolates were maintained in our laboratory in ddY mice and were recovered from the muscle by digestion method using artificial gastric juice. The following parasites were prepared using a method reported elsewhere (Saito *et al.*, 1994): *Ascaris suum* (As), *Toxocara canis* (Tc), *Trichuris vulpis* (Tv), *Dirofilaria immitis* (Di), *Diphyllobothrium latum* (Dl), *Cysticercus cellulosae* (Cc), *Fasciola gigantica* (Fg), *Clonorchis sinensis* (Cs) and *Schistosoma japonicum* (Sj).

Preparation of crude antigens: We followed a method described elsewhere (Yamashita *et al.*, 1989).

Production of MoAbs: We followed methods described elsewhere (Rojekittikhun *et al.*, 1991). Briefly, spleen cells from BALB/c mice immunized with Y-ML crude antigens were fused with P3X63-Ag8.653 myeloma cells. Approximately 2 weeks after fusion, antibody in the supernatants was examined by ELISA. After limiting dilution was carried out 4 times, antibody producing hybridoma cell lines were established.

ELISA: Details were reported elsewhere (Yamashita *et al.*, 1989). Briefly, ELISA multiplates (Sumitomo Co. Ltd., Tokyo, Japan) were coated with 0.3 μg/100 μl/well with ML crude antigen solutions and were then blocked with 5% skim milk in PBS. After addition of first antibody, peroxidase-conjugated anti-mouse immunoglobulins (DAKO-PATTS, Denmark) were added. O-phenylene diamine was used as the substrate. OD values at 492 nm were measured with an automatic spectrophotometer (Flow Lab., U.S.A.).

SDS-PAGE and Western immunoblotting analysis: Details were reported elsewhere (Rojekittikhun *et al.*, 1991; Yamashita *et al.*, 1989). Briefly, crude antigens mixed with the equal volume of sample solution (0.01M Tris-HCl, 25% glycerin, 0.05% Bromophenol blue, 3% SDS) were treated at 100°C for 5 min in the presence or absence of 5% ß-mercaptoethanol (2ME). One μg of the treated antigens was applied to the gel consisting of 5% stacking gel and 12.5% separating gel or 5–15% gradient gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corp. MA, U.S.A.). The membrane was then blocked with 5% skim milk at 4°C, overnight and incubated with MoAbs at 37°C for 60 min. After washing, it was incubated at 37°C for 60 min with peroxidase-labeled anti-mouse immunoglobulins diluted 1:300. It was then washed and treated with a substrate solution consisting of 0.01% DAB, 0.01% H_2O_2 in 50 mM Tris-HCl (pH7.6) at room temperature.

Treatment of ML crude antigens with periodate or trypsin: Details were reported elsewhere (Rojekittikhun *et al.*, 1991). ELISA plates coated with crude antigens were treated with 1–5mM sodium metaperiodate. After oxidation with 50mM sodium acetate buffer (pH4.5) in a dark room for 60 min, the reaction was stopped by adding 20mM sodium sulfate. For trypsin treatment, crude antigen-coated ELISA plates were treated with trypsin at concentrations of 1–1,000 μg/ml for 30 min at room temperature. The reaction was then stopped with trypsin inhibitor (Type I-S: From Soybean, Sigma Chemical Co., MO, U.S.A.).

Enzyme-immunostaining of tissue sections on slides: Details were reported elsewhere (Takagi *et al.*, 1989). Specimens fixed in 10% formalin and embedded in paraffin were cut into 4 μm sections. After deparaffinization and rehydration, the sections were exposed to MoAbs at 37°C for 2 hrs, incubated with peroxidase-conjugated anti-mouse immunoglobulins (DAKOPATTS, Denmark) at 37°C for 2 hrs, exposed to 3,3'diaminobenzidine (DAB-H_2O_2) (Dojin, Kumamoto, Japan) and finally counterstained with hematoxylin.

Results

Establishment of MoAb producing hybridoma cell lines: Anti Ts antibody producing wells were selected by ELISA. Cells in 19 wells were cloned twice and the supernatants from each clone were examined by Western immunoblotting. Three groups of immunoblotting patterns were observed. Four

clones, designated TY10, TY20, TY28, and TY64, from the three groups were established after two further clonings. The immunoglobulin subclass of TY10, TY28 and TY64 was IgG1 and that of TY20 was IgM.

Immunological specificity of the 4 MoAbs: Using ELISA, we examined the reactivity of four MoAbs, TY10, TY20, TY28, and TY64, with the P-ML and 9 heterologous antigens (As, Tc, Tv, Di, Dl, Cc, Fg, Cs, and Sj). TY10 and TY64 reacted with the P-ML antigen whereas both TY20 and TY28 reacted not only with P-ML but also with all heterologous antigens except Cc and Sj (Fig. 1).

In the following experiments, we examined two Ts specific MoAbs, TY10 and TY64, for their reactivity.

Western immunoblotting analysis of P-ML with MoAbs TY10 and TY64: When immunoblotting of P-ML was performed, TY64 reacted with a major band at 32kD and 2 other minor bands at 25kD and 14kD whereas TY10 reacted with 4 major bands at 42kD, 47kD, 65kD and 67kD as well as with several minor bands (Fig. 2).

Sensitivity of Ts ML antigens to periodate and trypsin treatment in terms of reactivity with MoAbs TY10 and TY64: To study the properties of antigens reactive with MoAbs TY10 and TY64, ELISA plates coated with antigens were treated with trypsin or periodate and residual reactivity with MoAbs was examined. As shown in Fig. 3a, reactivity with

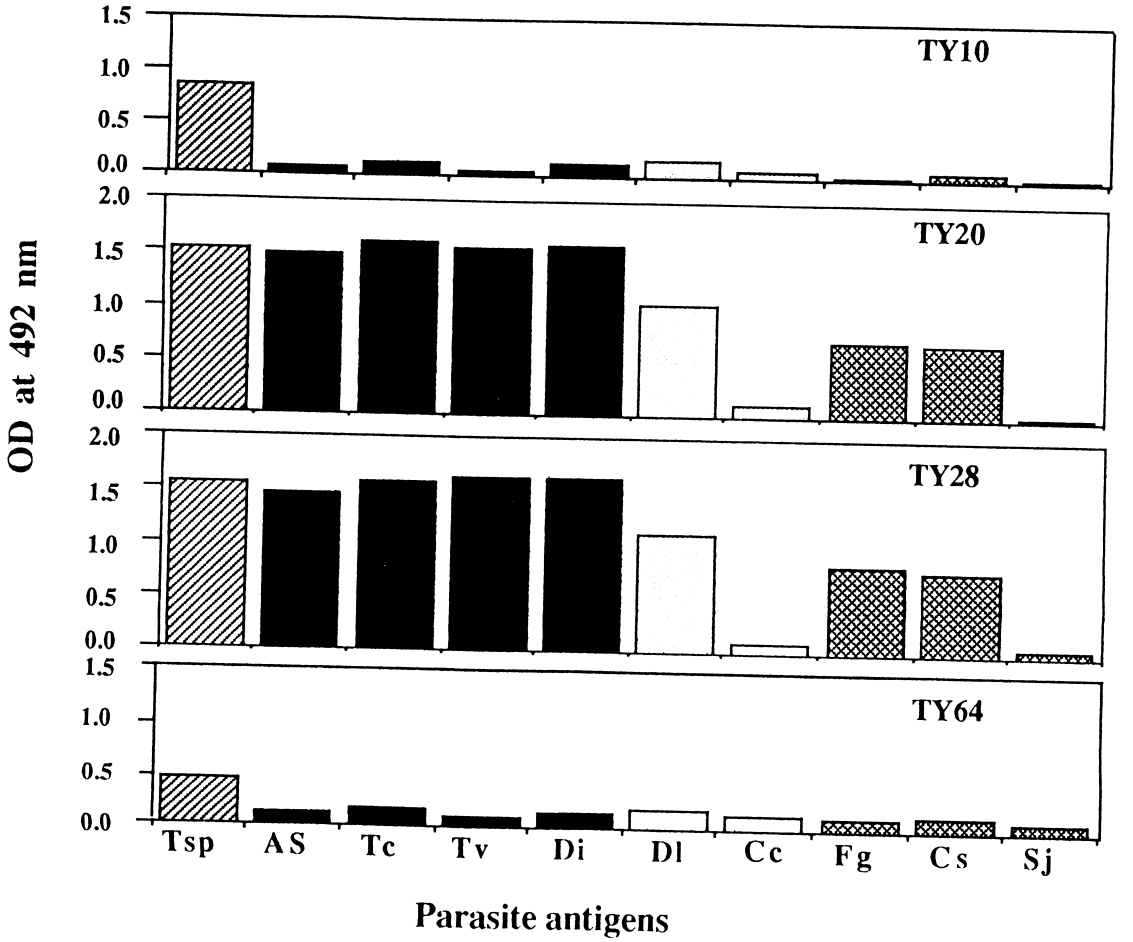

Fig. 1 Specificity of MoAbs to *T. spiralis* determined by ELISA. Tsp, *T. spiralis*; As, *A. suum*; Tc, *T. canis*; Tv, *T. vulpis*; Di, *D. immitis*; Dl, *D. latum*; Cc, *C. cellulosae*; Fg, *F. gigantica*; Cs, *C. sinensis*; Sj, *S. japonicum*.

TY64 decreased with trypsin treatment but that with TY10 was not affected. Reactivity with both TY10 and TY64 did not decrease after periodate treatment (Fig. 3b).

Localization of MoAb reactive antigens in Trichinella spiralis: We then examined the localization of MoAb reactive antigens in the worm. TY10 stained whole stichocytes, the surface of the worm, and the anterior portion of the gut (Fig. 4). However, no parts of the worm were stained with TY64.

Similarity of Western immunoblotting patterns between MoAbs and sera from trichinellosis mice. Western immunoblotting patterns were compared between those of TY10 and those of the sera from Ts infected mice obtained 1, 2, 4, 6, and 12 months PI. As shown in Fig. 5, TY10 showed a pattern almost identical with that of sera obtained at least 2 months PI. The 32kD band observed with TY64 was also seen with the sera collected more than 4 months PI.

Comparison of Western immunoblotting patterns among the 7 isolate and Tps antigens recognized with TY10 and TY64: 2 ME treated or untreated antigens obtained from 7 isolates of Ts and Tps were examined for their reactivity with TY10 and TY64 using Western immunoblotting. The reaction patterns obtained with TY10 using 2 ME untreated antigens, were classified into 4 groups. U, P, C, and T gave main bands of 42kD, 47kD, and 160kD; Y and I, of 44kD, 47kD, and 140kD; S, of 44kD, 47kD, and 135kD; and Tps, of 39kD and 60kD, respectively (Fig. 6a). 2 ME treated antigens were also able to be classified in the same 4 groups, although the M.W. of bands in each group were different from those observed in the 2 ME untreated preparations; that is, U, P, C, and T gave main bands of 42kD,

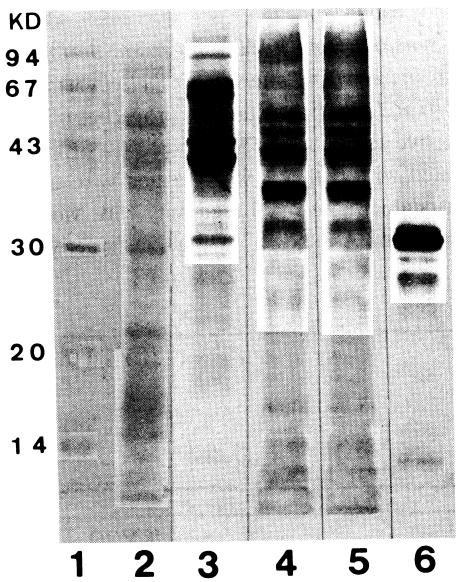

Fig. 2 Western immunoblotting analysis of *T. spiralis* ML antigen with anti-*T. spiralis* MoAbs, TY10 (lane 3), TY20 (lane 4), TY28 (lane 5), and TY64 (lane 6). Molecular weight markers (lane 1) and the antigen (lane 2) were stained by CBB.

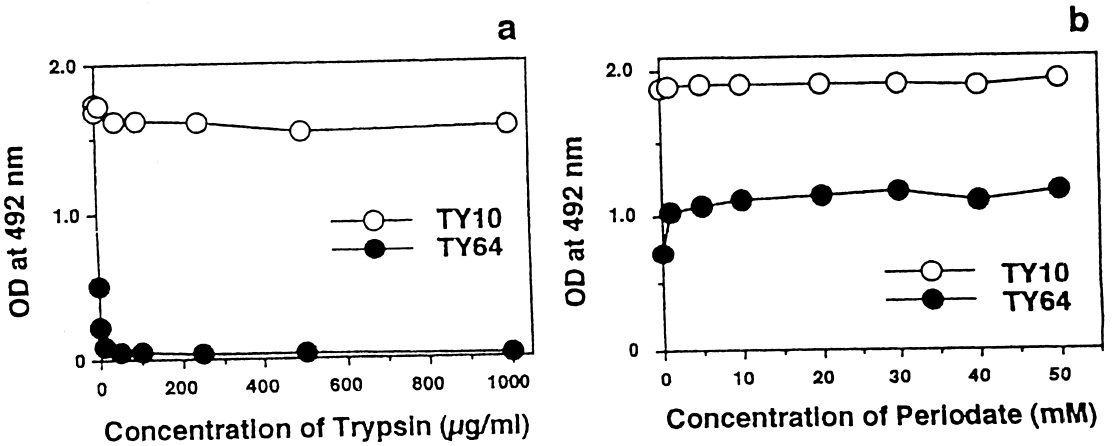

Fig. 3 Sensitivity to trypsin (a) and periodate (b) treatment of ML antigens in terms of reactivity with MoAbs TY10 and TY64. For details of trypsin and periodate treatment, see Materials and Methods.

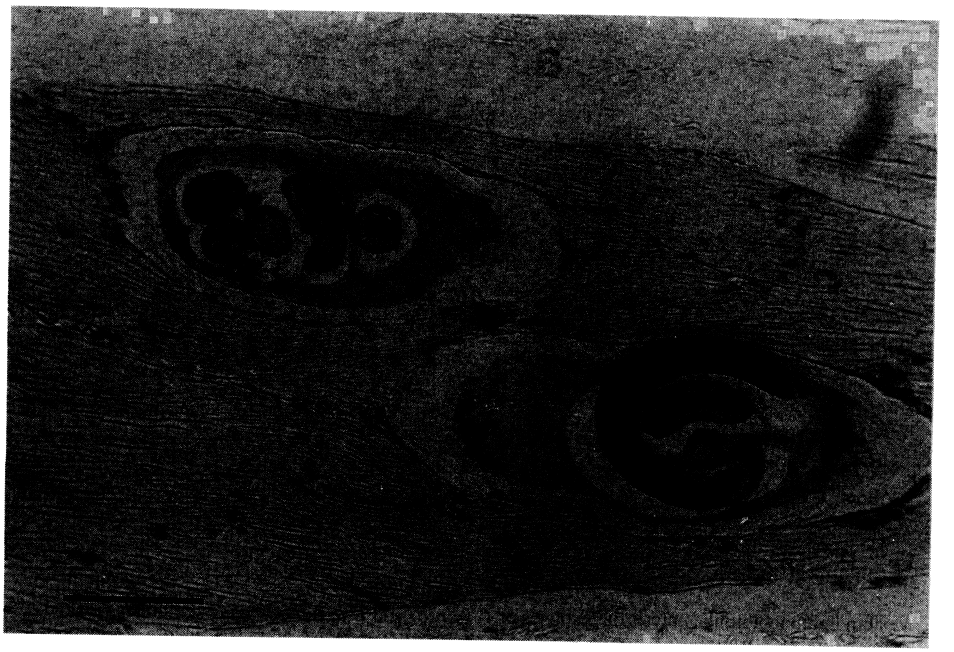

Fig. 4 Immunostaining of *T. spiralis* muscle larvae with a MoAb, TY10. Whole stichocytes, the surface and former portions of the gut were stained. Scale bar = 100 μm.

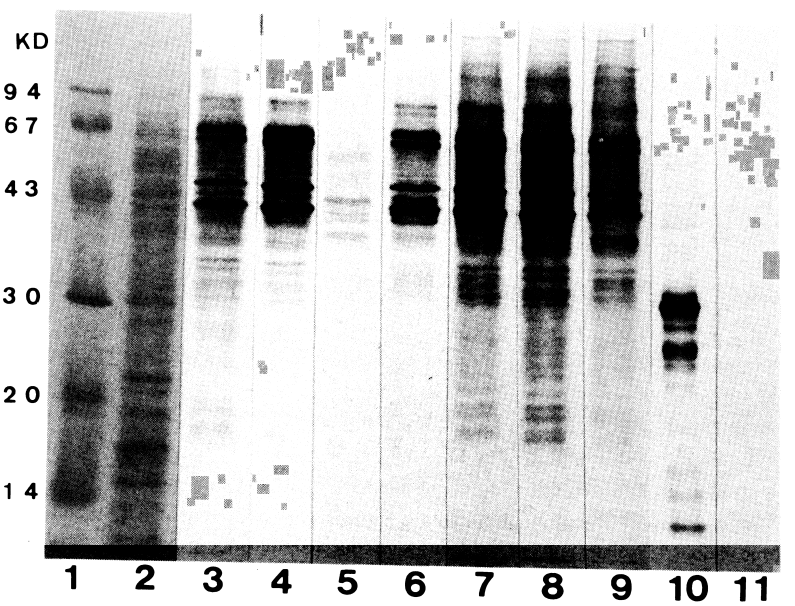

Fig. 5 Western immunoblotting analysis of *T. spiralis* ML antigen with anti-*T. spiralis* MoAbs, TY10 and *T. spiralis* infected sera. Lane 1 shows molecular weight markers. The antigen was stained by CBB (lane 2), treated with undiluted TY10 culture supernatant (lane 3) and TY10 ascites (1:1,000, lane 4), mouse sera (1:200) at 1 (lane 5), 2 (lane 6), 4 (lane 7), 6 (lane 8), and 12 (lane 9) months PI, TY64 culture supernatant (lane 10) and normal mouse serum (1:200, lane 11).

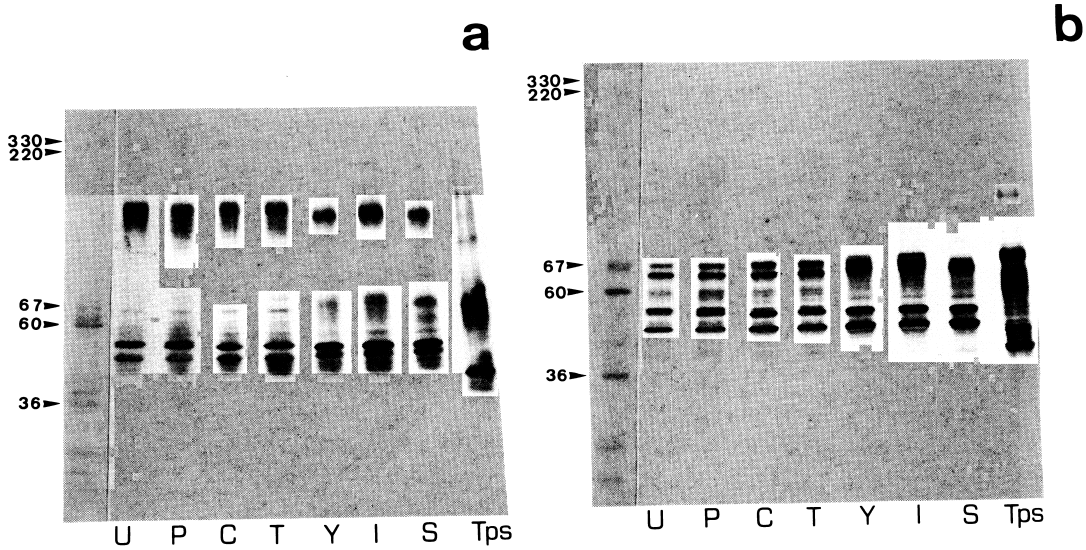

Fig. 6 Western immunoblotting analysis of 7 isolates of *T. spiralis* and *T. pseudospiralis* ML antigens treated in the absence (a), and presence of 2ME (b) using anti-*T. spiralis* MoAb, TY10. One μg each of treated antigens (U, P, C, T, Y, I, S and Tps) was applied to the gradient gel. The two leftmost lanes show high molecular weight markers stained by CBB.

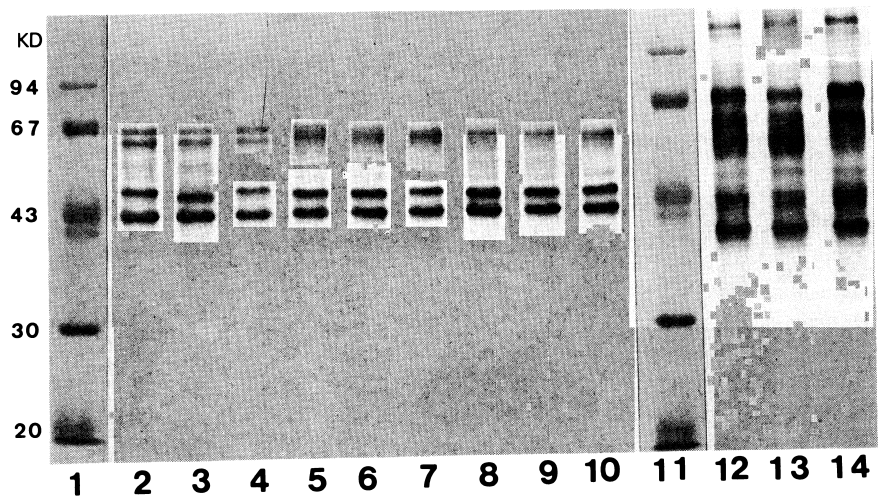

Fig. 7 Comparison of Western immunoblotting patterns among different lots of ML antigens obtained from various Ts isolates and Tps. Antigens were collected from mouse muscle every 1.5 months PI on various dates: P on Jun. 15, 1991 (lane 2), Jan. 14, 1992 (lane 3) and May 26, 1992 (lane 4); Y on Mar. 21, 1990 (lane 5), Dec. 18, 1991 (lane 6) and May 26, 1992 (lane 7); S on Mar. 21, 1990 (lane 8), Jun. 15, 1992 (lane 9) and May 26, 1992 (lane 10); and Tps on Mar. 21, 1990 (lane 12), Jun. 15, 1991 (lane 13) and Jun. 16, 1992 (lane 14).
Both lanes 1 and 11 show molecular weight markers stained by CBB.

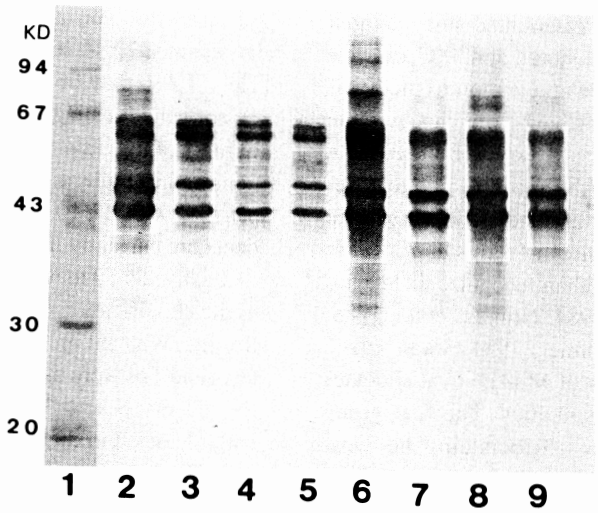

Fig. 8 Comparison of Western immunoblotting patterns of various Ts isolates obtained from different species of animals. Polish (lanes 2–5) and Yamagata (lanes 6–9) ML antigens were collected from mice (lanes 2, 6), rats (lanes 3, 7), hamsters (lanes 4, 8) and guinea pigs (lanes 5, 9). Lane 1 shows a molecular weight marker stained by CBB.

47kD, 65kD, and 67kD; Y and I, of 42kD, 47kD, and 67kD; S, of 42kD, 47kD, and 66kD; and Tps, of 39kD, 60kD, 70kD, respectively (Fig. 6b). Another MoAb, TY64, recognized a major band at 32kD in all 7 isolates and Tps in the presence or absence of 2 ME (data not shown).

Comparison of Western immunoblotting patterns among various lots of antigens obtained from mice or antigens from 4 species of infected hosts: In order to examine whether Western immunoblotting patterns of various isolates are stable, irrespective of the date of infection, patterns of worms isolated under various conditions were compared using TY10 as a probe. Western immunoblotting patterns of each isolate collected on various days were the same as shown in Fig. 7. Each isolate of ML antigen (P, Y) collected from mice, rats, hamsters, and guinea pigs 1.5 months PI also showed the same patterns (Fig. 8).

Discussion

We established 2 MoAbs which specifically react with Ts and characterized the molecular properties and localization of the corresponding antigens in the worm. With respect to MoAbs to Ts, several studies have been conducted from the stand point of protective immunity (Silberstein and Despommier, 1984; Despommier and Laccetti, 1981; Appleton *et al.*, 1988), localization of reactive antigens (Gamble and Graham, 1984; Niimura *et al.*, 1988; Ortega-Pierres *et al.*, 1984; Despommier and Muller, 1976; Capo *et al.*, 1986; Mclaren *et al.*, 1987), and molecular properties of reactive antigens (Gamble and Graham, 1984; Niimura *et al.*, 1988; Despommier and Laccetti, 1981; Appleton *et al.*, 1988; Ortega-Pierres *et al.*, 1984; Philipp *et al.*, 1980). All reports, except for those of Niimura *et al.* (1988) and Silberstein and Despommier (1984), demonstrated that MoAbs react with molecules of various sizes, which is consistent with our present results. Although the reasons for this phenomenon are still obscure, one possibility is that these MoAbs may recognize the same antigenic determinants in different molecules. The diversity of bands reacting with TY10 and TY64 can not be ascribed to the carbohydrate nature of the determinants because the corresponding antigens were resistant to periodate treat-

ment. Degradation of molecules during antigen preparation may also be discounted since antigen was extracted below 4°C, stored at –20°C and prepared immediately before application to the SDS-PAGE gel. TY10 reacts with whole stichocytes, the worm surface, and portions of the gut (Fig. 4). As for the localization of MoAbs reactive antigens reported previously, some antibodies detect surface antigens of the worm (Ortega-Pierres *et al.*, 1984; Mclaren *et al.*, 1987) and others recognize stichocytes (Gamble and Graham, 1984; Niimura *et al.*, 1988; Silberstein and Despommier, 1984; Capo *et al.*, 1986). There are reports of MoAbs to stichocytes with three different specificities. The first group recognizes ß-stichocyte (Silberstein and Despommier, 1984; Capo *et al.*, 1986); the second, α-stichocyte (Niimura *et al.*, 1988; Silberstein and Despommier, 1984; Capo *et al.*, 1986); and the third, whole stichocytes (Gamble and Graham, 1984). The specificity of TY10 seems to be similar to that reported by Gamble and Graham (1984), in that both react with whole stichocytes, however, inasmuch as they do not report in their paper that the MoAb reacts with the surface and the gut, it remains uncertain whether these MoAbs recognized the identical determinant. The finding that TY10 reacts not only with the stichocytes but also with the surface and the gut is not surprising since it has been reported that the 50/55kD antigen detected in α-stichocytes is occasionally observed in the gut or on the cuticle (Silberstein and Despommier, 1984).

The fact that reactivity to TY64 decreased with trypsin treatment suggests that the antigenic determinant recognized by this MoAb is protein in nature. On the other hand, reactivity to TY10 was not affected by either trypsin or periodate treatment. This may have been because the antigenic determinant was too small to be cleaved by trypsin. This may also explain the finding that many bands appeared in Western immunoblotting using TY10. We obtained similar results in our previous experiments with autoreactive MoAbs produced from spleen cells of parasite infected mice (Saito *et al.* 1994).

Our present results that Western immunoblotting patterns with TY10 and with sera collected at least 2 months PI were almost identical, is interesting in two respects. 1) It suggests that TY10 may recognize the major antigens detected by antibodies in Ts infected hosts. 2) Stichocytes may produce antigens which are the main targets recognized by Ts infected hosts, since TY10 recognizes a stichocyte antigen. Thus, TY10 may be a useful tool in purification of Ts antigens for diagnosis of human trichinellosis. Using Ts antigens purified in this manner, examinations of the similarities of Western immunoblotting patterns with TY10 and sera of trichinellosis patients are currently in progress.

Lastly, TY10 may be useful from the standpoint of the classification of Ts isolates. As shown in the Results, Western immunoblotting patterns of 7 isolates and Tps were divided into 4 groups, that is, U-P-C-T, Y-I, S and Tps, all of which were consistently detected in different antigen lots or host species. The present results are in good agreement with the previous reports showing that soluble protein profiles and isozyme patterns could be divided into these same 4 groups (Fukumoto *et al.*, 1987) and our previous report that a reproductive disturbance was observed among these groups (Saito and Sendo, 1989). Taken together, Ts isolates used for our experiments may consist of 3 species.

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