A panel of antigens of muscle larvae of *Trichinella spiralis* and *T. pseudospiralis* as revealed by two-dimensional Western blot and immunoelectron microscopy

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SUMMARY

This study characterized antigens of *Trichinella spiralis* and *T. pseudospiralis* muscle larvae recognized by mice infected with the worms. Two-dimensional (2-D) Western blot analysis revealed some profile of antigenic peptides including: (1) molecular weight (MW); (2) isoelectric points (pI), (3) reactivity to well-defined monoclonal antibodies (mAb) and (4) cross-reactivity between the 2 species. Antigenic peptides of *T. spiralis* consisted of about 100 spots. The MW ranged from 22 to 80 kDa, and pI ranged from 4 to 7. The mAb against TSL-1 stained most of the *T. spiralis* excretory–secretory (E–S) peptides migrating at 40, 45 and 50 kDa, and the mAb against TSL-4 stained non-E–S peptides. Antigenic peptides of *T. pseudospiralis* consisted of about 20 to 30 peptide spots. The MW ranged from 25 to 80 kDa, and pI ranged from 4 to 7. The mAb against TSL-1 stained most of the *T. pseudospiralis* E–S peptides migrating at 35 and 45 kDa, and the mAb against TSL-4 stained non-E–S peptides. Two-dimensional Western blots showed that the E–S products of *T. spiralis* and *T. pseudospiralis* were highly cross-reactive with each other. The non-E–S peptides were, however, not recognized by *T. pseudospiralis*-infected sera but were recognized by *T. spiralis*-infected sera. An immunoelectron microscopical study showed the similar result that stichocyte granules and cuticle surface (known to contain E–S antigen) had cross-reactive antigens between the two species. *T. pseudospiralis*-infected sera stained very weakly the cuticle inner layers and haemolymph (known to contain non-E–S antigen). This evidence implies that mice infected with *T. pseudospiralis* do not evoke antibodies against non-E–S antigen at the detectable level.

Key words: *Trichinella spiralis*, *Trichinella pseudospiralis*, antigen, two-dimensional Western blot, electron microscopy.

INTRODUCTION

The need to characterize antigens of *Trichinella* is 3-fold: firstly to identify target antigens of protective immunity; secondly to select the best antigens for an immunodiagnostic method and thirdly to identify peptides that may play a crucial role to transform muscle cells after the entrance of muscle cells by the parasite.

Trichinella spiralis muscle larvae have dominant antigens which are synthesized in the stichosome. These are excreted through the oesophagus and gut, and absorbed by the cuticle surface at least in part. The host immune response during *T. spiralis* infection is directed against this stichosome/surface antigen (Ortega-Pierres *et al*. 1996) and phosphorylcholine (PC) (Takahashi *et al*. 1993).

T. spiralis and *T. pseudospiralis* are independent species in the genus *Trichinella*. The former forms a typical cyst but the latter forms a very poor cyst (Xu *et al*. 1997). Since E–S products are transported to the nucleus of the nurse cell (Despommier *et al*. 1990; Lee *et al*. 1991; Vassilatis *et al*. 1992; Ko *et al*. 1994), E–S peptides received a great deal of attention. In fact *T. spiralis* and *T. pseudospiralis* share similarity, but not identity in peptide maps of E–S products, morphology of stichocyte granules (origin of the E–S products) and expression of messenger RNA encoding E–S peptides is concomitant to such phenotypic difference and similarity (Garate & Rivas, 1987; Wu, Nagano & Takahashi, 1998). Only 3 peptides of E–S products of 45 kDa are shared by the species, which may be responsible for the shared phenotype. Vassilatis *et al*. (1996) also suggested the 43 kDa glycoprotein (probably equivalent to our 45 kDa glycoprotein) may play a common role in the life-cycles of these two.

This study further characterized antigens of *Trichinella* using 2-D Western blot and immunoelectron microscopy. The parameters for characterization of antigens included MW, pI, localization, cross-reactivity and reactivity to the well-defined monoclonal antibodies, and thus provided comprehensive data about antigens of *T. spiralis* and *T. pseudospiralis* muscle larvae.

MATERIALS AND METHODS

Parasites, crude extracts and E–S products

Muscle-stage larvae of *T. spiralis* (ISS413) and *T. pseudospiralis* (ISS13) were isolated by pepsin–HCl digestion from mice 1 month after the infection. E–S

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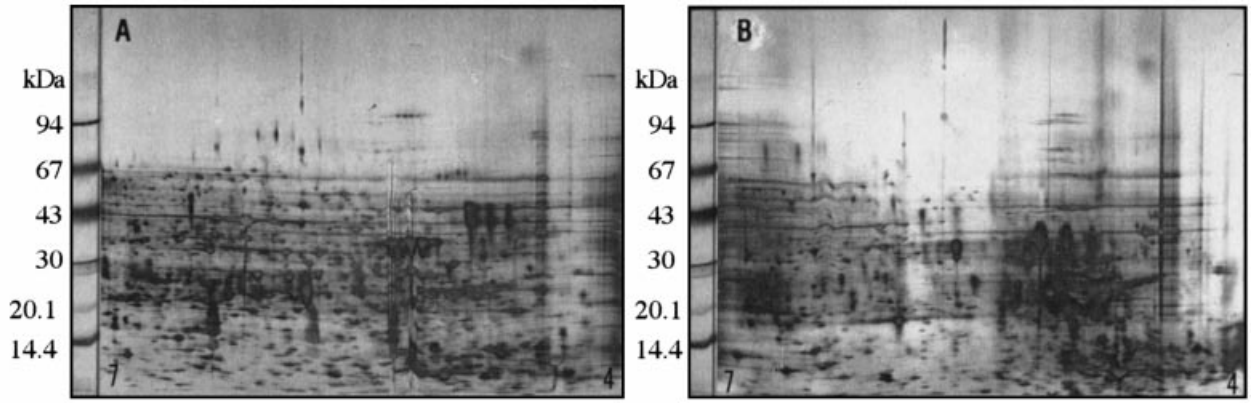

Fig. 1. The peptide maps of muscle larval crude extracts from *Trichinella spiralis* (A) and *T. pseudospiralis* (B) visualized by silver staining after 2-dimensional electrophoresis (pI ranging from 4 to 7).

products and larval crude saline extracts were prepared by the conventional methods (Wakelin *et al*. 1994; Wu *et al*. 1998).

Infected sera and monoclonal antibodies

Infected sera were obtained from mice infected with 200 larvae of *T. spiralis* or *T. pseudospiralis* after 3 months of the infection. MAbs 9E6 (against TSL-1 antigen) and 305 (against TSL-2 antigen) are kind gifts from Dr Appleton (Cornell University) and Ts2 (against TSL-4 antigen) is from Dr Lim (Chinese University of Hong Kong).

Two-dimensional electrophoresis

Two-D electrophoresis was done adapting Multiphor II (Pharmacia, Uppsala, Sweden) as reported previously (Wu *et al*. 1998). For silver staining, 50 μg of crude antigens were used. For Western blot, 100 μg of crude antigen or 30 μg of E–S antigen were loaded.

Silver staining

The silver staining was performed by our method published elsewhere (Wu *et al*. 1998), which was originally described by Henkeshoven & Dernicl (1985).

Western blot

The semi-dry transfer technique was employed in Western blot with Multiphor II NovaBlot electrophoretic transfer unit and MultiDrive XL 3·5 kV power supply (Pharmacia, Uppsala, Sweden). After the second dimension running, the gel was separated from a support film using Film Remover (Pharmacia, Uppsala, Sweden). The blot was carried out using an electrophoretic buffer (25 mM Tris, 192 mM glycine, 0·01 % SDS and 20 % (v/v) methanol, pH 8·3) and run under the condition of 0·8 mA/cm^2 of the trans unit for 1 h. After transfer, the nitrocellulose membrane was blocked with 5 % skim milk in Tris–borate saline buffer (TBS) for 1 h at room temperature (RT), and incubated with the primary antibody, diluted to the appropriate titre, in 5 % skim milk in TBS for 1 h at RT, or overnight at 4 °C.

The primary antibody included either *T. spiralis*-infected sera, *T. pseudospiralis*-infected sera, or the mAbs. E–S products and crude extracts from both species were reacted with these primary antibodies.

The membrane was washed 4 times in TBS with 0·3 % Tween–20 for 5 min each time, and then incubated with alkaline phosphatase-conjugated secondary antibodies in TBS for 1 h at RT and washed again. The membrane was treated with developing solution (0·1 M Tris, 0·1 M NaCl, 0·01 M $MgCl_2$, 0·15 mg/ml BCIP (5-bromo-4-chloro(3 indolyl) (10-toluidine) salt) and 0·3 mg/ml NBT (nitroblue tetrazolium), pH 9·5) for approximately 8 min, and the reaction was then terminated with a stop solution (0·02 M Tris, 1 mM EDTA, pH 2·9). Finally the membrane was washed with distilled water and dried.

Immunoelectron microscopy

Muscle larvae were processed for immunoelectron microscopy according to the method described previously (Takahashi *et al*. 1990). In brief, the isolated worms were fixed and embedded in LR White. Ultrathin sections were made and subjected to immunostaining. To detect cross-reactive antigen, *T. spiralis* sections were immunostained with *T. pseudospiralis*-infected sera (1:100 dilution), and the *T. pseudospiralis* sections were immunostained with *T. spiralis*-infected sera (1:100 dilution). The mAbs were used to detect each antigen.

RESULTS

Peptide map of crude extracts of T. spiralis *and* T. pseudospiralis

There were numerous peptide spots in crude extracts from muscle larvae of *T. spiralis* (Fig. 1A) and *T. pseudospiralis* (Fig. 1B). The spots ranged from 10 to 90 kDa in MW and from 4 to 7 in pI,

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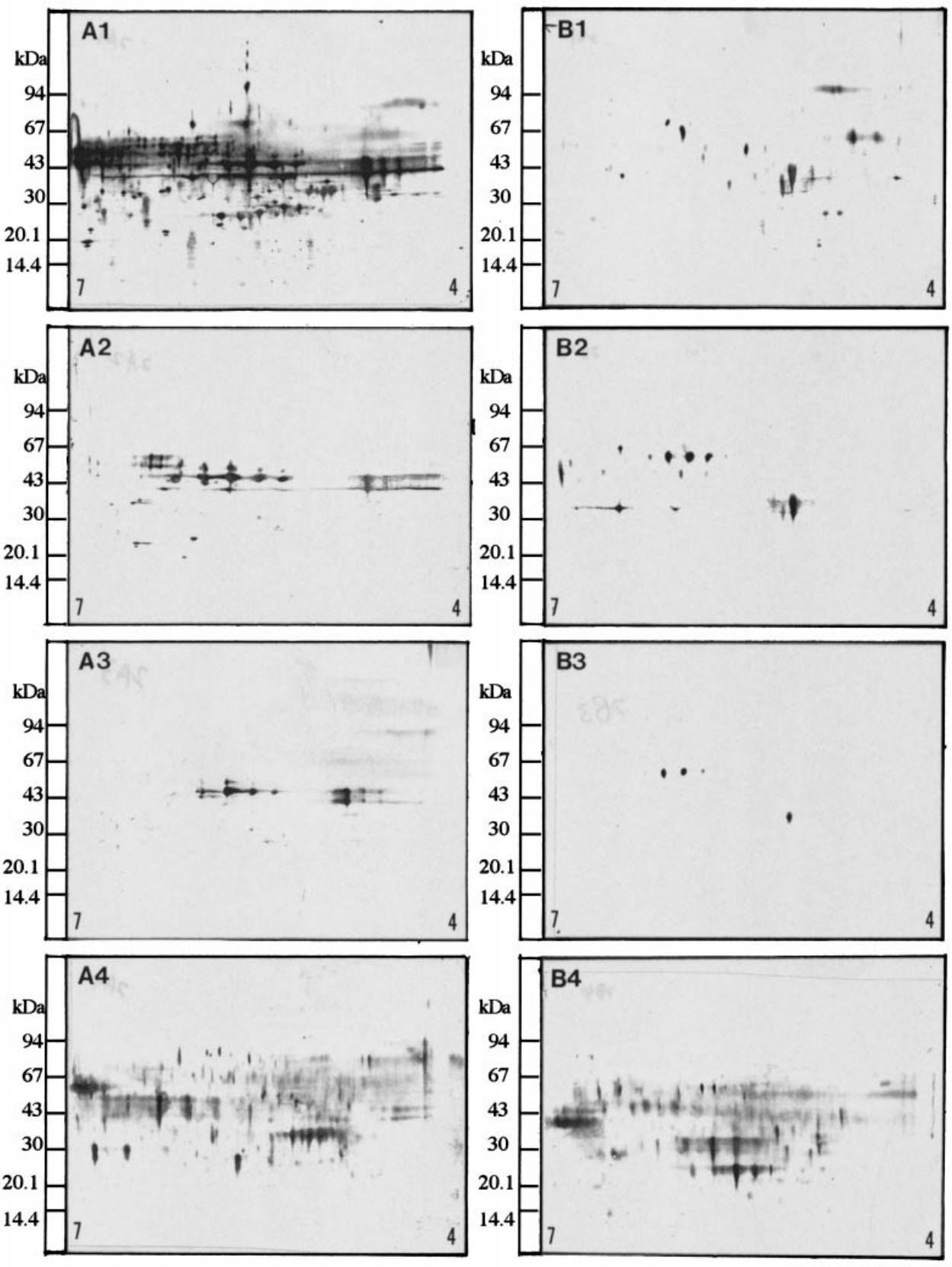

Fig. 2. Western blot analysis after the 2-dimensional electrophoresis (pI ranging from 4 to 7) of larval crude extracts. *Trichinella spiralis* specimens stained with *T. spiralis*-infected sera (A1), anti-TSL-1 mAb (A2), anti-TSL-2 mAb (A3), and anti-TSL-4 mAb (A4). *T. pseudospiralis* specimens stained with *T. pseudospiralis*-infected sera (B1), anti-TSL-1 mAb (B2), anti-TSL-2 mAb (B3), and anti-TSL-4 mAb (B4).

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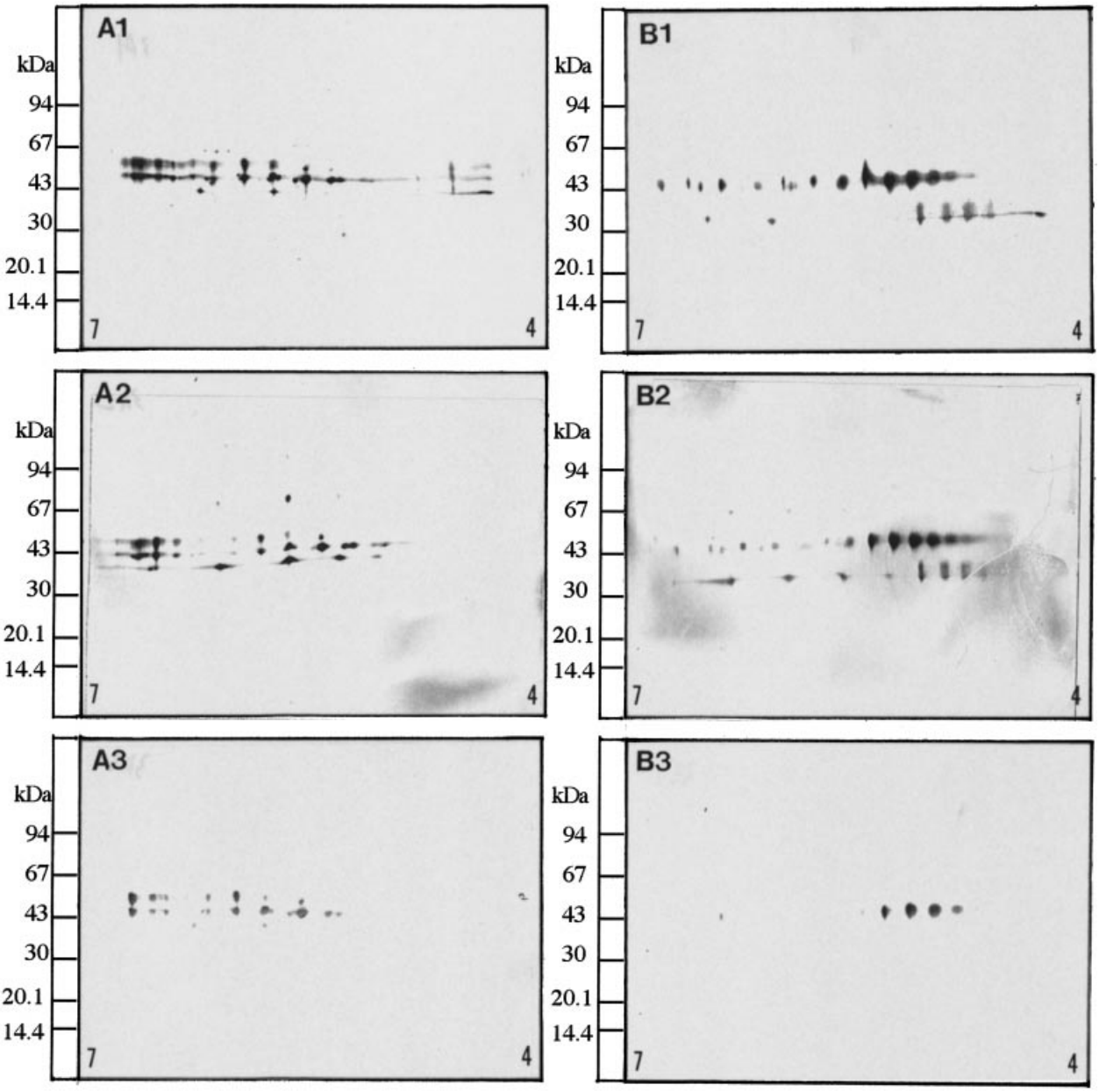

Fig. 3. Western blot analysis after the 2-dimensional electrophoresis (pI ranging from 4 to 7) of larval E–S products. *Trichinella spiralis* specimens stained with *T. spiralis*-infected sera (A1), anti-TSL-1 mAb (A2), and anti-TSL-2 mAb (A3). *T. pseudospiralis* specimens stained with *T. pseudospiralis*-infected sera (B1), anti-TSL-1 mAb (B2), and anti-TSL-2 mAb (B3).

although some of peptides may not be detected by this method. The peptide map of larval E–S products of both species was reported previously (Wu *et al.* 1998).

Western blot analysis of crude extracts from T. spiralis *muscle larvae*

Among the numerous peptide spots shown in Fig. 1A, only some of them (about 100) were positively stained with infected sera. These antigenic peptides migrated at 28–80 kDa as shown in Fig. 2A1. Among them, about 30 spots migrating at 28, 40, 45, 50 and 55 kDa were positively stained with anti-TSL-1 mAb (Fig. 2A2). Anti-TSL-2 mAb recognized fewer spots (about 10 spots migrating at 40, 45 50 kDa as shown in Fig. 2A3) than the anti-TSL-1 mAb. This notion is concomitant with the immunoprecipitation data (Appleton *et al.* 1991).

Anti-TSL-4 mAb positively stained many peptides migrating at 25–85 kDa (Fig. 2A4). The recognized spots did not correspond to peptides from E–S products (compare with Fig. 3A1).

Western blot analysis of crude extracts from T. pseudospiralis *muscle larvae*

Among numerous peptide spots in crude extracts from *T. pseudospiralis* muscle larvae only some were antigenic (20–30 spots as shown in Fig. 2B1). The

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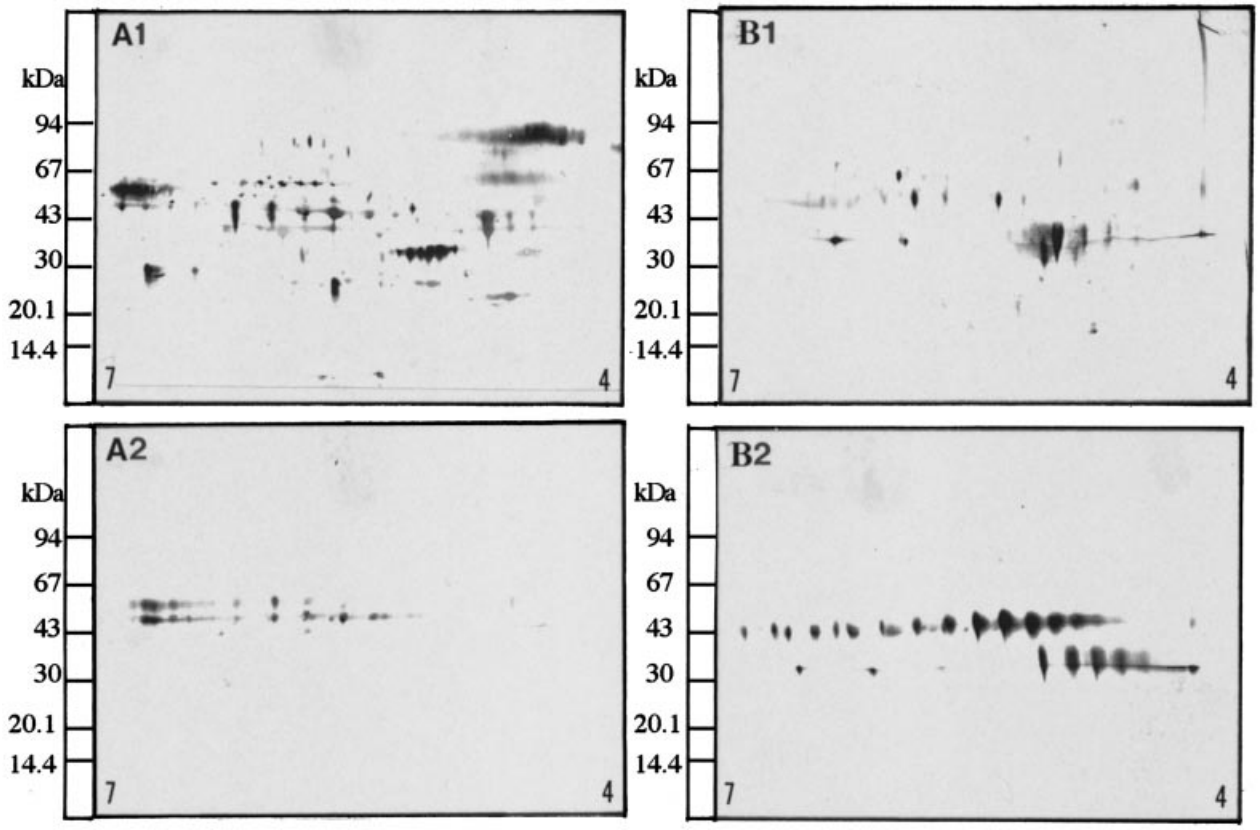

Fig. 4. Two-D Western blot analysis (pI ranging from 4 to 7) of cross-reaction among antigens of *Trichinella spiralis* and *T. pseudospiralis*. The crude extract (A1) and E–S extract (A2) from *T. spiralis* muscle larva were stained with *T. pseudospiralis*-infected sera. The crude extract (B1) and E–S extract (B2) from *T. pseudospiralis* muscle larva were stained with *T. spiralis*-infected sera.

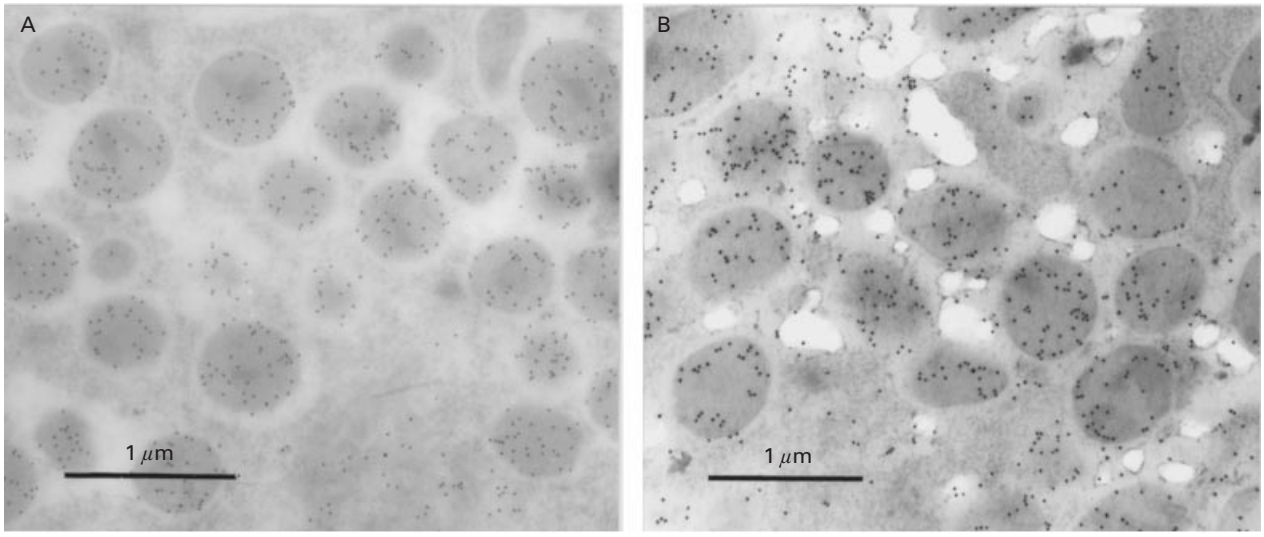

Fig. 5. (A and B) Immunoelectron micrographs showing cross-reactive antigens between the two species. (A) *Trichinella spiralis* section immunostained with *T. pseudospiralis*-infected sera showing intense staining on stichocyte granules. (B) *T. pseudospiralis* section immunostained with *T. spirals*-infected sera showing intense staining on stichocyte granules.

number of antigenic spots was apparently less than that of *T. spiralis*. These antigenic peptides migrated at 25–80 kDa, and most of them seemed to be E–S peptides (compare with Fig. 3B1). Similar spots (about 15) migrating at 35, 45 and 60 kDa were stained with anti-TSL-1 mAb (Fig. 2B2).

Anti-TSL-2 mAb stained a smaller number (4 spots) of peptides migrating at 35 and 60 kDa (Fig. 2B3). Anti-TSL-4 mAb recognized many peptides migrating at 25–60 kDa as shown in Fig. 2B4. These peptides were hardly stained with *T. pseudospiralis*-infected sera (Fig. 2B1).

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Western blot analysis of E–S products from T. spiralis *muscle larvae*

Fig. 3A1 shows antigenic peptide map of E–S products of *T. spiralis* recognized by *T. spiralis*-infected sera. Most of the peptides from E–S products are antigenic. Examples include spots migrating at 40, 45 and 50 kDa. These spots were positively stained with anti-TSL-1 and TSL-2 mAbs (Fig. 3A2 and A3, respectively). Anti-TSL-4 mAb failed to stain any peptides from E–S products.

Western blot analysis of E–S products of T. pseudospiralis *muscle larvae*

Fig. 3B1 shows antigenic peptide map of E–S products of *T. pseudospiralis*, recognized by *T. pseudospiralis*-infected sera. Most of the peptides from E–S products are antigenic. Examples include the spots migrating at 35 and 45 kDa. Anti-TSL-1 mAb stained most of 35 and 45 kDa peptides (Fig. 3B2), and anti-TSL-2 mAb only stained four 45 kDa spots of pI 5–5·5 (Fig. 3B3). Anti-TSL-4 mAb failed to stain any spots.

Western blot analysis of cross-reactive antigens

To check cross-reaction, *T. spiralis* antigens were immunostained with *T. pseudospiralis*-infected sera, and *T. pseudospiralis* antigens were immunostained with *T. spiralis*-infected sera. When the 2-D Western blot analysis results were compared between *T. spiralis* peptide spots immunostained with *T. spiralis*-infected sera and those with *T. pseudospiralis*-infected sera, some difference was noticed; that is, low MW peptide spots in *T. spiralis* crude extracts (mainly non-E–S peptides) were recognized by *T. spiralis*-infected sera (Fig. 2A1), but not recognized by *T. pseudospiralis*-infected sera (Fig. 4A1).

No essential difference was, however, noticed in cross-reactivity as to the E–S products of *T. spiralis* (compare Fig. 3A1 with Fig. 4A2), of the *T. pseudospiralis* crude extracts (compare Fig. 2B1 with Fig. 4B1), and of the E–S products of *T. pseudospiralis* (compare Fig. 3B1 with Fig. 4B2).

Three 45 kDa peptides (pI 5·88, 6·04, 6·54) shared by *T. spiralis* and *T. pseudospiralis* (Wu *et al*. 1998) were cross-reactive (Fig. 4A2 and B2) and also immunostained with anti-TSL-1 antibody.

Immunoelectron microscopy

The following structures in muscle larvae of *T. pseudospiralis* were positive for immunostaining with *T. pseudospiralis*-infected sera. Strongly positive staining was observed on structures that contain E–S antigens; cuticle surface, stichocyte granules, oesophagus occupying substance and brush border. These structures were also positively stained with the mAbs 9E6 and 305 (data not shown).

Very weakly positive, or no staining, was observed on structures that contain non-E–S antigen; cuticle inner layers, glycogen area and haemolymph. These structures were also positively stained with the mAb Ts2 (data not shown).

Thus the spectrum of structures recognized by infected sera was very similar to that reported for *T. spiralis* (Takahashi *et al*. 1990), although there were obvious differences in the intensity of immunostaining.

The structures with E–S antigens were cross-reactive because those structures in *T. spiralis* sections were positively immunostained with *T. pseudospiralis*-infected sera (Fig. 5A) and those in *T. pseudospiralis* sections also were positively immunostained with *T. spiralis*-infected sera (Fig. 5B).

DISCUSSION

T. spiralis *antigens*

Although there are numerous peptides in larval crude extracts of *T. spiralis*, only some of them are antigenic (about 100 spots). These antigenic peptides have a wide range of MW (22–80 kDa) and pI (4–7), and group I and group II antigens (Denkers *et al*. 1991) are likely included among these peptides. The former is recognized by anti-TSL-1 antibody and apparently the peptides in E–S products (Denkers *et al*. 1991). The latter is recognized by anti-TSL-4 antibody and is somehow related to phosphorylcholine (PC).

The E–S products of *T. spiralis* have mainly 3 kinds of antigenic proteins, migrating at about 40, 45 and 50 kDa, when analysed by 1-D Western blot (Gamble & Murrell, 1986; Appleton, Schain & McGregor, 1988; Bolas-Fernandez & Wakelin, 1990; Zhang, Lee & Smith, 1993). The present 2-D Western blot, however, revealed that these proteins with the same MW are composed of about 5–11 isoforms. Nearly all of these are recognized by anti-TSL-1 antibody, suggesting these peptides bear the same epitope (tyvelose) in spite of differences in MW and pI.

PC-related antigens were found in a variety of microorganisms, including bacteria, fungi and parasites (Faro *et al*. 1985; Maizels, Burke & Denham, 1987; Harnett *et al*. 1993). In *T. spiralis*, PC-associated antigens may be secreted from exocrine organs of adults and induce a rapid immune response as early as 2 weeks after infection (Takahashi *et al*. 1994). Anti-TSL-4 mAb does not recognize antigens on the cuticle surface nor stichocyte granules that are recognized by anti-TSL-1 antibodies (Takahashi *et al*. 1993). Our 2-D Western blot results further confirmed that there is no overlap of TSL-1 and TSL-4 antigens, because

anti-TSL-1 and anti-TSL-4 antibodies recognize different peptides. Anti-TSL-1 antibody stained only E–S peptides and anti-TSL-4 antibody stained only non-E–S peptides.

The anti-TSL-2 antibody recognizes TSL-1 type antigens (Appleton *et al*. 1991). The present 2-D Western blot revealed that anti-TSL-2 antibody recognizes a smaller number of peptide spots than anti-TSL-1 antibody does because anti-TSL-2 positive spots of *T. spiralis* crude extracts were always anti-TSL-1 positive, while some anti-TSL-1 positive spots were negative with anti-TSL-2 antibody. For example, 2 peptides migrating at 40 kDa and some peptides migrating at 50 kDa were recognized by anti-TSL-1 antibody, but not recognized by anti TSL-2 antibody.

T. pseudospiralis *antigens*

Only some of the peptides in crude extracts of *T. pseudospiralis* were antigenic, as was the case with *T. spiralis*. These antigenic peptides seemed to be grouped into 2 groups, (i) the peptides corresponding to E–S products (35 and 45 kDa) and (ii) the non-E–S products (14–90 kDa).

Using Western blot, the immunostaining by infected sera seemed to be weak, compared to that of *T. spiralis*. This weak staining does not mean that *T. pseudospiralis* muscle larvae have fewer antigens. *T. pseudospiralis* muscle larvae contain cross-reactive antigens at the comparative level where they can be detected by Western blot analysis and immunoelectron microscopy. Reportedly, *T. pseudospiralis* infection provokes a weaker immune response and intestinal inflammation (Bolas-Fernandez & Wakelin, 1990; Wakelin *et al*. 1994; Alford *et al*. 1998). Interestingly, the weak response was obvious in the response against non-E–S peptides, and the response against E–S peptides was equivalent to that of *T. spiralis*.

The E–S products of *T. pseudospiralis* mainly consisted of only 2 sets of peptides of MW 35 and 45 kDa. The majority of such peptides were acidic, which is in sharp contrast to the fact that E–S products of *T. spiralis* are rather neutral.

Cross-reactive antigens

Antigenic cross-reaction between *T. spiralis* and *T. pseudospiralis* has been well recognized. PC-related antigens are responsible for such cross-reactivity at least in part. E–S products also had cross-reactive antigens confirming previous reports (Kehayov *et al*. 1991; Zhang *et al*. 1993) although E–S products of *T. spiralis* muscle larvae have been known as species specific (Gamble & Graham, 1984; Boireau *et al*. 1997).

The carrier proteins of the cross-reactive antigen are likely different between the two species, because the Western blot analysis revealed that each species shared cross-reactive antigens with different MW and pI. And the immunoelectron microscopical analysis showed that cross-reactive antigens reside in stichocyte granules which are similar but different between the two species (Wu *et al*. 1998).

An additional cross-reactive protein may be 35 kDa of *T. pseudospiralis* E–S products, because most of these peptides were immunostained by *T. spiralis*-infected sera but not by anti-TSL-2 and TSL-4 mAbs.

Candidate peptides responsible for nurse cell transformation

T. spiralis and *T. pseudospiralis* display marked and interesting differences in various aspects. The most striking and unique difference is the host pathology associated with its parasitism. The E–S product is the most likely candidate responsible for important functions to establish such different parasitism (Despommier *et al*. 1990; Lee *et al*. 1991; Vassilatis *et al*. 1992; Ko *et al*. 1994; Jasmer, 1995). Among the many peptides of E–S products of *T. spiralis*, 40 and 50 kDa peptides are candidates for having a unique function for *T. spiralis* because no such peptides were found in *T. pseudospiralis* E–S. The same is true for 35 kDa peptides of *T. pseudospiralis* because the peptides were unique for that species according to the 2-D Western blot analysis.

On the other hand we identified 3 E–S peptides shared by *T. spiralis* and *T. pseudospiralis* in the previous paper (Wu *et al*. 1998), suggesting these play some role in parasitism common to the two. These peptides bear TSL-1 antigen and are cross-reactive between the two species. Vassilatis *et al*. (1996) suggested the 43 kDa glycoprotein is shared by the two species and plays a common role in the life-cycle.

Antibodies play a crucial role as probes of peptides in recent cell biological research. It is disappointing that there is to date no antibody against *Trichinella* that can stain single peptide/structure on 2-D Western blot and immunoelectron microscopy.

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