IMMUNOHISTOCHEMICAL LOCALIZATION OF *GNATHOSTOMA SPINIGERUM* LARVAL ANTIGENS BY MONOCLONAL ANTIBODIES: I. LIGHT MICROSCOPY

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Abstract. Immunohistochemical localization of antigens in advanced third-stage larvae of *Gnathostoma spinigerum* (GsAL3) was studied by indirect enzyme immunostaining using 7 *G. spinigerum* specific monoclonal antibodies, FS-3D11, SS-5H5, SK-6C4, SK-4E1, SK-7G6, SD-8D4 and SA-9B5. All these MAb belong to the IgG1 subclass and only FS-3D11 and SS-5H5 recognize carbohydrate determinants. Each MAb exhibited a different reaction pattern and staining intensity in sectioned GsAL3. FS-3D11 bound primarily to the intestinal brush border whereas SS-5H5 reacted with various tissues of the parasite including intestinal epithelium and brush border, lateral cords, muscle, pseudocoel, and cuticle. SK-6C4 predominantly stained muscle, however, SK-4E1 and SK-7G6 exhibited a lack of labeling. SD-8D4 bound to the cuticle and the lateral cords whereas SA-9B5 reacted primarily with the pseudocoel. These results suggest that antigens sharing common epitopes are present in various structures of the larvae with the intestine being the most antigenic site. The present data also suggest that certain GsAL3 antigens recognized by the MAb obtained in this study are sensitive to formalin fixation and/or paraffin embedding since for 2 out of the 7 MAb staining was negative.

INTRODUCTION

Little is known concerning the specific antigens responsible for host immune response in gnathostomiasis. Localization of the antigens within the infective larvae, and the manner in which these secreted substances are transported to the exterior are still poorly understood. It has been reported that *G. spinigerum* advanced third-stage larval extract is highly complex and is comprised of more than 40 polypeptides of which more than 20 are antigenic in humans (Nopparatana *et al,* 1989). In addition, it was found that the protein fraction is far more antigenic than the fat or sugar fraction (Ando, 1957). Ratanasiriwilai *et al* (1985) studied the *in vitro* production of excretory-secretory (ES) products from GsAL3 and found them to be enzymatic. In serodiagnosis of human gnathostomiasis, Maleewong *et al* (1988) reported that the somatic and ES antigens from GsAL3 produced similar results with ELISA. Rojekittikhun *et al* (1989) demonstrated the presence of antigens on the exterior portions of air-dried infective larvae by means of larval microprecipitation reactions. Using SDS-PAGE and Western blot analysis, Tapchaisri *et al* (1991) reported that the 24 kDa component of GsAL3 is a specific antigen with diagnostic potential for use in the immunodiagnosis of human gnathostomiasis. Nopparatana *et al* (1991) further demonstrated that this 24 kDa component is a protein with a pI of 8.5, and anatomically located in the body fluid, esophagus and intestine of the larvae (Nopparatana *et al,* 1992). In indirect fluorescent antibody assays, Morakote *et al* (1989) treated sections of GsAL3 with hyperinfected rabbit or human gnathostomiasis sera and demonstrated antigen in the anterior part of the esophagus, the surface of the cuticle and in the cytoplasmic granules of the intestine.

As far as is known, no work has been carried out utilizing *G. spinigerum* specific MAb to study localization of target antigens within infective larvae which might shed some light on the role these particular antigens play in the immune response in gnathostomiasis. The present communication reports findings on the immunohistochemical localization of *G. spinigerum* antigens by the immunoperoxidase method using formalin-fixed paraffin-embedded sections of GsAL3 and 7 MAb specific to the parasite.

MATERIALS AND METHODS

Parasite preparation

GsAL3 were obtained from laboratory infected mice 3 - 4 weeks post-infection. They were

washed with PBS, fixed with 10% formalin, embedded in paraffin, and cut to 3 μm in thickness. Some larvae collected from naturally infected eels were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, and were then subjected to frozen section. The fixed-frozen specimens were cut to 8 μm in thickness.

Monoclonal antibody production

To obtain MAb for use in this study, two fusion experiments were performed. Three specific MAb were established from the first fusion as reported by Rojekittikhun *et al* (1991). For the second fusion, spleen cells were harvested from BALB/c mice which had previously received 5 successive injections of crude GsAL3 antigens. The immunization protocol is described elsewhere (Chaicumpa *et al,* 1991). Cell fusion was undertaken in the presence of polyethylene glycol 4000 (Nakarai Chemicals Ltd, Japan) using P3-X63-Ag8. 653 myeloma cells as the fusion partner. Hybridomas secreting antibodies reactive with crude GsAL3 antigens were cloned 3 times by limiting dilution in the presence of hybridoma cloning factor (HCF) (Bokusui Brown, Japan). The details of the characterization and establishment of the specific MAb to *G. spinigerum* were as described in the previous report (Rojekittikhun *et al,* 1991).

Indirect enzyme immunostaining

After deparaffinization and rehydration, the sections were treated with freshly prepared 1% H_2O_2 in methanol for 20 minutes and then with 70% methanol for 5 minutes. Sections were washed twice with distilled water (DW) and twice with PBS for 5 minutes each and then blocked with skimmed milk-PBS for 1 hour at room temperature. After washing 3 times in PBS, undiluted culture supernatant containing MAb, or supernatant of X63 myeloma cells as a negative control, were applied to the section. They were then left in a humid chamber for 1 hour at room temperature and washed 4 times with PBS before the next 1 hour incubation with 1:300 diluted horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts, Denmark). After washing in PBS for 20 minutes with 5 changes, sections were incubated with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.02% 3,3' - diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 for 15 minutes at room temperature. The reaction was stopped by rinsing with DW. The sections were counterstained with hematoxylin, washed, dehydrated, cleared in xylol and mounted (Nakane. 1967).

The fixed-frozen section were washed 3 times in PBS before treatment with 1% H_2O_2 in methanol. The remaining steps were performed as for the paraffin-embedded sections.

RESULTS

Three specific MAb, FS-3D11, SS-5H5 and SK-6C4, were obtained from the first fusion experiment. In the second trial, 4 additional MAb, SK-4E1, SK-7G6, SD-8D4 and SA-9B5, were isolated. Some of the properties of these 7 MAb are summarized in Table 1. All of them belong to the IgG1 subclass. Two MAb, FS-3D11 and SS-5H5, are anti-carbohydrate antibodies, as revealed by periodate oxidation. Since they both recognized carbohydrate moieties on the GsAL3 antigens, the two MAb showed similar patterns of multiple diffuse bands on the Western blot (data not shown). However, all remaining anti-protein antibodies, except SD-8D4, were shown to react with several antigenic components as well. SK-6C4, SK-4E1 and SK-7G6 were found to bind to major target proteins of similar molecular weight (Table 1).

In the immunoperoxidase study, there were some variations in staining reactivity of each of the MAb which differed from experiment to experiment as well as variation among each individual larva in the same section. The overall results are summarized in Table 2. The two anti-carbohydrate antibodies showed strong binding to the intestine. FS-3D11 reacted firmly with the intestinal brush border (Fig 1) whereas SS-5H5 stained several tissues including epithelium, brush border, muscle, lateral cords, pseudocoel and cuticle. The most intense staining was seen in the epithelial cells and no labelling was observed in the esophagus (Fig 2). SK-6C4 showed moderate reactivity in the muscle, weak binding in the lateral cords, and irregular binding in the brush border and pseudocoel (Fig 3). Surprisingly, MAb SK-4E1 and SK-7G6, which had very similar patterns to that of SK-6C4 in the Western blot (data not shown), exhibited negative staining (Fig 4). SD-8D4, which recognized a 16 kDa molecule in

Table 1

Some properties of the 7 monoclonal antibodies specific to *G. spinigerum*.

MAb	Isotype	Periodate sensitive	Major target molecules (kDa)
FS - 3D11	IgG1	+	30 – 94
SS - 5H5	IgG1	+	22 – 94
SK - 6C4	IgG1	–	17,31,38,67,85
SK - 4E1	IgG1	–	16,31,38,67,85
SK - 7G6	IgG1	–	16,31,38,67,85
SD - 8D4	IgG1	–	16
SA - 9B5	IgG1	–	38,85

Table 2

Immunoperoxidase staining reactivities of the 7 monoclonal antibodies and mouse immune sera in paraffin-embedded sections of *G. spinigerum* advanced third-stage larvae.

MAb	Cuticle	Muscle	Lateral cord	Brush border	Epithelium	Pseudocoel	Esophagus
FS - 3D11	–	–	–	+ + / + + +	– / + +	–	–
SS - 5H5	– / + +	– / + +	– / + +	+ + / + + +	+ + / + + +	– / +	–
SK - 6C4	–	– / + +	– / + +	– / +	–	– / +	–
SK - 4E1	–	–	–	–	–	–	–
SK - 7G6	–	–	–	–	–	–	–
SD - 8D4	– / + +	–	– / + +	–	–	–	–
SA - 9B5	–	–	– / +	–	–	– / + +	–
Immune sera	– / + +	– / +	– / + +	– / + + +	– / + +	– / + +	– / +

+ + + strong, + + moderate, + weak, – negative staining reactivities

the GsAL3 extract, was localized in the cuticle and lateral cords (Fig 5) whereas SA-9B5 was confined mostly to the pseudocoel (Fig 6). Mouse immune sera (1:500 dilution) was incorporated for comparison. Positive labeling ranging from slight to intense was observed over entire parasite sections (Table 2, Fig 7).

Fixed-frozen sections were also included in parts of this study. The results are tabulated in Table 3. FS-3D11 showed a typically strong reaction in the intestinal brush border (Fig 8). SS-5H5 bound strongly to the epithelium, but lost binding to most of the other tissues, as observed in paraffin-embedded sections. Intense staining was also noted along the intestinal lumen, rectum, anus and cuticle at the posterior end of the larvae (Fig 9). SK-6C4 gave moderate staining in the muscle and weaker staining in the lateral cords and pseudocoel (Fig 10). These results are somewhat similar to those obtained with the paraffin-embedded sections.

DISCUSSION

Among 7 MAb used in the present study, FS-3D11 and SS-5H5 were shown to recognize carbohydrate moieties of the antigens. Therefore, it is not surprising that both gave strong positive staining over the intestinal epithelium and brush

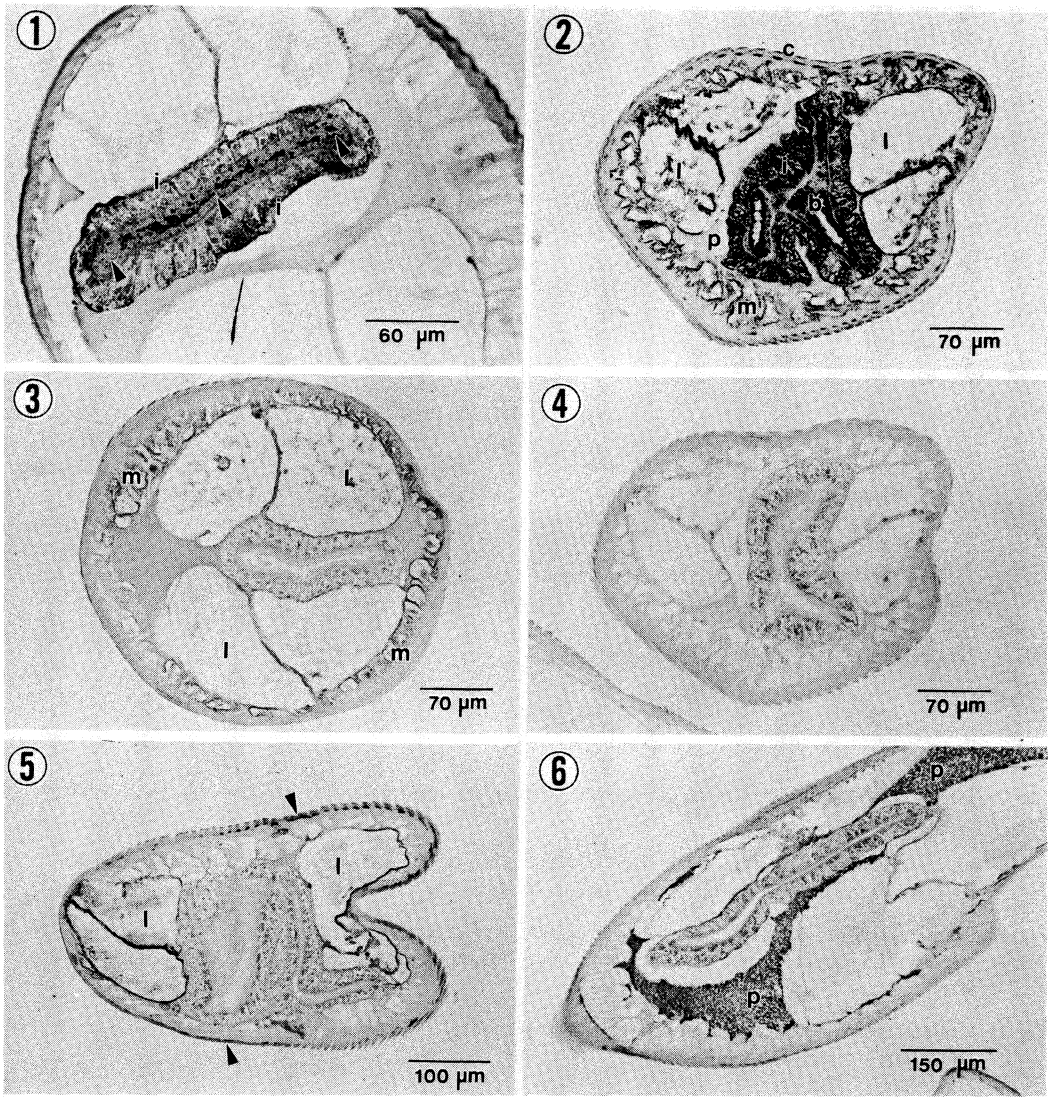

Fig 1—Immunoperoxidase localization of antigens in a section of *G. spinigerum* advanced third-stage larva by MAb FS - 3D11. Strong and weaker reactions in the intestinal brush border (arrow head) and the epithelial cells (i), respectively, can be seen.

Fig 2—Localization of antigens in *G. spinigerum* larva by MAb SS - 5H5. Positive immunostaining in various tissues; cuticle (c), muscle (m), lateral cord (l), epithelium (i), brush border (b) and pseudocoel (p) can be seen.

Fig 3—Localization of antigens in *G. spinigerum* larva by MAb SK - 6C4. Note the moderate staining in the muscle cells (m) and the lateral cords (l).

Fig 4—Negative immunostaining in a section of *G. spinigerum* larva when probed with either MAb SK - 4E1, SK - 7G6 or the culture supernatant of X63 myeloma cells (negative control). Note the much paler color as compared to Fig 2.

Fig 5—Binding of MAb SD - 8D4 to the cuticle (arrow) and the lateral cords (l) of *G. spinigerum* larva.

Fig 6—Positive staining of MAb SA - 9B5 within the pseudocoel of *G. spinigerum* larva (p).

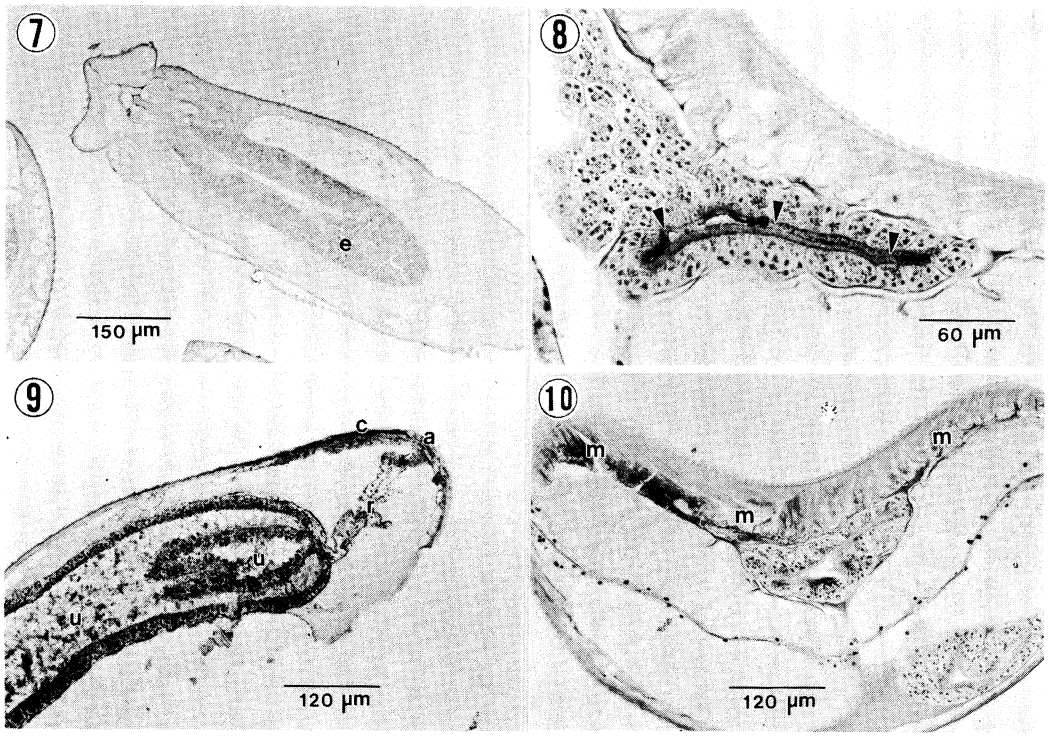

Fig 7—Localization of antigens in *G. spinigerum* larva by mouse immune sera. Note the weak reactivity of the esophagus (e).

Fig 8—Immunoperoxidase staining on a fixed-frozen section of *G. spinigerum* larva with MAb FS - 3D11. Strong labelling in the intestinal brush border (arrow head) can be seen.

Fig 9—Immunoperoxidase staining of a fixed-frozen section of *G. spinigerum* larva with MAb SS - 5H5. Positive reactivity in the intestinal lumen (u). rectum (r), anus (a), and the cuticle at the tail end of the larva (c) can be seen.

Fig 10—Immunoperoxidase staining of a fixed-frozen section of *G. spinigerum* larva with MAb SK - 6C4. Note the moderate reaction in the muscle (m).

Table 3

Immunoperoxidase staining reactivities of 3 monoclonal antibodies in fixed-frozen sections of *G. spinigerum* infective larvae.

Tissues	FS - 3D11	SS - 5H5	SK - 6C4
Cuticle	−	− / +	−
Epithelium	−	+ + +	−
Brush border	+ + / + + +	+ +	−
Lateral cord	−	−	− / +
Pseudocoel	−	−	− / +
Muscle	−	−	+ +

+ + + strong, + + moderate, + weak, − negative reactivities

border of the larvae since it has often been found that antigens of a predominantly carbohydrate nature are associated with the gut (Ruppel *et al,* 1987). However, FS-3D11 bound predominantly to the brush border whereas SS-5H5 reacted as well with most other tissues which were also recognized by certain other MAb. These results suggest a wide distribution of antigens sharing common epitopes in various tissues of the parasite (McLaren *et al,* 1987; Hanna *et al,* 1988).

The main target antigens of SK-6C4 are localized in the muscle and the lateral cords. These may possibly represent antigenic moieties other than glycoproteins secreted by the larvae. The existence of such antigens in these organs was demonstrated by Fujino and Ishii (1991) who reported that most of the nematodes they examined showed moderate enzyme activity for glycosidases in the hypodermis, especially in the lateral cords, which is a metabolically active part of the round worm. The function of glycosidases in these tissues might be related to secretion or maintenance of the cuticle.

Although SK-6C4, SK-4E1 and SK-7G6 all exhibited very similar patterns on the Western blot, the latter 2 MAb gave negative reactions in the sectioned larvae. Unfortunately, there are no available data from frozen sections to confirm this negative result, which may reflect the fragility of certain antigens recognized by MAb to formalin fixation and/or paraffin embedding. It has been reported by Naritoku and Taylor (1982) that 6 of 9 MAb lost their ability to recognize prostatic acid phosphatase on formalin-fixed paraffin-embedded tissues, although all were capable of staining frozen tissues. The reason for this lack of labelling may be explained by a weaker avidity of these 6 MAb compared to conventional antiserum, as well as by the destruction or masking of some antigenic sites during fixation or embedding. Loss of 1 or 2 epitopes on the antigen molecule might be of crucial significance with a MAb that recognizes only one determinant (Naritoku and Taylor, 1982).

The polyclonal mouse immune sera recognized almost all tissues of the sectioned larvae including the esophagus. There have been several reports stating that substances secreted from the esophagus and intestine might represent ES antigens of gnathostome larva (Morakote *et al,* 1989); that esophagus is the most antigenic organ of *G. spinigerum* (Ando, 1957); and that proteins in the extracts of esophagus and intestines are more immunogenic in man than proteins of other portions of the parasite (Nopparatana *et al,* 1992). However, none of the 7 MAb used in the present study stained the esophagus. This might be due to a low quantity of the corresponding antigens in the GsAL3 homogenates. The continuous release of these antigens from migrating larva could conceivably elicit an immune response in the infected hosts (Ruppel *et al,* 1987). When using human infected sera as a probe, positive staining was observed in both the esophagus and the brush border. In contrast, mouse infected sera gave positive staining only in the brush border (data not shown). Data from further investigations are needed to confirm and explain this diversity.

SS-5H5 and SD-8D4 both recognize the cuticle, as well as other sites. The origin and role of these cuticular antigens may be somewhat analogous to those postulated for other parasites as follows. The cuticle itself might play a role in secretion (Page *et al,* 1992) or have independent antigenic activity (Silberstein and Despommier, 1984). Alternately, cuticular antigens might be released from ES-associated glands, exteriorized, and then may somehow become incorporated into the surface covering (Silberstein and Despommier, 1984; McLaren *et al,* 1987; Blanton *et al,* 1991; Page *et al,* 1992).

Binding of SA-9B5 was virtually restricted to the pseudocoel. Nopparatana *et al* (1992) also found that the diagnostic antigen (24 kDa) of GsAL3 was located in the body cavity, as well as in other organs. This indicates heterogeneity and strong antigenicity of the substances within the larval pseudocoel.

The results obtained with fixed-frozen sections were somewhat similar to those obtained with paraffin-embedded sections, except in the case of SS-5H5 which lost positive staining in some tissues, especially in the muscle and the lateral cords. One reason which may account for this is that the antigenicity of those antigens was destroyed by glutaraldehyde fixation (McLean and Nakane, 1974). However, the intense staining of SS-5H5 in the intestinal lumen, rectum, anus and the cuticle that was noted at the tail end of the larvae might throw some light on the potential role of intestinal secretions as major immunogenic substances of GsAL3.

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