[Jpn. J. Parasitol., Vol. 40, No. 3, 260–266, June 1991]

Further Characterization of the Excretory and Secretory Antigens of *Trichinella spiralis* Muscle Larvae

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(Accepted for publication; May 22, 1991)

Abstract

Excretory and secretory (ES) antigens of *Trichinella spiralis* muscle larvae were injected with Freund's adjuvant to mice and antibodies against the ES antigen was obtained. The reactivity of the antibodies was examined by immuno-gold staining on ultrathin sections of LR White-embedded larvae. The antibodies intensely stained so-called "specific" antigens such as the cuticle surface, stichocyte granules, esophagus occupying substance (EOS) and midgut occupying substance (MOS), and weakly stained so-called "non-specific" antigens such as the cuticle inner layer, hypodermis, cord, hemolymph and glycogen aggregates. The results revealed that the host immunized with the ES antigens produces antibodies not only against antigens reported to have species specificity, stage specificity and protection provoking ability, but also against antigens reported to have less species specificity. It seems to be important to predict possible reactions due to the immunized sera with diverse specificities in evaluating the reactions of anti ES antibodies.

Key words: *Trichinella spiralis,* excretory and secretory antigen, immunoelectron microscopy

Introduction

Excretory and secretory (ES) products of helminths are known to contain potent antigens towards the host. Since the ES antigens are endowed with stage specificity (Berntzen, 1974; Mackenzie *et al.*, 1978; Parkhouse and Clark, 1983; Parkhouse and Almond, 1985; Wynne *et al.*, 1981), species specificity (Kaushal *et al.*, 1984) and protection provoking ability (Thorson, 1954, 1956; Campbell, 1955; Despommier, 1977; Goose, 1978; Stromberg, 1978; Despommier and Laccetti, 1981; Maizels *et al.*, 1982, 1984; Silberstein and Despommier, 1984, 1985a, b; Akao, 1985), they have received a great deal of attention.

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This work was partially supported by a Grant-in-Aid for Scientific Research (02454173) from the Ministry of Education, Science and Culture, Japan.

Trichinella spiralis also releases ES products. As encysted larvae of this nematode remain viable for prolonged period in host muscle tissues, it is reasonable to assume that immunogenic stimulation would persist throughout this time and provide the host with a consistent level of immunity (Despommier, 1975). Since the ES products of this nematode can be easily collected from the culture medium, their biochemical properties have been analysed; the ES products are composed of both proteins and glycoproteins (Mills and Kent, 1965; Gold *et al.*, 1990) which are separated into four fractions by ion-exchange chromatography and three fractions by cellulose acetate electrophoresis (Crandall and Zam, 1968). The ES antigens are stage-specific. Berntzen (1974) collected the ES products at 3, 12, 20 hrs and 8 days after mediation of muscle larvae and demonstrated by immuno-electrophoresis that these samples contain 3–9 specific antigens for each stage. Furthermore, Silberstain and Despommier (1984) demonstrated that 48kd and 50–55kd antigens from the ES products are

those that induce a protective response in mice.

In spite of those earlier auther's informative reports, further characterization of the ES antigens is necessary for elucidation of the immunological relationship between the host and parasites. For example, the origin of the ES antigens should be defined to understand the target organs and mode of host immune attack. Thus far only fragmentary data are available as to the ES antigens of *T. spiralis* muscle larvae.

In this study, antisera against the ES antigens were obtained by immunizing mice with the ES products collected by a 3 hour incubation of *T. spiralis* muscle larvae in vitro and identified *in situ* localization of the corresponding antigens on muscle larvae by immunoelectron microscopy.

Materials and Methods

Parasites

T. spiralis (Polish islate) was kindly supplied by Emeritus Professor T. Yamaguchi, University of Hirosaki School of Medicine, Japan, and maintained in ICR mice.

ES products

ES products were collected from supernatant of muscle larvae suspension in phosphate buffered saline (PBS). In brief, muscle larvae were recovered from infected muscles of Fischer rats by pepsin-HCl digestion. After being washed several times with PBS, the exocysted larvae were incubated in PBS at 37°C for 3 hrs. Significant amount of ES products can be collected in the initial 3 hours of incubation (Mills and Kent, 1965). The supernatant was dialysed against distilled water and then lyophilized.

Immunized sera against ES antigens

C3H (n = 5) and NIH (n = 5) mice were used for immunization with the ES antigens. The two strains of mice are known to show different antibody response against *T. spiralis* infection (Almond and Parkhouse, 1986). Each mouse was subcutaneously injected with 30 μg of the ES products emulsified with Freund's complete adjuvant followed by the booster injection of 10 μg of the ES products emulsified with Freund's incomplete adjuvant after 2 and 3 weeks. Sera were collected one week after the final booster injection, frozen and stored until use.

Post-embedding immuno-gold method

Immuno-gold staining was carried out on ultrathin sections of muscle larvae embedded in LR White resin (London Resin Co., Ltd., UK) according to our previous method (Takahashi *et al.* 1990a, b). In brief, the ultrathin sections were incubated with sera (anti ES immunized sera for experiments and normal sera for controls) diluted at 1:200 with 1% bovine serum albumin in PBS (BSA-PBS) at room temperature for 30 minutes. After being washed three times with BSA-PBS, sections were treated with the biotin tagged anti-mouse IgG antibody (Vector Laboratories, Inc., CA., USA) diluted at 1:200 with BSA-PBS. After 30 minutes of incubation at room temperature, the sections were washed and treated with avidin-colloidal gold complex. After immunostaining, the sections were counter stained with uranyl acetate.

Results

We examined two kinds of immunized sera against ES antigens raised in two distinct strains, C3H mice which are susceptible against *T. spiralis* infection and NIH mice which are less susceptible (Jungery and Ogilvie, 1982; Bell *et al.* 1982, 1985; Almond and Parkhouse, 1986). Since no significant difference among the two sera was obtained, the following results were based on the outcome from staining with both sera.

The immunized sera against the ES antigens reacted against all of the antigenic structures that have been listed in our previous reports (Takahashi *et al.*, 1989a). They reacted against cuticle surface, the inner layer (Fig. 1, 2), stichocyte α0, α1 (Fig. 1), α2 (Fig. 3), β (Fig. 2) and γ granules, esophagus occupying substance (EOS, Fig. 4) and midgut occupying substance (MOS, Fig. 5), hypodermis (Fig. 1, 2), cord, hemolymph (Fig. 1, 2), glycogen aggregates (Fig. 1, 2, 5) but not against muscle fibril (Fig. 1, 2) and nucleus. Among these structures there were

noticeable differences in the intensity of immuno staining. The cuticle surface, stichocyte $\alpha 0$, $\alpha 1$, $\alpha 2$, β and γ granules, EOS and MOS were strongly stained. The staining was more intense than that with infected rat sera published elsewhere (Takahashi *et al.*, 1989a; 1990a), which confirmed that effective response of the immunized sera against the structures. However, the staining of the cuticle inner layer, hypodermis, cord, hemolymph, glycogen aggregates were much weaker.

Control sera did not stain any structures confirming specificity of the method employed.

Discussion

Since the post-embedding immuno-gold method allows the localization of antigens at the electron microscopic level, the precise localization of antigens recognized by immunized sera against the ES antigens of muscle larvae can be demonstrated by the method. The results was that a wide variety of structures including "specific" antigens such as the cuticle surface, stichocyte granules, EOS and MOS (Seawright *et al.*, 1983; Arriaga *et al.*, 1989; Takahashi *et al.*, 1990c) and "non-specific" antigens such as the cuticle inner layer, hypodermis, cord, hemolymph and glycogen aggregates (Takahashi *et al.*, 1990c) were recognized by the immunized sera against the ES antigens, which will be basic but important information for future investigation of the origin and/or immunological nature of the ES antigens when the immunized sera was used.

We have previously demonstrated the presence of antigenic substances in the alimentary tract of *T. spiralis* muscle larvae, namely the EOS and MOS (Takahashi *et al.*, 1989a, b), hypothesizing these substances partially account for the origin of the ES antigens. Stichocyte granules have been also implicated as the origin of the ES antigens, because antigens extracted from these granules are cross-reactive with the ES antigens (Despommier and Muller 1976). This hypothesis is further strengthened by the fact that stichocyte granules are secreted into the esophagus lumen through the canalicular tree (Bruce 1970, Despommier 1974, 1983). Our present data, which demonstrates anti ES immunized sera intensely stained stichocyte granules, the EOS and MOS, do not contradict the hypothesis of the origin and subsequent secretion route of the ES antigens.

The cuticle surface was also intensely stained by immunized sera against ES antigens. This result is interesting in terms of identification of the ES antigen on the cuticle surface, since the cuticle surface shares the same antigenicity with the stichocytes granules, the EOS and MOS (McLaren *et al.*, 1987, Takahashi *et al.*, 1990b).

The major origin of ES antigens thus seems to be the cuticle surface, stichocyte granules, the EOS and MOS. Antigens on these structures are endowed with species specific (Takahashi *et al.*, 1990c) and protection inducing ability (Silberstein and Despommier, 1984; Grencis *et al.*, 1986), therefore, the ES antigens were regarded as practical antigen for serodiagnosis and protection against oncoming infection. However, it is noticeable that the immunized sera against ES antigens also stained cuticle inner layer, hypodermis, cord, hemolymph and glycogen aggregates which contain comparatively unimportant antigens in terms of serodiagnosis and protective immunity (Despommier, 1981; Silberstein and Despommier, 1984). This result may raise two possibilities; one is colocalization of both

Immunoelectron micrographs of *T. spiralis* muscle larvae stained with immunized sera against ES antigens.

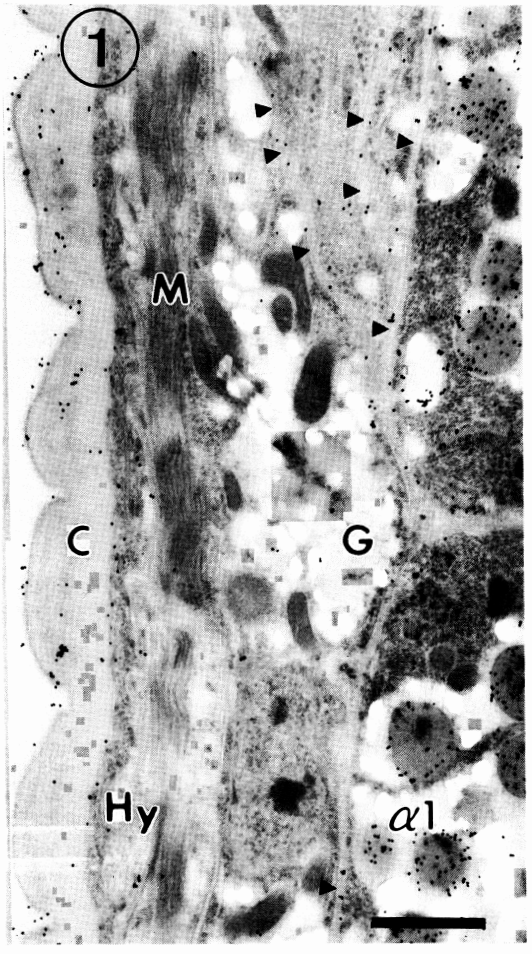
Fig. 1 The cuticle (C) surface and stichocyte $\alpha 1$ granules ($\alpha 1$) are strongly positive and the cuticle inner layer, hypodermis (Hy), glycogen aggregates (G) and hemolymph (arrow heads) are weakly positive by immunostaining. M: myofibril, Bar = 1 μm.

Fig. 2 The cuticle (C) surface and stichocyte β granules (β) are strongly positive and the cuticle inner layer, hypodermis (Hy), glycogen aggregates (G) and hemolymph (arrow heads) are weakly positive by immunostaining. M: myofibril, Bar = 1 μm.

Fig. 3 Stichocyte $\alpha 2$ granules ($\alpha 2$) are strongly positive by immunostaining. Bar = 1 μm

Fig. 4 Esophagus occupying substance (E) is strongly positive by immunostaining. Bar = 1 μm

Fig. 5 Midgut occupying substance (M) is strongly positive and glycogen aggregates (G) are weakly positive by immunostaining. Bar = 1 μm


1
M
C
G
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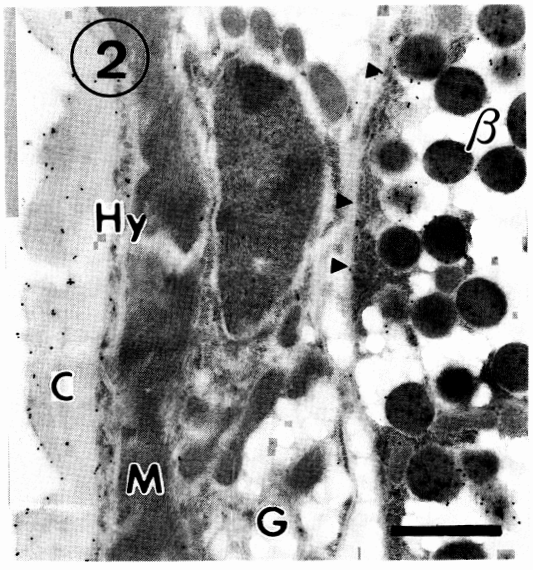
α1


2
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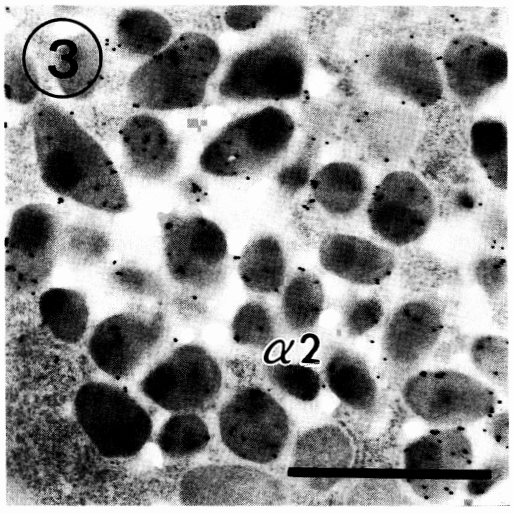
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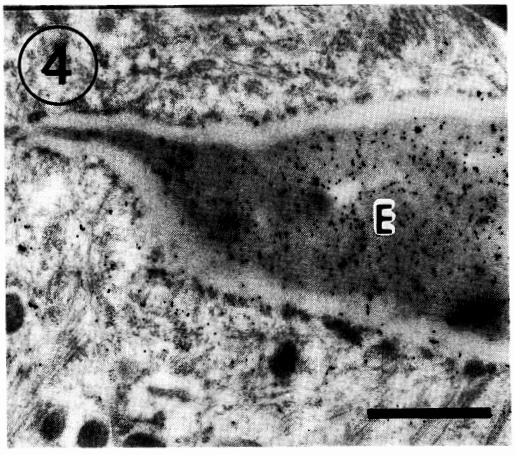
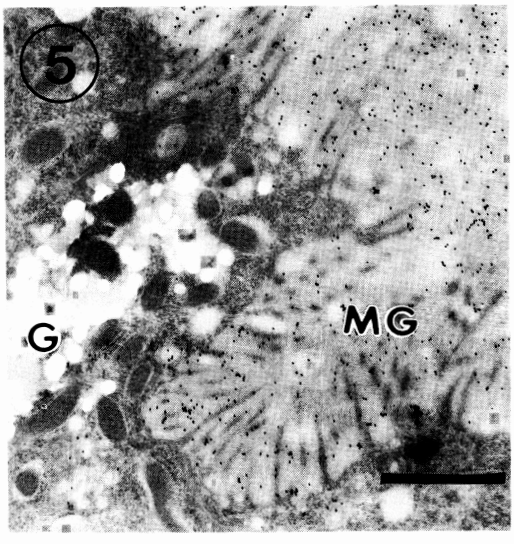
α2


4
E


5
G
MG

"specific" and "non-specific" antigens in the cuticle inner layer, hypodermis, cord, hemolymph and glycogen aggregates, and the other is contamination of the ES products with "non-specific" antigens. If the latter is the case, the ES antigens likely looses its value. Our preliminary result, however, denied this possibility; the compositional heterogeneity of the ES antigens was examined by indirect ELISA using monoclonal antibodies. A monoclonal antibody, which recognizes "specific" antigens, reacted against the ES products used in this experiment, but other monoclonal antibody which recognizes "non-specific" antigens did not react (our unpublished data).

Although we have no ready explanation for diverse specificity of anti ES antibodies, it is noteworthy that nematode antigens with less specificity contain antigenic determinants like phosphoryl choline against which antibodies are easily produced in the host (Pery *et al.*, 1974; Clafin *et al.*, 1974; Faro *et al.*, 1985; Gualzata *et al.*, 1986, 1988; Pengo *et al.*, 1987; Lerio *et al.*, 1988: O'keefe *et al.*, 1990).

In conclusion, the hosts immunized with the ES antigens of muscle larvae produce antibodies not only against the cuticle surface, stichocyte granules, EOS and MOS but also against diverse structures which are devoid of species specific antigens, although ES products are likely endowed with only "specific" antigens. The evidence must be kept in mind in any immunological analysis using sera raised by immunization with the ES antigens.

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