PRODUCTION OF MONOCLONAL ANTIBODIES TO THE CUTICLE OF ADVANCED THIRD-STAGE LARVA OF *GNATHOSTOMA SPINIGERUM*

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Abstract. This study has demonstrated that sera from Balb/c mice infected with live advanced third-stage larvae (aL3), but not those immunized with crude larval extract, immunoprecipitated the 25-kDa protein from surface-iodinated extract of aL3. Hybridoma cell lines derived from spleen cells of an infected mouse secreted antibodies that reacted with several tissue of aL3 including the esophagus, intestine, muscle and cuticle by immunofluorescence assay. However, none of the cuticle-positive hybridoma cell lines produced antibodies that recognized surface-iodinated protein of aL3 by immunoprecipitation. Western blot analysis showed that monoclonal antibodies (MAbs) secreted by clones derived from one of the cuticle-positive hybridoma lines recognized proteins of molecular weights ranging from 55-96 kDa. The MAbs most likely reacted with the collagenous component of the cuticle.

INTRODUCTION

Human gnathostomiasis, which is caused by the spirurid nematode of the genus *Gnathostoma*, is endemic in many Asian countries, particularly Thailand and Japan. Many species of *Gnathostoma* have been described, however, the only species found infecting Thai people is *Gnathostoma spinigerum.* Man is believed to receive this infection principally by eating inadequately cooked fresh-water fish or other paratenic hosts containing encapsulated infective advanced third-stage larvae (aL3). In human host, they migrate aimlessly and produce mild to severe clinical diseases depending on the organs affected (Boongird *et al,* 1977; Punyagupta *et al,* 1990; Teekhasaenee *et al,* 1986). Many parasitic nematodes migrate through tissues of their hosts evoking host immune responses that have little or no detrimental effects on themselves. Various mechanisms of evasion of host immune responses have been proposed but still remain unclear in many situations. The nematode surface, being the interface between the parasite and its host, may have important roles that determine the host-parasite interaction. Unlike that of trematodes and cestodes, the nematode surface has acellular layer named the cuticle.

Ultrastructurally, the basic structure of the nematode cuticle comprises distinct layers of the epicuticle, corticular, median and basal layers, which overlay the hypodermis (Wright, 1987). The outermost covering of the nematode is not only the epicuticle but also has an external amorphous envelope called carbohydrate-rich glycocalyx (surface coat). The protective role of the surface coat against immune response is found in *Toxocara canis,* in which host antibody or leukocyte attachment stimulates rapid shedding of the coat, allowing the larva to escape, leaving behind it abandoned glycocalyx to which host leukocytes remain attached (Blaxter *et al,* 1992; Page *et al,* 1992). Like *Trichinella spiralis,* the antigens of the adult and larva that react with monoclonal antibody (MAb) are also localized in the surface coat (Philipp and Rumjanek, 1984; McLaren

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et al, 1987). The epicuticle, which is trilaminar in appearance, contains lipids. The rapid changes in epicuticle lipids generally occur when parasitic infective larva enters the definitive host. The cuticle is composed of disulfide cross-linked collagens and insoluble structural protein or cuticlin. They are hidden from the host during infection and are not recognized by post-infection sera (Pritchard *et al,* 1988). The noncollagenous cross-linked protein, cuticlin, is resistant to solubilization by SDS and reducing agents (Maizels *et al,* 1993). Cuticular proteins of nematode parasites not only serve as exoskeleton but are also shown to be either the source of induction or the targets of host protective immune response (Philipp *et al,* 1988). Unlike the collagens, whose solubilization requires chaotropic and reducing agents, high temperatures, and denaturing detergents, surface associated molecules can be solubilized from the cuticle by mild procedures involving homogenization in purely aqueous buffer solutions or in nondenaturing detergents (Storey and Philipp, 1992).

Although the cuticle is a target of host antibody in *Gnathostoma* infections (Morakote *et al,* 1989; Anantaphruti *et al,* 1991; Rojekittikhun *et al,* 1993), the nature of the antigens associated with *G. spinigerum* cuticle has not been elucidated. The present study was carried out in order to produce MAbs to surface antigens of aL3 but they turned out to be possibly against cuticular collagens.

MATERIALS AND METHODS

The parasite

Encysted aL3 were collected from livers of infected fresh-water eels. The encysted larvae were used to prepare paraffin sections (Morakote *et al,* 1989). The larvae excised from the cysts were used for the preparation of crude somatic antigens, and surface labeling with ^{125}I. Live larvae were used to infect Balb/c mice by oral feeding.

Preparation of crude somatic antigens

The aL3 were homogenized in phosphate buffer saline (PBS) containing protease inhibitors (0.05 mg/ml leupeptin-antipain, 10 mM EDTA) with a tissue grinder and then sonicated on ice by ultrasonic disintegrator (Branson) at 15 Watts for 4 minutes according to the method described by Tuntipopipat *et al (*1989). The protein concentration of the homogenate was determined by Lowry method (Lowry *et al,* 1951).

Induction of antibodies against aL3 in mice

Four to five week-old Balb/c mice were each immunized by intraperitoneal injection with 100 μg of crude aL3 antigens in Freund's complete adjuvant. The injection was repeated two weeks later with the same amount of the antigens in Freund's incomplete adjuvant. On the other hand, some mice were infected by oral feeding with 5 live aL3 suspended in normal saline solution (NSS). The same number of live larvae in NSS was fed to the mice two weeks later. Three days prior to fusion, the final immunization was done by intravenous injection of 50 μg of the antigens into the tail vein.

Hybridoma production

Splenocytes from immunized or infected mouse were fused with myeloma cells P3-X63-Ag8.653 (American Type Culture Collection CRL 1580) by the standard method with some modifications (Zola, 1987). Two weeks after fusion, culture supernatants were screened for antibodies to aL3 by ELISA, IFA and immunoprecipitation. Positive hybridomas were expanded and cloned twice by limiting dilution at cell densities of 10, 3, 1 and 0.3 cells/well. The MAb producing clones were repeatedly screened and expanded for further characterization. Isotypes of the MAb were identified using a commercial ELISA kit (Sigma).

Enzyme-linked immunosorbent assay (ELISA)

This was performed as previously described (Morakote *et al,* 1987). Briefly, 5 μg/ml of the crude somatic aL3 antigen was coated in each well of microtiter plate. Antigen-coated wells

were incubated with hybridoma supernatants or diluted mouse sera (1:200) for 30 minutes at 37ºC. Following washing step, the horseradish peroxidase-conjugated goat anti-mouse immunoglobulin A+G+M (1:20,000) was applied to each well and incubated at 37ºC for 30 minutes. The plate was washed and freshly prepared substrate solution (using *o*-phenylenediamine) was added to each well. After 30 minutes, the reaction was stopped and the optical densities were monitored by an ELISA reader at 490 nm. All samples were done in duplicates.

Immunofluorescence assay (IFA)

The procedure was the same as described in detail previously (Morakote *et al,* 1989). Briefly, deparaffinized sections of encapsulated aL3 were blocked with 1% bovine serum albumin (BSA) for 10 minutes and incubated with one drop of undiluted hybridoma supernatant or diluted immunized mouse serum (1:20) for 30 minutes at 37ºC in a humidified chamber. After washing twice with PBS, FITC-conjugated goat anti-mouse IgA+G+M diluted (1:20) in PBS containing 0.2% Evans blue was applied and incubated for 30 minutes. Finally, the sections were washed extensively, mounted in buffered glycerol and examined under a fluorescence microscope (Olympus).

Surface labeling of aL3 with ^{125}I

Surface proteins of *G. spinigerum* aL3 were iodinated using IODOGEN method (Scott *et al,* 1988). Glass vials were coated with 100 µg of IODOGEN. Five to 10 live aL3 were washed with PBS, suspended in 0.2 ml PBS and transferred to an IODOGEN-coated vial. The vial was then added with 2 µl (0.2 mCi) of Na^{125}I (Amersham, UK), mixed gently and incubated for 15 minutes at room temperature. After the labeling step, the larvae were washed 6 times with PBS. The surface-iodinated larvae were extracted in DOC extraction solution containing 1% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA in PBS and left on an ice bath overnight. The supernatant was collected by centrifugation and measured for the radioactivity by a gamma counter.

Immunoprecipitation and SDS-PAGE

For immunoprecipitation, either 100 µl of undiluted hybridoma supernatant of a mixture of 3 µl serum and 100 µl NETT buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.6, 0.5% Triton X-100) was incubated with 100,000 cpm of ^{125}I-labeled extract at 4ºC overnight. The reaction was then added with 75 µl of 25% protein A-Sepharose beads and mixed by rocking for 1 hour. The beads were washed twice with each of NETTS (500 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.6, 0.5% Triton X-100), NETT and NET (150 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.6) buffers. The immune complexes were solubilized from the beads by adding 20 µl of sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 5% SDS, 0.004% bromophenol blue and 5% 2-mercaptoethanol) and heated at 100ºC. The solubilized proteins were resolved by 8-20% gradient SDS-PAGE according to the method described (Laemmli *et al,* 1970). The gels were stained with Coomassie blue, dried with a slab gel dryer and exposed to Kodak XAR-5 films at -80ºC for autoradiography.

Western blotting

Following gel electrophoresis, the separated proteins were transferred onto nitrocellulose paper (NCP, pore size 0.45 µm) by electroblotting as described by Towbin *et al* (1979). The NCP strips were incubated with 1:200 diluted mouse serum or undiluted hybridoma culture supernatant for 1 hour at room temperature. After three washes, the strips were reacted with peroxidase-conjugated goat anti-mouse IgA+G+M (diluted 1:20,000) for 1 hour at room temperature. The strips were extensively washed and immersed in luminol enhancer (Pierce) for 5 minutes and immediately exposed to an X-ray film for 30 seconds before developing.

Monoclonal antibody

The MAb GN6/24 was kindly provided by Dr Wanpen Chaicumpa of the Fuculty of

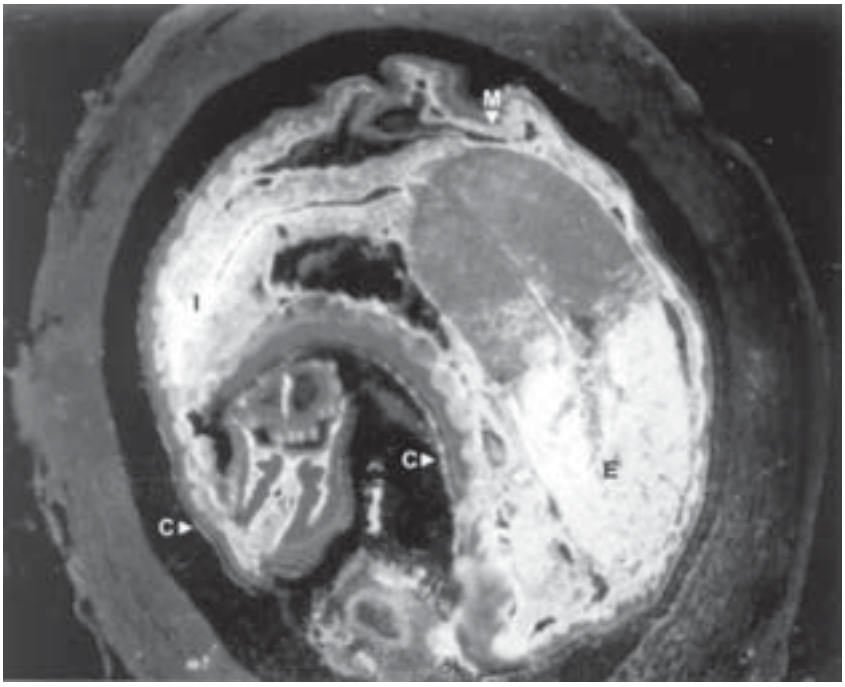

Fig 1–Immunofluorescence assay of infected mouse serum on a paraffin section of encysted advanced third-stage larva of *Gnathostoma spinigerum*. Greenish fluorescence was observed at the cuticle (C), esophagus (E), intestine (I), and muscle (M) of the larva. Magnification 100x.

Tropical Medicine, Mahidol University. The MAb is specific to the 24-kDa protein of *G. spinigerum* aL3 (Chaicumpa *et al*, 1991).

RESULTS

The sera of immunized and infected mice were all positive for antibodies against aL3 by ELISA and IFA. The IFA showed that the antibodies bound to the surface of the cuticle, esophagus, intestine and muscle on paraffin sections of aL3 (Fig 1). However, when tested by immunoprecipitation, only the sera from mice infected with aL3 precipitated the ^{125}I-labeled 25-kDa protein from the larval extract whereas none of those from immunized mice reacted with the surface-iodinated protein (Fig 2). The total DOC extract of ^{125}I-labeled aL3 contained prominent iodinated proteins with apparent molecular weights of 25 and 12 kDa and other relatively weak bands at 44, 23, 21, 18, 17 and 14 kDa (Fig 2).

Since antibodies to surface-iodinated proteins were observed only in infected mice, the production of monoclonal antibodies was performed on spleen cells from these mice. In one attempt, there were 17 hybridoma cell lines that produced antibodies reactive to aL3 sections by IFA. Four of them gave antibodies that bound to the esophagus and cuticle, one reacted with the intestinal cells and cuticle, and the other 12 hybridoma lines reacted with the cuticle only. However, when tested by immunoprecipitation, none of them reacted with the surface-iodinated proteins of aL3. One of the hybridoma lines that reacted with the cuticle only was cloned twice by limiting dilution. The supernatants of cloned lines showed fluorescent staining with the entire thickness of the cuticle of aL3 (Fig 3). The MAbs produced by these cloned lines were further tested with crude extract of aL3 by Western blot analysis.

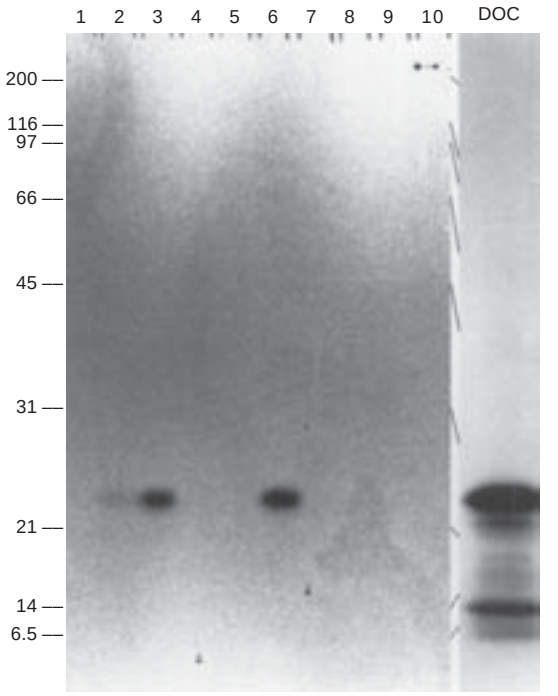

Fig 2–Immunoprecipitation of DOC extract of surface-iodinated advanced third-stage larva of *Gnathostoma spinigerum* by mouse antisera. Autoradiography of iodinated larval extract immunoprecipitated by normal mouse serum (Lane 1), infected mouse antisera (Lanes 2, 3 and 6) and immunized mouse antisera (Lanes 4, 5, 7, 8, 9 and 10) and resolved on 8-20% gradient SDS-PAGE. Total DOC extract is also shown for comparison. On the left margin are broad range protein molecular weight markers in kilodaltons (Bio-Rad).

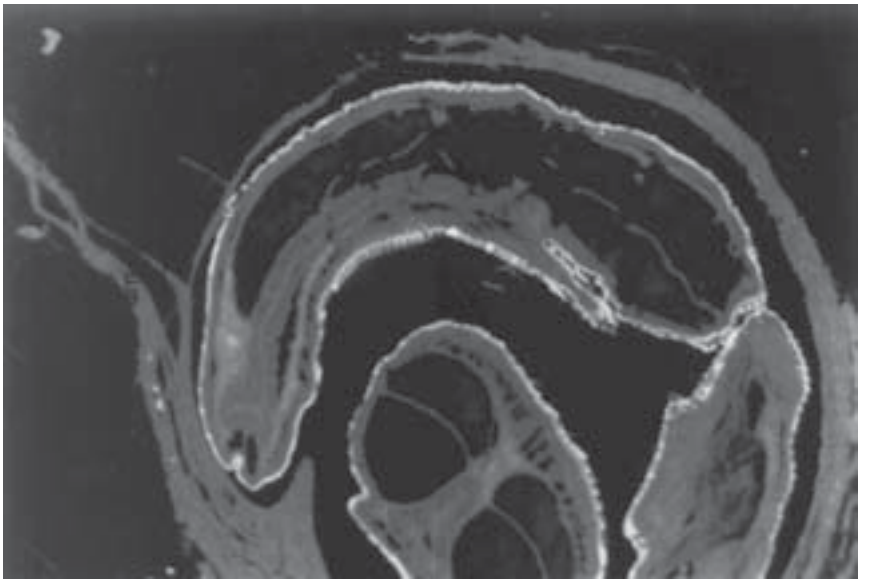

Fig 3–Immunofluorescence assay of a monoclonal antibody on a paraffin section of advanced third-stage larva. The monoclonal antibody S-7G7 showing greenish fluorescence at the cuticle. Magnification 100x.

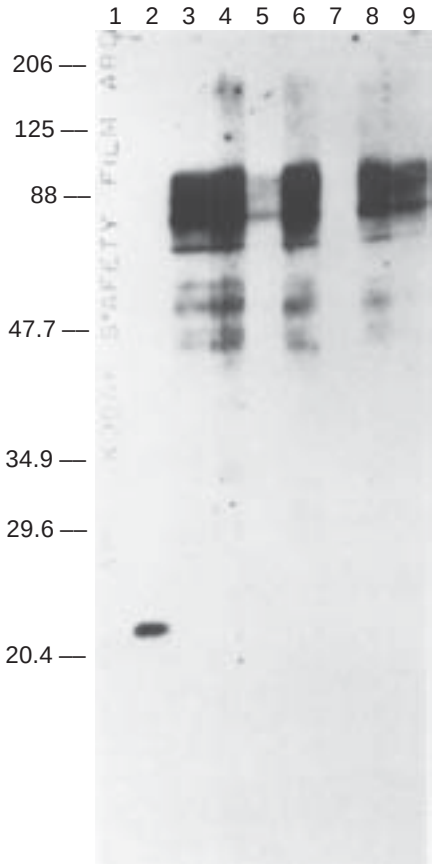

Fig 4–Western blot analysis of the monoclonal antibodies. Nitrocellulose strips of crude larval extract separated by 8-20% gradient SDS-PAGE were probed with normal hybridoma supernatant (lane 1), mAb GN6/24 (lane 2), and mAbs S-6A1, S-7G7, T-1C11, T-1C6, T-2D2, T-2D4 and T-3F9 (lanes 3-9, respectively) and finally developed by chemiluminescence substrate. Prestained protein molecular weight markers in kilodaltons are on the left margin (Bio-Rad).

As shown in Fig 4, the MAbs secreted by clones S-6A1, S-7G7, T-1C11, T-1C6, T-2-D4 and T-3F9 recognized serveral proteins with molecular weights ranging from 55 to 96 kDa (Fig 4, lanes 3, 4, 5, 6, 8, and 9). Discrete bands were observed at 85 and 75 kDa. On the contrary, clone T-2D2 did not give any visible band on the blot (Fig 4, lane 7). As a positive control, the MAb GN6/24 clearly showed the specificity to the 24-kDa protein of the larval extract (Fig 4, lane 2). All of the MAbs to the cuticle of aL3 were of IgM isotype.

Four more fusion experiments using spleen cells from infected mice were carried out to produce MAbs to surface-iodinated proteins of aL3 and similar results were observed in all attempts. Immunoprecipitation showed that most hybridomas secreted antibodies to the iodinated 25-kDa protein in the primary screening of the supernatants (data not shown). However, these supernatants were negative by IFA. After limiting dilution, the supernatants of cloned hybridomas did not precipitate any protein from the iodinated extract.

DISCUSSION

This study has shown that mice infected with aL3 *G. spinigerum* produced antibodies to a major 25-kDa surface protein of aL3. This observation could be explained by the fact that infected mice would be exposed mainly to the surface proteins, the proteins shed from the surface coat, and the excretory/secretory (ES) protein of the parasite. In contrast, immunized mice having been exposed to all protein components of the crude extract would mount immune responses to major or more immunogenic proteins. The absence of reactivity to iodinated extract of the sera from immunized mice suggests that the surface proteins constitute minor components of the crude extract or they are relatively less immunogenic. Immune responses to live aL3 in infected mice should also mimic natural infection because human gnathostomiasis antiserum gave the same immunoprecipitation pattern with ^{125}I-lableled extract of aL3 (data not shown). The MAbs obtained in this study were thus derived from spleen cells of an infected mouse. The IFA showed that the MAbs reacted with the whole thickness of the cuticle, which made it very difficult to discern whether they reacted to the surface proteins since they reside in a very thin layer of the surface coat covering the epicuticle. However, these MAbs gave negative immunoprecipitation results with ^{125}I-labeled extract of aL3 suggesting that they did not

recognize the surface proteins. Therefore, it is most likely that the MAbs recognized immunogenic components of the cuticle proper. Basically, the composition of the cuticle consists of the collagens and cuticlin. The collagens, major cuticle components, are solubilized from the cuticle with reducing agent such as 2-mercaptoethanol (2-ME). The collagenous molecules can be iodinated only following the removal of surface coat by cetyltrimethylamonium bromide (CTAB) and collagen-rich fraction can be obtained by homogenizing whole worm in buffers containing SDS and 2-ME, followed by harvesting the supernatant (Pritchard *et al,* 1988). Cuticlin is the material remaining after treating the cuticle with denaturing detergent SDS and 2-ME. It is noncollagenous protein with nonreducible highly cross-linked bonds (Politz and Philipp, 1992). The recognition of aL3 proteins by the MAbs as shown by Western blot analysis fits to the notion that the collagens are solubilized by 2-ME. Moreover, cuticlin, being resistant to SDS and 2-ME, is the unlikely target of these MAbs. The MAbs were also found to react with the cuticle of adult *G. spinigerum* by IFA (data not shown) suggesting that the target antigen was common to different developmental stages. Our assumption was also supported by the previous study (Politz and Philipp, 1992) showing that collagen genes in nematodes are part of a large multigene family. It has been shown that the *col-1* gene family is common to all nematodes echoing the well-known antigenic cross-reactivity among proteins and glycoproteins of parasitic nematodes. The nature of this skeptical protein can be elucidated by performing immunoprecipitation between the MAbs and ^{125}I-labeled collagenous molecules after removing the surface coat by CTAB. On the other hand, the characterization of the genes and expressed proteins can be accomplished by immunoscreening of the cDNA library of *G. spinigerum* aL3 using one of these MAbs.

Direct screening by immunoprecipitation in later fusions showed that the 25-kDa surface protein was recognized by most supernatants from growing hybridomas. Disappointingly, after expanding the hybridomas, none of the supernatants precipitated the 25-kDa protein. The observation led to the suspicion that the antibody recognizing the 25-kDa proteine might be released from unhybridized immune lymphocytes. This argument was proven not to be the case since the supernatants collected from several wells with no growth of hybridomas did not contain antibody to the 25-kDa protein (data not shown). It was suggested that these hybridomas were actually secreting desired antibodies but became unstable and discontinued producing the antibodies afterwards.

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