Recognition of deglycosylated larval proteins of *Gnathostoma spinigerum* by a monoclonal antibody and human gnathostomiasis antiserum

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Abstract

The study on the recognition of ^{35}S-labelled somatic antigens of *Gnathostoma spinigerum* advanced third-stage larva (aL3) has revealed that the mAb GN6/24 immunoprecipitated 26- and 24-kDa proteins from the undigested and N-glycosidase F-digested larval extracts, respectively. The recognition of the deglycosylated form of the glycoprotein indicated that the mAb reacted with the peptide epitope on the 26-kDa protein. Human gnathostomiasis antiserum immunoprecipitated most of the N-glycosidase F-digested larval proteins including the deglycosylated 26-kDa protein.

Introduction

Gnathostomiasis, a disease caused by the nematode of the genus *Gnathostoma*, is endemic in many Asian countries especially Thailand and Japan. In Thailand, the only species causing human infection is *Gnathostoma spinigerum*. Humans usually acquire infection by eating raw or undercooked meat especially from freshwater fish containing infective third-stage larvae. The pathology of gnathostomiasis is caused by the invasion of migrating larvae in various organs. This includes the involvement of skin and subcutaneous tissues producing intermittent migratory swellings (Daengsavang, 1980; Rusnak & Lucey, 1993) or the more severe involvement of the eye and central nervous system leading to permanent damage or even death in some cases (Boongird *et al.*, 1977; Teekhasaenee *et al.*, 1986; Punyagupta *et al.*, 1990). In clinical practice, the diagnosis of gnathostomiasis often relies on the clinical presentation and a history of diet supported by blood eosinophilia and serological tests. The most widely used serological test for gnathostomiasis is the ELISA using crude somatic antigens of advanced third-stage larvae (aL3) with varying sensitivity from 87 to 100% (Suntharasamai *et al.*, 1985; Dharmkrong-At *et al.*, 1986; Maleewong *et al.*, 1988; Anantaphruti, 1989). However, the assay suffers from cross-reactivities of the antigens used with sera from other parasitic infections especially angiostrongyliasis (Suntharasamai *et al.*, 1985). Later studies identified a unique 24-kDa protein of aL3 recognized only by sera from confirmed cases of gnathostomiasis (Tapchaisri *et al.*, 1991). Evaluations of the partially purified 24-kDa protein by ELISA have shown that the antigen preparation is highly sensitive and specific for gnathostomiasis (Nopparatana *et al.*, 1991; Tuntipopipat *et al.*, 1993). Despite its diagnostic potential, the preparation and purification of the 24-kDa protein from natural sources for diagnostic purposes may not be cost-effective (Nopparatana *et al.*, 1991; Tuntipopipat *et al.*, 1993). The situation warrants an alternative strategy employing the recombinant DNA technology to produce the desired antigen. This approach would involve the construction of a cDNA library of *G. spinigerum* aL3 for immunoscreening by a monoclonal antibody to the 24-kDa protein. The production of a monoclonal antibody (mAb GN6/24) to the 24-kDa protein has already been

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described (Chaicumpa *et al*., 1991). The library could also be screened by human antisera from parasitologically confirmed cases of gnathostomiasis to identify other clones expressing possibly unique proteins. Since the conventional cDNA library is expressed in *E. coli*, which lacks the machinery to add oligosaccharide to the translated proteins, it is necessary to demonstrate whether the antibodies that will be used in immunoscreening of the library could recognize the target proteins in deglycosylated forms. This work aims to study the reactivities of the mAb GN6/24 and human gnathostomiasis antiserum with crude somatic antigens of aL3 after treatment with N-glycosidase F or O-glycosidase.

Materials and methods

Cysts of *G. spinigerum* aL3 were isolated from the livers of eels and rinsed in 70% ethanol. The aL3 were aseptically excised from the cysts, transferred to an RPMI-1640 medium supplemented with 10% FCS, 5 μg ml^{-1} gentamicin and 10 μg ml^{-1} amphotericin B and maintained at 37°C in an atmosphere of 5% CO_2 (Maleewong *et al*., 1995). The culture medium was changed every 3 days. The aL3 of 1 to 5 months old in culture were metabolically labelled with [^{35}S]methionine. Five aL3 were washed three times with methionine-free RPMI 1640 (Gibco BRL) and resuspended in 1 ml of methionine-free RPMI 1640 containing 50 μCi of [^{35}S]methionine (Amersham, >1000 Ci mmol^{-1}, 15 mCi ml^{-1}). After 72 h, the larvae were washed three times with phosphate-buffered saline (PBS) and ground in 0.5 ml PBS containing 1% sodium dodecyl sulphate (SDS), 2 mM phenylmethylsulphonyl fluoride (PMSF), and 5 mM ethylenediaminetetraacetic acid (EDTA). The larval somatic extract was centrifuged at 10,000 × *g* for 30 min at 4°C and the supernatant collected for further analyses. The amount of radioactivity in the somatic extract was determined by a β-counter following trichloroacetic acid (TCA) precipitation.

For deglycosylation, the ^{35}S-labelled somatic extract in 60 μl of PBS containing 1% SDS was denatured by boiling for 10 min. The volume of the reaction was increased to 300 μl such that it contained 25 mM Na_2HPO_4, 1% Triton X-100, 0.2% SDS, and 1 unit of N-glycosidase F (Boehringer Mannheim). The reaction was incubated at 37°C overnight. The labelled extract was also digested overnight with 1 unit of O-glycosidase (Boehringer Mannheim) at 37°C in 200 mM sodium cacodylate buffer in the presence of 1% Triton X-100 and 0.2% SDS. Equivalent amounts (~200,000 TCA-precipitable cpm) of the undigested, N-glycosidase F-digested, and O-glycosidase-digested extracts were incubated overnight at 4°C with 100 μl of the mAb GN6/24 (culture supernatant, ten times concentrated by ultrafiltration with PM10 membrane (Amicon)), 3 μl of human gnathostomiasis antiserum or 3 μl of rabbit antiserum previously infected subcutaneously with 100 aL3. Seventy-five μl of 25% protein A-Sepharose (Pharmacia Fine Chemicals) suspension was added to the reaction and mixed by rocking at room temperature for 1 h. The beads were washed twice with each of NETTS (0.5 M NaCl, 1 mM EDTA, 10 mM Tris, 0.5% Triton X-100, pH 7.6), NETT (0.15 M NaCl, 1 mM EDTA, 10 mM Tris, 0.5% Triton X-100, pH 7.6), and NET (0.15 M NaCl, 1 mM EDTA, 10 mM Tris, pH 7.6) buffers. The washed beads were suspended in 20 μl of sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2.5% SDS, 0.004% bromophenol blue, and 5% β-mercaptoethanol) and boiled for 5 min. The supernatants of the suspensions were resolved by 8–20% gradient SDS-PAGE. The Coomassie blue-stained gels were soaked in 1 M sodium salicylate for 1 h, dried and exposed to Kodak XAR-5 films for fluorography at –70°C.

Results and Discussion

As expected, deglycosylation of ^{35}S-labelled somatic antigens of *G. spinigerum* aL3 by N-glycosidase F revealed shifts in the M_r of several proteins (fig. 1, lane 2). Among these, the M_r of the prominent 46-, 45-, 42-, and 26-kDa proteins decreased to 45, 44, 41, and 24 kDa, respectively. Digestion with O-glycosidase did not produce discernible effects on the larval somatic antigens indicating the absence of O-linked sugars in *Gnathostoma* glycoproteins (fig. 1, lane 3). When subjected to immunoprecipitation, the mAb GN6/24 precipitated the 26- and 24-kDa proteins from the labelled somatic extract before and after digestion with N-glycosidase F, respectively (fig. 1, lanes 4 and 5). The mAb GN6/24 reacted with the protein of 26 kDa in this study in contrast to 24 kDa as originally observed by Western blot analysis (Chaicumpa *et al*., 1991). This discrepancy could be due to variations among laboratories. The present results clearly showed that the 26-kDa protein was a glycoprotein and that the 24-kDa protein was the deglycosylated form of the 26-kDa protein. The fact that the deglycosylated form of the 26-kDa protein was still recognized by the mAb indicated that the mAb reacted with a peptide epitope on the protein.

Immunoprecipitation by human gnathostomiasis antiserum showed that most if not all of the labelled larval proteins including the 26-kDa protein were recognized by the antiserum (fig. 1, lane 6). The antiserum still immunoprecipitated most of the proteins in the extract digested with N-glycosidase F (fig. 1, lane 7). The recognition of the 24-kDa protein in the digested extract by the human antiserum was also observed, albeit with much lower reactivity. The lower reactivities were evident with some of the proteins larger than 65 kDa in the digested extract when compared with those in the undigested extract. The smaller amounts of the 24-kDa protein precipitated by the human antiserum when compared with its glycosylated form may indicate that there are arbitrarily two populations of antibodies to the protein in the antiserum, one against the peptide sequences and the other against the carbohydrate moieties. The two populations of antibodies may act synergistically in the precipitation of the 26-kDa protein. Once the carbohydrate moieties have been removed, the immunoprecipitation of the deglycosylated protein is affected only by antibodies to the peptide epitopes resulting in smaller amounts of the 24-kDa protein being precipitated. Antiserum from the infected rabbit produced patterns of immunoprecipitation similar to the human antiserum except that the 33- and 35-kDa proteins were more strongly recognized by the antiserum from the infected rabbit (fig. 1, lanes 8 and 9). The immunoprecipitation profiles of O-glycosidase-digested extract by the mAb,

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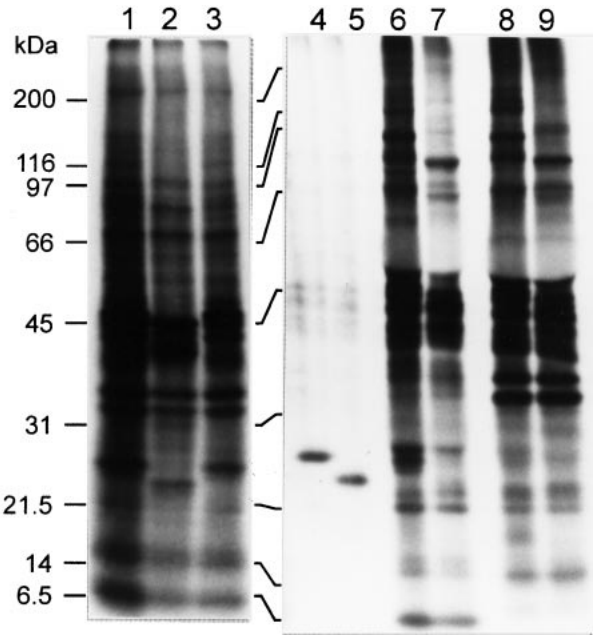

Fig. 1. Immunoprecipitations of ^{35}S-labelled somatic extracts of *Gnathostoma spinigerum* aL3. Total somatic extracts (lanes 1–3), somatic extracts immunoprecipitated by the mAb GN6/24 (lanes 4 and 5), human gnathostomiasis antiserum (lanes 6 and 7), and antiserum from infected rabbit (lanes 8 and 9) were analysed by 8–20% gradient SDS-PAGE. Lanes 1, 4, 6 and 8, undigested extract; lanes 2, 5, 7 and 9, extract digested with N-glycosidase F; lane 3, extract digested with O-glycosidase. The markers (in kilodaltons) on the left margin are broad range protein molecular weight markers (Bio-Rad).

human antiserum, and antiserum from the infected rabbit were similar to those of the undigested extract (data not shown).

From the above results, it is likely that the 26-kDa proteins recognized by the mAb and human gnathostomiasis antiserum are the same protein. However, it does not exclude the possibility that they are different proteins co-migrating at the same position of the gel. An immunodepletion experiment was performed to clarify whether the mAb and human antiserum recognized the same 26-kDa protein. The labelled extract reacted with the human antiserum and the antigen-antibody complexes were removed from the extract by incubating with protein A-Sepharose beads. The procedure was repeated on the remaining extract for ten cycles to ensure complete removal of the proteins reactive to the human antiserum. Equal amounts of the final remaining extract were subjected to immunoprecipitation by the mAb or the same human gnathostomiasis antiserum. The results clearly demonstrated that the 26-kDa proteins recognized by both antibodies were the same protein since there was no 26-kDa protein left in the final immunodepleted extract for immunoprecipitation by the mAb (fig. 2).

In summary, *G. spinigerum* aL3 harbour several N-linked glycoproteins. The deglycosylated forms of these glycoproteins are recognized by the human gnathostomiasis antiserum. One such specific protein recognized by the mAb GN6/24 is the 26-kDa protein (previously designated the 24-kDa glycoprotein (Chaicumpa *et al.*, 1991)). The implication of these findings is that a pool of human gnathostomiasis antisera or this particular mAb will be useful in the immunoscreening of the conventional cDNA library. Further analyses may lead to identification of a cloned gene(s) expressing a protein(s) of diagnostic potential.

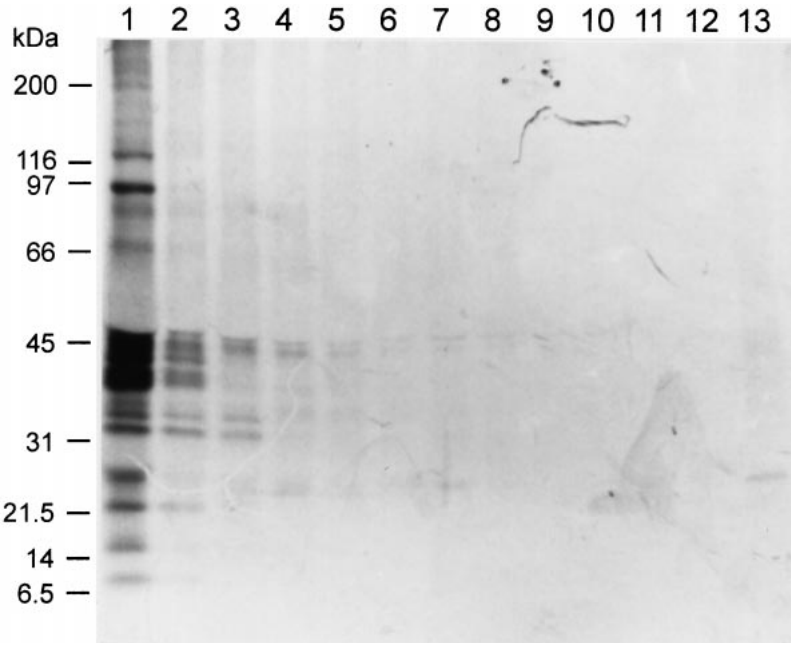

Fig. 2. Immunodepletion of proteins reactive to human gnathostomiasis antiserum. Proteins reactive to human gnathostomiasis antiserum were sequentially removed from ^{35}S-labelled larval somatic extract as described in the text (lanes 1–10). The final immunodepleted extract was immunoprecipitated by the same human gnathostomiasis antiserum (lane 11) or the mAb GN6/24 (lane 12). The extract immunoprecipitated by the mAb GN6/24 served as a positive control (lane 13). The markers (in kilodaltons) on the left margin are broad range protein molecular weight markers (Bio-Rad).

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