ANALYSIS OF ANTIGENS FROM DIFFERENT DEVELOPMENTAL STAGES OF *ANGIOSTRONGYLUS CANTONENSIS*

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INTRODUCTION

Angiostrongylus cantonensis is a rat lungworm that is a causative agent of eosinophilic meningoencephalitis in man in Southeast Asian countries and Pacific islands (Alicata and Jindrak, 1970; Cross, 1979; Punyagupta, 1979). The disease is clinically distinct from eosinophilic myeloencephalitis which is caused by *Gnathostoma spinigerum* (Punyagupta *et al.*, 1968). Currently there is no satisfactory immunological technique to distinguish between these two distinct but closely related clinical entities. Antibodies to adult worm antigens have been detected in the serum and cerebrospinal fluid of patients with eosinophilic meningoencephalitis (Tungkanak *et al.*, 1972; Kamiya, 1975). However, these antigens also gave positive reaction with samples from some patients with eosinophilic myeloencephalitis (Tharavanij, 1979; Tungkanak, 1971). Cross reaction is most likely due to the complex antigenic mosaic of the parasite (for review, Sirisinha *et al.*, 1977). As many as 25 different antigenic components have been detected in the crude extract of adult *A. cantonensis* (Bouthemy *et al.*, 1972; Kamiya *et al.*, 1973). Many of these adult-stage antigens also cross react with those of *Ascaris*, *Caenorhabditis*, *Panagrellus* and *Dirofilaria* species (Bouthemy *et al.*, 1972).

Little information is currently available on the antigens of the earlier developmental stages of *A. cantonensis*. Infected rat and human sera reacted with crude extract of young adults collected from brain and subarachnoidal space of infected rats (Cross, 1978; Dharmkrong-at *et al.*, 1978). These antigens cross reacted also with antiserum to adult worm extract (Sirisinha *et al.*, 1977). Bouthemy *et al.*, (1972) were able to detect as many as 11 antigenic components in the crude extract of fourth-stage larvae (L_4). Techasoponmani and Sirisinha (1980) previously demonstrated that fourth-stage larval somatic antigen gave a reaction of identity in immunodiffusion with ES antigen of adult female worms, suggesting the presence of some common antigens between L_4 and adult worms. Yong and Dobson (1982) presented evidence suggesting that antibodies to adult worms cross-reacted with antigens of third-stage larvae (L_3). In developing a specific and sensitive method for the diagnosis of angiostronglyliasis, it seems more advantageous if one could use a less complicated larval antigen. It is also more logical to use the larval antigen for this parasitic infection as the parasites do not normally develop into adult stage in human (Alicata and Jindrak, 1970). To enhance our understanding on the immunology and serology of angiostrongyliasis, we studied the protein profiles of extracts from different developmental stages by polyacrylamide gel electrophoresis and tried to identify and characterize antigens by radioimmunoprecipitation technique.

MATERIALS AND METHODS

Parasites and hosts: The strain of *A. cantonensis* used in the present study was originally obtained from the Faculty of Tropical Medicine of Mahidol University and has been propagated for several years in our laboratory by cycling it through adult rats

and snails *(Biomphalaria glabratus)*. Procedures for the infection of rats with L_3 and of snails with 1st-stage larvae (L_1) have been described (Techasoponmani and Sirisinha, 1980; Dharmkrong-at, 1977).

Antigens from various developmental stages

Adult worms: Adult worms were obtained from the pulmonary arteries of rats that had been infected with *A. cantonensis* larvae for at least 6 weeks. Male and female worms were separated according to the criteria previously described by Mackerras and Sandars (1955). The worms were homogenized in the presence of small volume of 0.85% NaCl (saline) in a ground glass tissue grinder and were then sonicated in an Ultrasonic Disintegrator (Soniprep 150, MSE Scientific Instrument, Manor Royal, England) set to operate at the maximum amplitude at 1-min intervals. Sonication was continued until most intact cells were broken, as judged from microscopic examination. The sonicate was then centrifuged at 10,000 rpm for 30 min and supernatant fluid was kept at -20°C. All steps of antigen extraction were carried out at 4°C.

First-stage larvae: Pooled faeces from infected rats was loaded on a Baerman apparatus filled with warm saline (37°C.). The larvae that migrated toward the tip of the funnel were collected and were centrifuged at 1,800 rpm for 15 min. The pellet containing larvae and insoluble faecal debris in a conical centrifuge tube was then centrifuged at 600 rpm for 5 min. Under this condition, the faecal debris would settle to the bottom-most portion of the tube, leaving the larvae at the interfacial region. The larvae were gently removed and the procedure was repeated twice more. The larvae prepared as such would also be minimally contaminated with the intestinal flora which under this condition remained in the supernatant fluid. Somatic antigen was extracted from the larvae essentially as described for the adult worms.

Third-stage larvae: Both somatic and metabolic antigens were prepared from L_3 obtained from infected snails as follows. Snails that had been infected with L_1 for approximately 3 weeks were first decontaminated by soaking in 2 changes of distilled water containing penicillin and streptomycin at final concentrations of 500 I.U. and 500 μg per ml respectively. To minimize further contamination, all glasswares and reagents were sterile and all steps were carried out under aseptic condition as much as possible. The snails were crushed and loaded on a Baerman apparatus filled with warm (37°C) digesting fluid (1% pepsin-HCl, pH 2-3). The larvae collected 30 min later were separated from the digesting fluid by gravitational sedimentations at room temperature and were washed several times with saline containing antibiotics before culturing in Eagle's basal medium (BME; GIBCO, Grand Island, N.Y.) at a ratio of 1,800 larvae per ml of medium. The larvae were cultured at 37°C in 5% CO_2 for 7 days with gentle shaking twice a day. At the end of the incubation priod, the cultured fluid was centrifuged at 2,000 rpm for 15 min. The pellet was used for the preparation of somatic antigen (L_3S) by the procedure described for adult worms. The supernatant fluid was concentrated under a nitrogen atmosphere by ultrafiltration, using UM-2 Diaflo membrane (Amicon, Lexington, Mass.). Large volume of saline was added to the concentrated fluid and reconcentrated twice more to get rid of residual culture medium. The concentrated metabolic products (L_3ES) and L_3S were kept frozen at -20°C.

Immune sera: Antisera to different *A. cantonensis* antigens were prepared by infecting rats with a sublethal dose of infective L_3 as well as by immunizing them with somatic antigens prepared from adult female worms (FACE) or third-stage larvae (L_3S), Chronically infected rat serum (CIS) was obtained

from animals that had been infected with *A. cantonensis* for at least 20 weeks. Anti-FACE and anti-L_3S were prepared by first immunizing the animals intraperitoneally with 1,300 µg and 142 µg of antigen in complete Freund's adjuvant respectively. Three weeks later the animals were boosted orally via a stomach tube on 2 consecutive days, each time with 650 µg and 71 µg of antigens in 1.3% $NaHCO_3$ respectively. All animals were bled from retroorbital plexus 1 week later. The anti-*A. cantonensis* activity in these sera were determined by immunodiffusion (Crowle, 1961) and by indirect hemagglutination (IHA, Hirata and Brandriss) techniques. The IHA titer of CIS and anti-FACE against FACE were 1 : 80 and 1 : 20 respectively and that of anti-L_3S against L_3S was 1 : 320.

Polyacrylamide gel electrophoresis (PAGE): Slab gel electrophoresis was carried out in the presence of 1% sodium dodecyl sulfate (SDS) essentially as described by Laemmli (1970). A 15-7.5% linear gradient was prepared and allowed to polymerize at room temperature. A 5% stacking gel was formed on the top of the gradient immediately before the samples were added and the current was applied. Both the unknown and standard proteins were prepared in sample buffer containing 0.0625 M Tris, 1% SDS, 10% glycerol and 5% mercaptoethanol and were heated in boiling waterbath for 2½ min. The gel was stained with 0.25% Coomassie Brilliant Blue; silver stain (Merril *et al.*, 1981) was used when increased sensitivity was required. A combination of SDS-PAGE and autoradiography of radiolabelled antigens was also performed and the patterns obtained with different developmental stages were compared. To calibrate the gel for molecular weight determination, the following protein markers were used; bovine serum albumin (67,000), heavy chain of rabbit IgG (50,000), egg albumin (43,000), myoglobin (16,000), cytochrome C (12,500). Approximate molecular weights were determined by the method of Weber and Osborn (1969).

Radioimmunoprecipitation: Immunoprecipitation of ^{125}I-labelled *A. cantonensis* antigens was performed essentially as described by Ivarie and Jones (1979), using staphylococcal protein A precipitation technique. Antigens were radioiodinated with ^{125}I(Amersham Radiochemical Center, England) by the chloramine-T technique, using a ratio of 2 µCi radioactive iodine per µg protein (modified from Greenwood *et al.*, 1963). *Staphylococcus aureus* Cowan stain I used as immunosorbent were prepared and used in the assay essentially as described by Kessler (1976). To minimize non-specific binding of radioactive antigens to serum proteins and to staphylococcal suspension, all antigens were preadsorbed by incubating the sample with pooled normal rat serum (0.1 µl/µg antigen) and 10% suspension of *S. aureus* (0.5 µl/µg antigen). Autoradiography was performed by exposing Kodak X-ray film (Kodak XRP-1) to the dried gels for different time intervals which depended on the original radioactivity counting.

Protein determination: Protein concentration was determined by a micromodification of the Folin-Ciocalteau tyrosine method (Kabat and Mayer, 1967) using bovine serum albumin as a standard.

RESULTS

Protein profiles: The somatic extracts of first-and third-stage larvae and of adult worm, as well as the metabolic products of third-stage larvae were subjected to SDS-PAGE under both reduced and unreduced conditions. The electrophoretic patterns of the somatic extracts of adult worms were highly complex containing at least 25-30 protein bands with molecular weights ranging

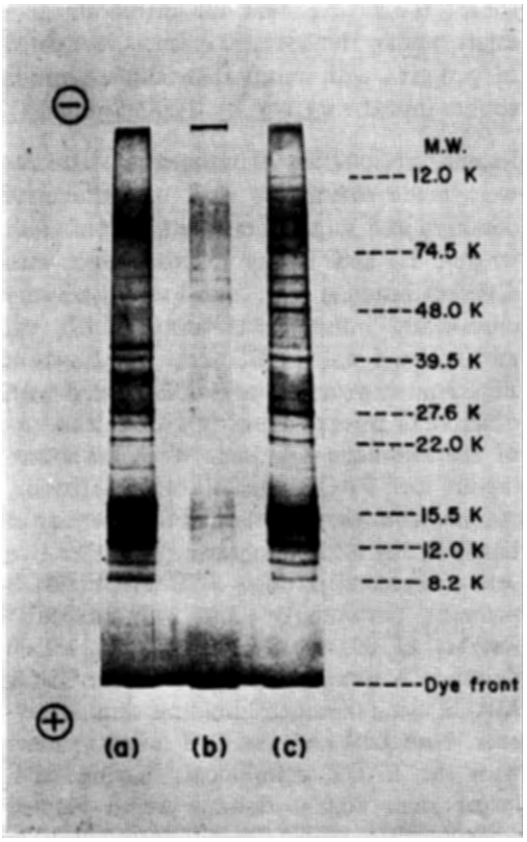

Fig. 1—SDS-polyacrylamide gel electrophoretic patterns of male adult crude extract (MACE, a), third-stage larval extract (L_3S, b) and female adult crude extract (FACE, c). Molecular weight (M.W.) estimations of some major components shown on the right of the patterns were determined as described in Materials and Methods.

from 120,000 to 8,200 daltons (Fig. 1). Some of the major components common to both female and male worms were those with molecular weights of 66,000, 39,500, 27,600, 22,000, 14,200 and 12,000 daltons. However, the peptides with molecular weights below 15,500 daltons predominated, judging from the intensity of staining. The autoradiographic patterns of electrophoresed ^{125}I-labelled extracts were similar to those of the stained unlabelled preparations, both in the number and the relative intensity of bands, and again the proteins with molecular weights below 15,500 daltons were heavily labelled. When the patterns of reduced and unreduced preparations were compared, only slight differences were observed suggesting that the majority of adult worm proteins are composed of single polypeptide chains. However, it is difficult to be certain as in both reduced and unreduced forms the patterns were highly complex.

While most components appeared to be common among the male and female worm extracts, sex-associated components existed. For instance, the female worms possessed many components including those with MW of 48,000, 74,500 and larger than 100,000 daltons that were either absent or present in only trace quantities in the male worms. On the other hand, the male worms possessed 15,500 dalton peptide which was present only in trace quantity in the female worms.

The SDS-PAGE patterns of both unlabelled and ^{125}I-labelled L_1S were surprisingly similar to those of the adult worms. However, the relative intensity of the corresponding bands of the 2 stages differed. The major L_1S components included those with MW of 43,500, 39,500 31,000, 22,000, 17,700 and 11,000 daltons, and many of these components corresponded with the major components found in the adult worms. The latter were those with MW of 39,500 and 22,000 daltons. However, in contrast to the adult worms, L_1S lacked components with MW larger than 100,000 daltons.

The electrophoretic patterns of L_3S were in general similar to those of adult worms (Fig. 1). Many components with identical molecular sizes were present in both stages, including those with 53,000, 43,500, 39,500, 22,000, 15,500 and 8,200 daltons. The one major difference between the 2 developmental stages, particularly obvious when one compared the autoradiographic patterns of labelled preparations, was that the components with MW below 15,500 daltons were not the

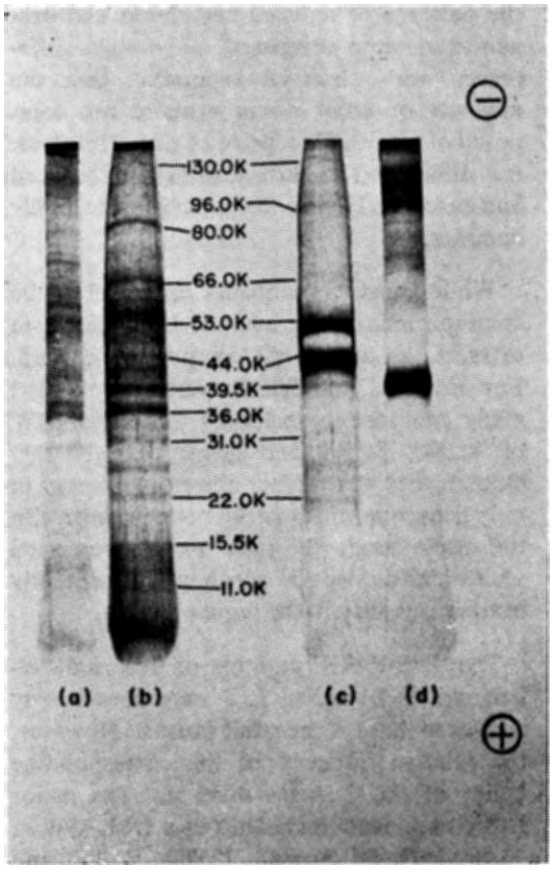

Fig. 2—SDS-polyacrylamide gel electrophoretic patterns of unreduced (a) and reduced (b) third-stage larval somatic extract (L_5S), and of unreduced (d) and reduced (c) metabolic products (L_3ES) of third-stage larvae, see legend to Fig. 1.

predominant components in L_3 stage. The major L_3S component were those with MW of 53,000 and 22,000 daltons. In contrast to the somatic extract, the patterns of both reduced and unreduced metabolic products of L_3 were relatively simple. As shown in Fig. 2, the unreduced L_3ES composed largely of those with MW of approximately 100,000 and 39,500 daltons. However, these 2 major components disappeared upon reduction but 2 new major bands and several minor ones appeared. The new major bands had MW of 53,000 and 43,500 daltons. It appeared from these observations that the metabolic products, unlike the somatic extracts, consisted of proteins with more than single peptide chains joined together by disulfide bond(s).

Immunoprecipitation of antigens: Attempts were made to identify and to characterize common and stage-specific antigens of adult worms, L_3 and L_1 by reacting them with different immune sera. The 3 sera used were chronically infected rat serum (CIS), rat anti-L_3S and anti-FACE sera. As shown in Fig. 3 most proteins in FACE reacted with different degrees of intensity with at least one of the 3 immune sera used. While the homologous anti-FACE reacted most intensely, judging from the number and the intensity of bands in the autoradiograms, the other two sera also reacted strongly with several FACE proteins, particularly those with molecular weights of 80,000, 74,500, 48,000, 15,500 daltons. A similar finding was observed for MACE using the same 3 immune sera. However, both CIS and anti-L_3S failed to react with the FACE components having MW larger than 80,000 daltons which reacted strongly with anti-FACE. Proteins with MW

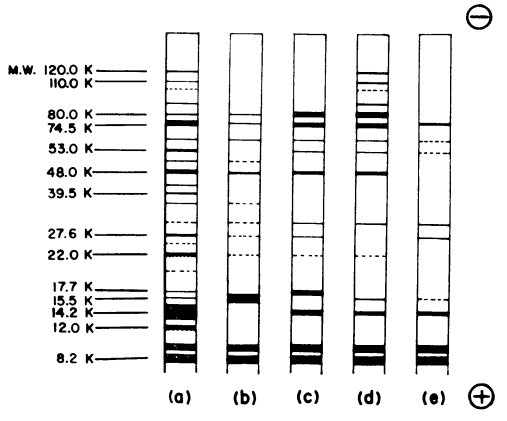

Fig. 3—Diagrams of autoradiographic patterns of ^{125}I-labelled female adult crude extract (a) specifically precipitated with chronically infected rat serum (b), rat anti-L_3S (c) and rat anti-FACE (d). Non-specific adsorption to normal rat serum and *S. aureus* is shown in the right pattern (e).

smaller than 10,000 daltons reacted non-specifically with all 3 immune sera and pooled normal rat serum. One of the proteins in normal rat serum that reacted non-specifically with FACE was IgG (data not shown). The FACE also reacted considerably with the *Staphylococcus aureus* suspension used as a source of protein A.

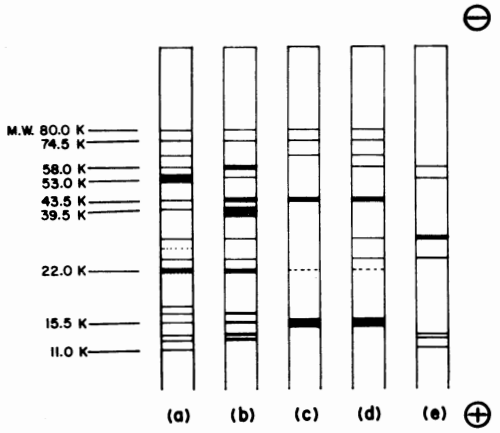

Fig. 4—Diagrams 'of autoradiographic patterns of ^{125}I-labelled third-stage larval extract (a) specifically precipitated with chronically infected rat serum (b), rat anti-L_3S (c) and rat anti-FACE (d). Non-specific adsorption to normal rat serum and *S. aureus* is shown in the right pattern (e).

Unlike FACE, L_3S reacted most intensely with CIS. As shown in Fig. 4, the CIS reacted with most of the labelled L_3S proteins. The strongest reaction was noted with the 39,500 component which surprisingly did not react with either anti-L_3S or anti-FACE. The latter two immune sera on the contrary reacted most strongly with the 15,500 component which reacted only weakly with CIS. There were several other components that reacted with all 3 immune sera and these included the 80,000, 74,500, 43,500 and 22,000 daltons. The low molecular weight L_3S components did not react to a significant degree with any immune or normal sera used. Unlike L_3S, L_3ES reacted strongly with normal rat serum (data not shown). One of the 2 major components, i.e., 53,000 reacted strongly with both immune and normal sera. The 43,500 daltons on the other hand did not react with any serum.

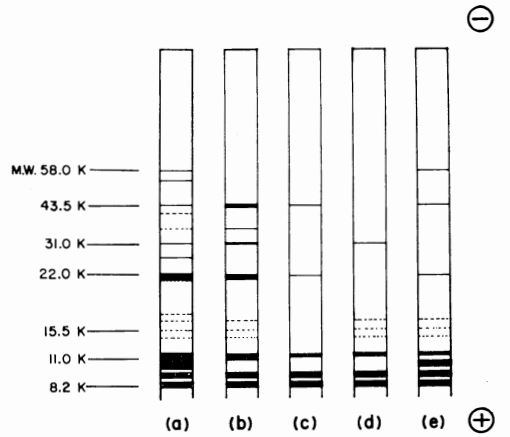

Fig. 5—Diagrams of autoradiographic patterns of ^{125}I-labelled first-stage laval extract (a) specifically precipitated with chronically infected rat serum (b), rat anti-L_3S (c) and rat anti-FACE (d). Non-specific adsorption to normal rat serum and *S. aureus* is shown in the right pattern (e.)

Analysis of L_1S was more difficult as no anti-L_1S was available. However, CIS should have a considerable amount of antibodies reactive with this antigen as L_1 should be continuously present in rat lungs from wk 6 onward. As shown in Fig. 5 CIS reacted strongly with the 43,500, 34,000, 31,000 and 22,000 dalton components. The other 2 immune sera reacted weakly with L_1S. Some of these components reacted also with normal serum. It should be noted that like the low MW components of FACE, the L_1S components with molecular weight less than 11,000 daltons also reacted non-specifically with all sera.

DISCUSSION

Results presented in this study show that somatic extracts of various developmental stages of *A. cantonensis* were highly complex

and that both common and stage-specific antigens could be readily demonstrated. This is not entirely unexpected for a nematode parasite, as similar observations have been documented for serveral other nematodes that have been previously characterized (Pery and Luffau, 1979). Bouthemy *et al.*, were able to distinguish at least 25 different (1972) antigenic components in adult worm extract by immunoelectrophoresis. In the present study we were able to demonstrate by radioimmunoprecipitation a similar number of antigens in adult male and female worms. Using the same technique, it was surprising to find that the antigenic make up of both first-and third-stage larvae were almost as complicated as that of the adults. It was previously reported that at least 11 antigenic components were present in fourth-stage larvae (Bouthemy *et al.*, 1972). The metabolic products produced and secreted by L_3 were on the other hand considerably less complicated than their somatic extract. We had previously reported that the metabolic products of adult female worms were also less complicated than the somatic counterpart (Techasoponmani and Sirisinha, 1980). For both cases, most of the antigens found in the *in vitro* culture medium could also be detected but to a lesser extent in the corresponding somatic extracts, suggesting that they are not stored in any appreciable quantity by living parasites.

Circumstantial evidence previously reported by Dharmkrong-at *et al.*, (1978) and by Yong and Dobson (1982) suggest the presence in *A. cantonensis* of some common antigens among certain developmental stages. In the present study we have shown direct evidence that several common antigens were present in more than one developmental stages. For example, the 22,000 and 39,500 dalton components could be detected in adults as well as in both larval stages, and the 80,000 dalton one was present at least in adult and L_3. Evidence for a cross reaction of antibody to adult worms with L_3 had been present (Yong and Dobson, 1982). The 15,500 dalton protein which was present mainly in L_3S is of particular interest as it stimulated a considerable amount of antibody response (Fig. 4). As this was the preparation that also gave protective immunity in rats (manuscript in preparation), it is possible that this protein represents one of the main functional antigens of this nematode. It should be noted also that the 39,500 dalton L_3S component reacted only with CIS. Because infected human would most likely be exposed to the early-stage antigens more than the adult-stage antigens, it would be of interest to see if the sera from patients with angiostrongyliasis would react with these components and to determine if they could be used for immunodiagnosis.

SUMMARY

Protein profiles of somatic extracts from different developmental stages and excretory and secretory products of third-stage larvae of *Angiostrongylus cantonensis* were analyzed and characterized by polyacrylamide gel electrophoresis in sodium dodecyl sulfate under both reduced and unreduced conditions. Immunological identification of common and stage-specific antigens was determined by radioimmunoprecipitation using chronically infected rat serum and antisera from rats immunized with somatic extracts of third-stage larvae and adult female worms. The somatic extracts of first-and third-stage larvae and of adult worms were found to be highly complex, each consisting of more than 20 different peptides with molecular weights varying from higher than 130,000 to 8,200 daltons. Most proteins were composed of single polypeptide chains. Polypeptides with molecular weights of 80,000, 39,500 and 22,000 daltons were present in more than one developmental stages and were antigenically

related. Those with molecular weight of 15,500 dalton, was present primarily in L_3S and its possible usefulness in immunodiagnosis of infection that occurs in man was discussed.

ACKNOWLEDGEMENTS

This investigation was supported in part by a research grant from the National Research Council of Thailand and by Mahidol University.

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