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Isolation of specific antigens from *Angiostrongylus cantonensis* 1. Preparative flatbed isoelectric focusing

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Abstract. Electrophoresis on SDS gel and analytical isoelectric focusing showed that a crude extract of *Angiostrongylus cantonensis* consisted of at least 40 protein components with molecular weights ranging from 13000–70000 and isoelectric points of pI values ranging from 3.7–10.0. Crossed-immunoelectrophoresis with a hyperimmune antiserum to *A. cantonensis* showed at least 40 different antigenic components in the crude worm extract which were cross-reactive with those of *Ascaris suum, Metastrongylus apri* and *Toxocara canis*. Using preparative isoelectric focusing, the somatic worm preparation was divided into 13 equal fractions, of which 3, 4 and 5, with pI values of 3.7, 4.0 and 4.45 respectively, were later shown by immunoelectrophoretic techniques and enzyme-linked immunosorbent assay to contain antigens specific to *A. cantonensis*.

Introduction

Angiostrongylus cantonensis is an important aetiological agent causing eosinophilic meningoencephalitis in man in Southeast Asia and the Pacific islands. At present, the diagnosis of angiostrongyliasis is generally presumptive especially in cases of light infections when the physician could only rely upon the clinical course of the disease, eosinophilia and epidemiological evidence because the parasite can only infrequently be recovered from the cerebrospinal fluid (CSF) of patients. No significant progress has been made in the development and improvement of serological techniques for an affirmative diagnosis of this zoonosis since the antigens generally employed in laboratories are based on crude worm extracts which invariably cross-react with those of some other helminths (Kamiya 1970; Suzuki et al. 1975). It is obvious that the specificity and sensitivity of an immunodiagnostic system depend mainly on the quality of the antigen used. Therefore, we attempted to isolate specific antigenic components from the crude worm

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extract of adult *A. cantonensis* by the preparative isoelectric focusing techniques.

Materials and methods

The somatic antigenic composition of *A. cantonensis* was initially examined by immunoelectrophoretic techniques, enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT) and immunocytochemical studies. Using these methods, an attempt was also made to determine whether this metastrongyloid nematode shares common antigenic determinants with some other common helminths. The preparative flatbed isoelectric focusing technique (PIEF) was then employed to isolate specific antigens from the crude extract of *A. cantonensis*. To determine the specificity of the isolated fractions, both immunoelectrophoretic techniques and ELISA were used. The molecular sizes and the pH profiles of the antigenic preparations were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analytical isoelectric focusing (AIEF) respectively.

To ascertain the purity of the immunospecific fractions, two New Zealand albino rabbits were hyperimmunized with the purified fractions according to an immunization schedule consisting of a series of five intramuscular and subcutaneous injections into multiple sites of an equal mixture of the appropriate purified fraction at 10-day intervals.

Third-stage *A. cantonensis* larvae obtained by the pepsin digestion of infected snail (*Achatina fulica*) were administered to Wistar albino rats by stomach intubation. Adult male and female worms were recovered from the lungs of infected rats 46 days post-infection. Adult male and female *Ascaris suum, Metastrongylus apri* and *Stephanurus dentatus* were collected from local slaughterhouses whereas *Toxocara canis* were obtained from naturally infected cats. Different stages of *Syphacia obvelata* were obtained from infected laboratory rats.

The crude antigenic saline whole worm extract was prepared as follows: After washing several times in 0.8% saline, 1 g (wet weight) of washed worms was homogenized in 2 ml of 0.8% normal saline using a glass homogenizer immersed in an ice-bath. The suspension was centrifuged at 25000 *g* for 30 min in a refrigerated ultracentrifuge and the supernatant was collected and stored in small aliquots at $-20°$ C.

The infected serum for *S. obvelata* were obtained from infected laboratory rats. The antisera against the whole worm extracts of *A. cantonensis, A. suum, T. canis* and *M. apri* were raised in rats according to an immunization schedule consisting of a series of three i.p. infections of an equal mixture of the appropriate antigenic extracts at 2-week intervals.

The standard normal rabbit serum was prepared from blood obtained from normal albino New Zealand rabbits. The antisera against the normal rat serum and the whole worm extracts of *A. cantonensis, A. suum, T. canis, M. apri* and *S. dentatus* were raised in rabbits according to an immunization schedule consisting of a series of four intramuscular and subcutaneous injections at intervals of 10 days into multiple sites of an equal mixture of the appropriate antigenic extracts.

Immunoelectrophoresis. This was performed according to Grabar and Williams (1953) and Grabar and Burtin (1964). One percent agarose in Tris-barbital-lactate buffer at pH 8.6 on a $8 \times 10 \times 0.2$-cm glass plate was used. Electrophoresis was carried out at 100 V for 2 h. After electrophoresis, antiserum was pipetted into the antiserum trough and immunodiffusion was allowed to proceed for 48 h at room temperatures in a humid chamber. Unreacted proteins were removed by washing overnight with several changes of saline containing sodium azide. The dried gel was stained with 0.5% Coomassie brilliant blue R-250. Excessive stain was removed by washing the plates with a solution of methanol, acetic acid and distilled water in a ratio of 5:1:5.

Rocket immunoelectrophoresis. This was performed according to Svendson (1973) with 1% agarose in Tris-barbital-lactate buffer at pH 8.6. The glass plate was $8 \times 10 \times 0.2$ cm in size. Electrophoresis was carried out at a potential of 25 V for 20 h.

Cross-immunoelectrophoresis. This was performed according to Weeke (1973) with 1% agarose in Tris-barbital-lactate buffer at pH 8.6. The glass plate was $8 \times 10 \times 0.2$ cm in size. Electrophor-

esis in the first dimension was carried out at a potential of 100 V for 2 h. A thin strip of agarose (1 × 8 cm) encompassing the electrophoresed antigens, in the direction of electrophoresis, was left on the plate. Antibody-incorporated agarose solution was poured onto the glass plate to surround the antigen-containing strip. Electrophoresis in the second dimension was performed at a potential of 25 V for 20 h.

Enzyme-linked immunosorbent assay. This was performed basically according to Engvall and Perlmann (1972) and Voller et al. (1976). Anti-rat and human IgG peroxidase-conjugates were purchased from Miles Laboratory (England). The absorbance values were read at 492 nm using a microElisa reader (MSE, England). Flat-bottomed polystyrene microtitre plates (Linbro, Flow Laboratories) were used.

Indirect fluorescent antibody testing. This was performed according to Kawamura (1969). Frozen sections of lung stage (34 days post-infection) of adult *A. cantonensis* were used as antigen. Antisera against various antigenic worm extracts were used to test the cross-reactivity of *A. cantonensis* with other helminths. Fluorescein-isothiocyanate (FITC) conjugated anti-rat IgG was purchased from Wellcome Reagents Ltd. The prepared slides were examined by a Carl-Zeiss epifluorescence microscope equipped with a high-pressure mercury lamp HB050W. The intensity of fluorescence was graded into four classes: − 0 (no fluorescence), 1 (faint fluorescence), 2 (moderate fluorescence) and 3 (intense fluorescence). The highest dilution of antiserum with a grade 2 fluorescence was regarded as the end point. Immunocytochemical analysis was performed according to McLaren et al. 1975; McLaren et al. 1978. Antigen slides were prepared as described for the IFAT procedures. The enzyme-substrate reaction was allowed to proceed at room temperatures for 30 min. Bound enzyme visualized microscopically as a dense brownish deposit was regarded as positive.

Isoelectric focusing. Following the method of Rodola (1973) PIEF was performed on 250 mg protein of *A. cantonensis* in a 3–10 pH gradient for the fractionation of the crude extract. Sephadex IEF gel, Pharmalyte, flatbed apparatus and a PIEF kit were supplied by Pharmacia. Focusing was performed at 30 W constant power for 6 h. Thirteen consecutive fractions of equal volume were cut from the gel and eluted with distilled water and a pH measurement was made on each fraction. The protein solution of each fraction was concentrated and separated from the gel and Pharmalyte by means of a combination of electrophoresis and filtration using an electrophoretic sample concentrator (Isco). Each protein fraction was then stored at −20 C.

Analytical isoelectric focusing. This was performed according to Vesterberg (1972). Polyacrylamide gel rods with an acrylamide concentration of 28% were used. Focusing was carried out at a constant power of 1 W per tube for 6 h at 4 C. After focusing, the gel was fixed in 10% TCA for 1 h. After washing in distilled water, staining was accomplished using a solution of 0.2% (w/v) Coomassie brilliant blue R-250 dissolved in a mixture of ethanol, water and glacial acetic acid (9:10:1, v/v). Destaining was carried out in a solution of ethanol, water and acetic acid (8:11:1, v/v).

SDS-PAGE. This was performed according to Weber and Osborn (1969); 50 µl of protein sample was applied to the gel. Electrophoresis was performed at a constant current of 8 mA per gel for 4 h at 4 C. Coomassie brilliant blue was used for staining (1.25 g in 454 ml of 50% methanol and 46 ml of glacial acetic acid). Overnight destaining was carried out using a solution containing acetic acid, methanol and water (3:2:35).

Results

Cross-reactivity of crude somatic antigens

Immunoelectrophoresis, rocket immunoelectrophoresis and crossed-immunoelectrophoresis revealed that the crude adult extract of *A. cantonensis*

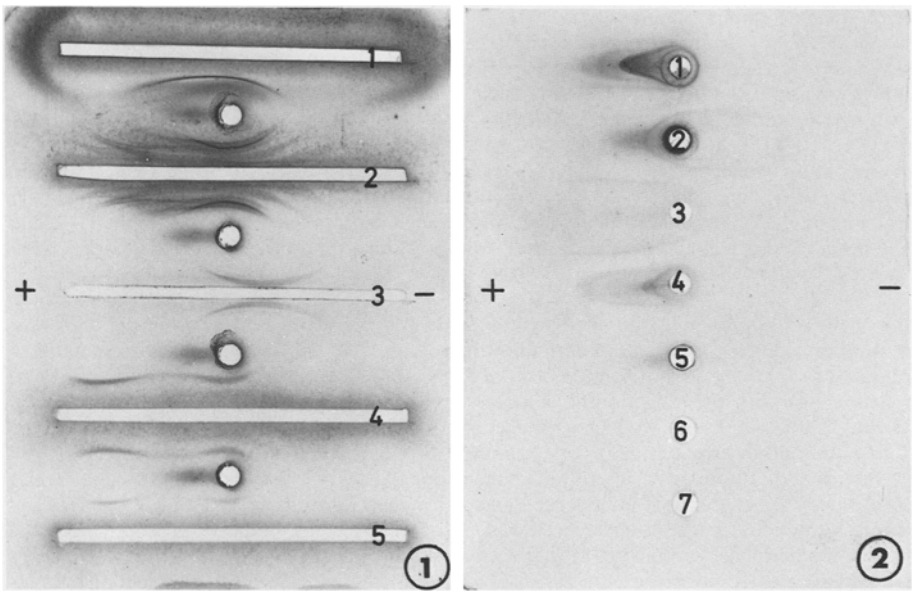


Fig. 1. Immunoelectrophoretic analysis of the antigenic extract of adult *A. cantonensis*. Antigen wells: crude extract of adult *A. cantonensis*. Antiserum troughs, from top to bottom: (*1*) anti-normal rat; (*2*) anti-*A. cantonensis*; (*3*) anti-*A. suum*; (*4*) anti-*T. canis*; (*5*) anti-*M. apri* rabbit antisera

Fig. 2. Rocket immunoelectrophoretic analysis using rabbit anti-*A. cantonensis* as the developing serum. Sample wells, from top to bottom: (*1*) *A. cantonensis*; (*2*) *A. suum*; (*3*) normal rat serum; (*4*) *M. apri*; (*5*) *T. canis*; (*6*) *S. dentatus*; (*7*) *S. obvelata*

shared some common antigenic determinants with normal rat serum, *A. suum, T. canis* and *M. apri* (Figs. 1–3). The crude worm extract of adult *A. cantonensis* was used as antigen while the rabbit antisera against the various worms and normal rat serum were used as antibodies.

Data obtained by ELISA also indicated that *A. cantonensis* actually contained some host (rat) antigens as well as sharing some common antigenic determinants with *A. suum, M. apri* and *T. canis*. The mean ELISA absorbance values at optimal conditions using *A. cantonensis* as antigen and rabbit antisera against the following: normal rat serum, *A. cantonensis, A. suum, T. canis, M. apri* and *S. dentatus* were 0.714, 1.34, 0.645, 0.616, 0.848 and 0.163 respectively. The cut-off value is 0.179 and the negative control is 0.159.

Using cryosections of adult male and female *A. cantonensis* as antigen, rabbit anti-*A. cantonensis* serum as antibody, FITC-conjugated goat anti-rabbit IgG gave a strong positive apple-green fluorescence both at the cuticle and in the internal musculature of the worm (Fig. 4). Positive reactions with specific moderate fluorescence were also observed both at the cuticular surface as well as in the internal musculature of the worm when tested with rabbit anti-normal mouse serum. Using rabbit anti-*A. suum,* rabbit

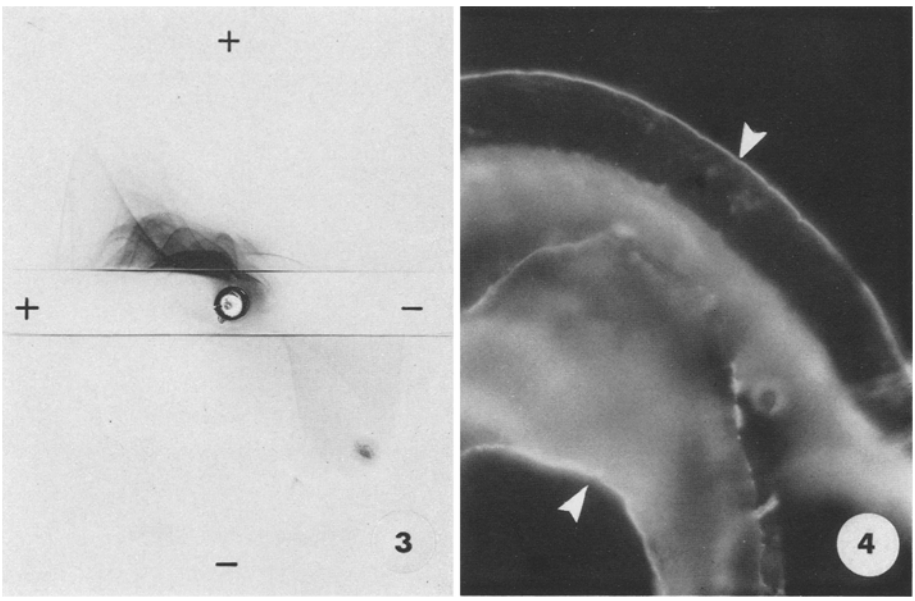


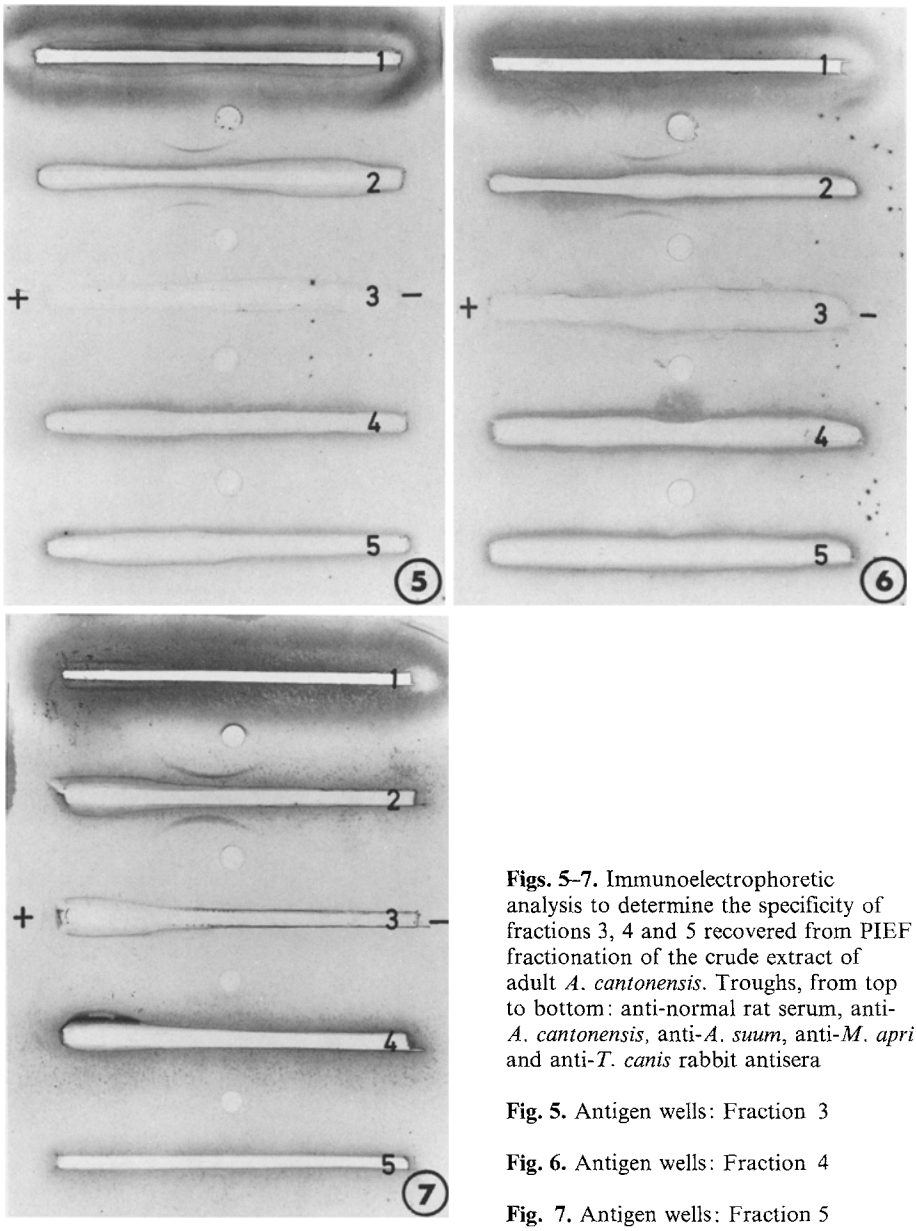
Fig. 3. Crossed-immunoelectrophoretic analysis of the crude worm extract of *A. cantonensis* using anti-*A. cantonensis* as the developing serum, showing at least 40 immunoprecipitin peaks (35 towards the anode and five towards the cathode)

Fig. 4. Indirect fluorescent antibody test using cryosections of *A. cantonensis* adult worms as antigen, rabbit anti-*A. cantonensis* was used as the testing antiserum. Strong positive fluorescence was observed both at the cuticle and in the internal musculature of the worm (*arrows*)

Table 1. The pH values and amount of yields of the 13 fractions obtained by PIEF fractionation of 250 mg protein of antigenic extract of adult *A. cantonensis* in pH 3–10 Pharmalyte

Fraction	pH	Amount yield (mg)
1	3.0	0.06
2	3.45	0.23
3	3.70	3.68
4	4.0	5.28
5	4.45	5.85
6	4.80	8.10
7	5.20	8.40
8	5.90	7.06
9	6.55	8.34
10	7.20	5.40
11	7.85	8.64
12	8.35	8.08
13	10.0	6.10

anti-*M. apri* and rabbit anti-*T. canis* as antibodies, positive reactions with a moderate fluorescence were detected at the internal musculature of worms. Negative reactions were obtained when tested with rabbit anti-*S. dentatus* as well as normal rabbit serum.



Figs. 5–7. Immunoelectrophoretic analysis to determine the specificity of fractions 3, 4 and 5 recovered from PIEF fractionation of the crude extract of adult *A. cantonensis*. Troughs, from top to bottom: anti-normal rat serum, anti-*A. cantonensis*, anti-*A. suum*, anti-*M. apri* and anti-*T. canis* rabbit antisera

Fig. 5. Antigen wells: Fraction 3

Fig. 6. Antigen wells: Fraction 4

Fig. 7. Antigen wells: Fraction 5

Using adult *A. cantonensis* as antigen, rabbit anti-*A. cantonensis* serum as antibody, dense peroxidase deposits were observed both at the cuticular surface and the internal musculature of worms. Similar positive reactions were also noted using rabbit anti-normal rat serum, rabbit anti-*A. suum*, rabbit anti-*M. apri* and rabbit anti-*T. canis* as antibodies. A negative reaction was obtained when tested with rabbit anti-*S. dentatus* serum.

Isolation of specific somatic antigens

Of the 250 mg protein of *A. cantonensis* applied for fractionation by PIEF, the total amount recovered after focusing was 75.22 mg (30.09% yield). Thirteen fractions with different pH and varying amounts of yield were obtained (Table 1).

One immunoelectrophoretic arc was observed each with fraction 3, 4 and 5 when tested with rabbit anti-*A. cantonensis* serum. Using rabbit antisera against normal rat serum, *A. suum, M. apri* and *T. canis* as testing antibodies, no immunoelectrophoretic arc was observed for fractions 3, 4 and 5 (Figs. 5–7).

The crossed-immunoelectrophoretic pattern of fraction 3 using anti-*A. cantonensis* serum indicated the presence of at least two antigenic components. At least three immunoprecipitin peaks were observed using fraction 4 as the antigen and anti-*A. cantonensis* serum as antibodies while fraction 5 was found to have at least six immunoprecipitin peaks.

Further analysis of fractions 3, 4 and 5 using ELISA demonstrated that if these fractions were used as antigens, positive mean absorbance values of 0.483, 0.579 and 0.652 respectively were obtained when tested with rabbit anti-*A. cantonensis* serum. However, negative values were obtained when these fractions were tested against rabbit anti-*A. suum,* rabbit anti-*M. apri,* rabbit anti-*T. canis* and rabbit anti-normal rat serum. The cut-off value is 0.179 and the negative control is 0.159.

Rocket immunoelectrophoretic analysis using hyperimmune rabbit antiserum against fraction 3 revealed at least three rockets each with *A. cantonensis* and fraction 3 as the testing antigens. At least three and six immunoprecipitin rockets were observed using *A. cantonensis* and fraction 4 respectively as antigens and hyperimmune rabbit antiserum against fraction 4 as antibodies. *Angiostrongylus cantonensis* and fraction 5 each revealed the presence of at least six immunoprecipitin rockets when tested with hyperimmune rabbit anti-fraction 5 antibodies. In all instances, no rocket was observed when tested with normal rat serum, *A. suum, T. canis, M. apri, S. dentatus* and *S. obvelata.*

The SDS-PAGE profile of the crude worm extract of *A. cantonensis* revealed the presence of at least 40 protein bands with molecular weights ranging from 13000–70000 (Fig. 8). The SDS-PAGE pattern of fraction 3 showed one protein band having a molecular weight of 31500; for fraction 4, three protein bands corresponding to molecular weights of 18500, 22000 and 23000; for fraction 5, seven protein bands with molecular weights ranging from 10000–51000 (Fig. 8).

The AIEF profile of the crude worm extract of *A. cantonensis* using polyacrylamide gel rods also showed at least 40 protein bands with pI values in the range 3.7–10.0. Fraction 3 and 4 each revealed the presence of two protein bands with pI values of 3.7 and 4.0 respectively while fraction 5 revealed the presence of at least three protein bands having a pI of 4.45.

The ELISA results of tests of the specificity and sensitivity of the purified fractions using sera and CSF samples of a two-year-old boy with clinical

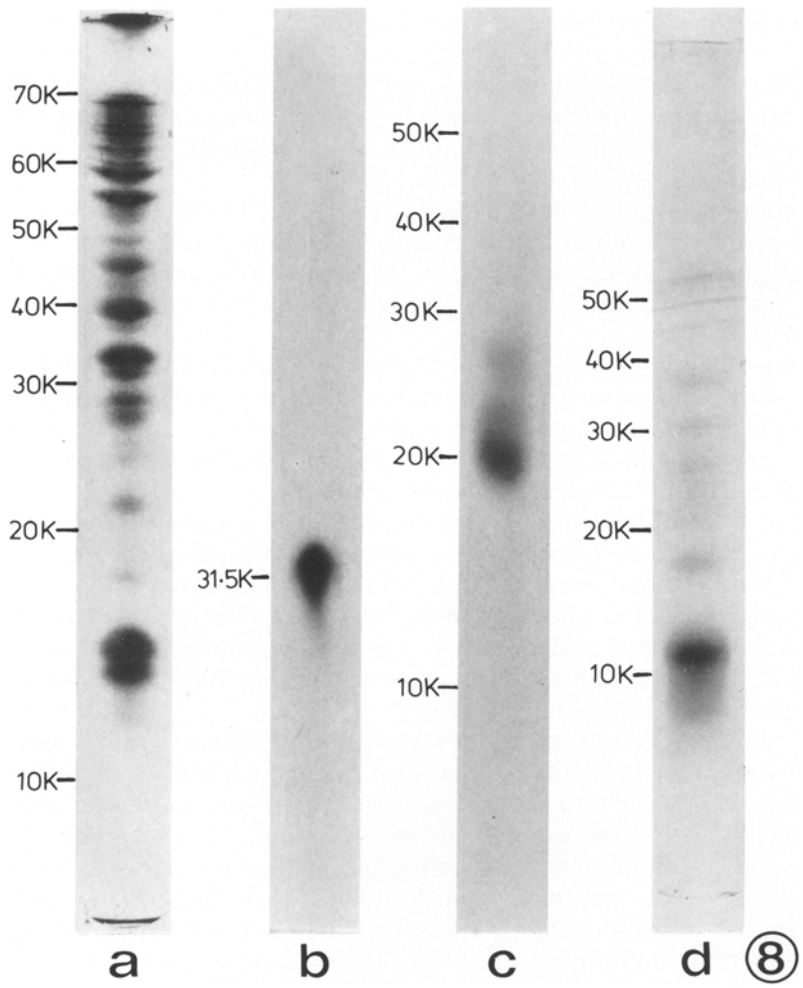


Fig. 8. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis to determine the molecular weights of adult *A. cantonensis,* and fractions 3, 4 and 5 recovered from PIEF fractionation of the crude extract of adult *A. cantonensis.* (*a*) Adult *A. cantonensis,* showing the presence of at least 40 protein bands with molecular weights ranging from 13500 to 70000; (*b*) Fraction 3, showing the presence of one protein band having a molecular weight of 31500; (*c*) Fraction 4, showing the presence of three protein bands corresponding to molecular weights of 18500, 22000 and 23500; (*d*) Fraction 5, showing the presence of seven proteins bands with molecular weights ranging from 10000 to 51000

manifestations of eosinophilic meningoencephalitis are summarized in Table 2. As compared to the crude antigens, the three fractions provided markedly lower but positive absorbance values. For a given sample, the mean absorbance values recorded for all fractions were similar. The values were slightly higher in the sera samples collected on days 20 and 59 than that taken on day 10. The CSF samples also yielded lower absorbance values.

Table 2. Results of the enzyme-linked immunosorbent assay (ELISA) for IgG antibodies using purified (fractions 3, 4 and 5) and crude antigens against the sera and cerebrospinal fluid samples (CSF) of a 2-year-old boy with clinical manifestations of eosinophilic meningoencephalitis

Sample No.	Time collected (days after admission into hospital)	Antigens[a] used (3 μg/ml)	Mean absorbance values (OD) 1:		
			1,600[b]	3,200	25,600
1S[c]	10	C	1.57	1.4	0.356
		Fr.3	0.52	–	–
		Fr.4	0.538	–	–
		Fr.5	0.573	–	–
2S	20	C	1.82	1.45	0.379
		Fr.3	0.626	–	–
		Fr.4	0.681	–	–
		Fr.5	0.693	–	–
3S	59	C	over 2	1.9	0.476
		Fr.3	0.642	–	–
		Fr.4	0.696	–	–
		Fr.5	0.765	–	–
Negative control		C	0.134	0.118	0.115
		Fr.3	0.060	–	–
		Fr.4	0.052	–	–
(10)[d]		Fr.5	0.057	–	–
1CSF	20	C	0.523	0.272	0.115
		Fr.3	0.409	–	–
		Fr.4	0.435	–	–
		Fr.5	0.489	–	–
2CSF	36	C	0.312	0.210	0.103

[a] C, crude; Fr, fraction
[b] Dilution of samples
[c] S, serum
[d] No. of samples tested

The tests were carried out simultaneously with normal Chinese sera and those from individuals known to be infected with other parasitic diseases (such as trichinellosis, clonorchiasis, ascariasis, hookworms and trichomoniasis); negative results were obtained for the latter (Table 3). Due to limited quantities, the purified fractions were only used for the serum and CSF samples diluted to 1:1600.

Discussion

The present study shows that the crude soluble somatic antigenic extract of *A. cantonensis* is highly heterogeneous, containing components capable of eliciting immune responses with varying specificity in rabbits and rats.

Ideally for antigenic cross-reactive studies of *A. cantonensis*, other closely related metastrongyloid nematodes or other parasites of human tissue

Table 3. Comparison of enzyme-linked immunosorbent assay (ELISA) for IgG antibodies; results between patients infected with various parasitic diseases using purified *A. cantonensis* antigens. (Serum dilution at 1:1600)

Serum samples	Antigens[a] (3 μg/ml)	Mean absorbance values (OD)
Angiostrongyliasis (1)[b]	C	1.57
	Fr.3	0.52
	Fr.4	0.538
	Fr.5	0.573
Clonorchiasis (19)	C	0.589
	Fr.3	0.064
	Fr.4	0.082
	Fr.5	0.094
Trichinellosis (18)	C	0.523
	Fr.3	0.052
	Fr.4	0.070
	Fr.5	0.084
Ascariasis (9)	C	0.569
	Fr.3	0.082
	Fr.4	0.091
	Fr.5	0.098
Hookworms (1)	C	0.378
	Fr.3	0.073
	Fr.4	0.084
	Fr.5	0.112
Trichomoniasis (3)	C	0.495
	Fr.3	0.067
	Fr.4	0.088
	Fr.5	0.097

[a] C, crude
[b] No. of samples tested

should be compared. However, due to the difficulty in obtaining significant quantities of such materials, the present study was unfortunately restricted to the only readily available local species. Nevertheless, our observations demonstrate that the crude extract of *A. cantonensis* shared some common antigens with *A. suum, T. canis* and *M. apri.* The occurrence of host (rat) antigens in the worm extract is probably due to the fact that the adult *A. cantonensis* feeds on blood. The absence of cross-reactions between *A. cantonensis, S. obvelata* and *S. dentatus,* on the other hand, probably indicates that they have different phylogeny and biology. Bouthemy et al. (1972) and Suzuki et al. (1975) also reported cross-reactions with *Caenorhabditis, Pangrellus, Dirofilaria, Ascaris, Anisakis* and *Toxocara.*

The results of both cytochemical and IFAT studies revealed that the parasite antigens and host components occurred on the cuticular surface and internal structures of *A. cantonensis* whereas antigens in common with *A. suum, T. canis* and *M. apri* were located only in the somatic musculature.

Therefore it appears that surface antigens of *A. cantonensis* may be exploited for specific identification. However, further studies are required to validate such an interpretation especially since little is known concerning the origin of cuticular antigens in nematodes.

As demonstrated by the various immunoelectrophoretic patterns, ELISA and hyperimmunization of rabbits, fractions 3, 4 and 5 contain specific antigenic determinants although it is not known which fraction contains a larger number of protection-inducing antigens. The SDS-PAGE profile showed more protein bands in fractions 5 than 3 and 4 suggesting that fraction 5 contains antigenic determinants possessing a wider range of molecular weights. It is possible, however, that several groups of antigens may possess protection-inducing activities as suggested by Despommier (1981) who analyzed the antigens of *Trichinella spiralis*.

The reliability of fractions 3, 4 and 5 for use in serodiagnosis was successful as confirmed in the test using the sera and CSF samples of a local two-year-old boy with clinical manifestations of eosinophilic meningoencephalitis. As compared to the crude extracts, all the fractions yielded a lower but distinctly positive ELISA mean absorbance value. Low negative values were obtained when the fractions were tested against sera from persons infected with other parasitic diseases, thus indicating a good specificity. It was unfortunate that a larger number of proven angiostrongyliasis cases were not available. Until 1983, such a disease was thought to be absent (Ko et al. 1984) from this area.

References

Bouthemy F, Capron A, Afchain D, Wattre P (1972) Structure antigenique du Nematode *Angiostrongylus cantonensis*. Aspects immunologiques des relations hôte-parasite. Ann Parasitol Hum Comp 47:531–550

Despommier DD (1981) Partial purification and characterization of protection-inducing antigens from the muscle larva of *Trichinella spiralis* by molecular sizing chromatography and preparative flatbed isoelectric focusing. Parasite Immunol 3:261–272

Engvall E, Perlman P (1972) ELISA: quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. J Immunol 109:129–135

Grabar P, Burtin P (1964) Immunoelectrophoretic analysis. Applications to human biological fluids. Elsevier Pub Co New York NY, pp 30–57

Grabar P, Williams CA (1953) Methods permettant l'étude conjugue' des proprietes électrophoretiques et immunochemique d'un mélange de proteins; application au serum sanguin. Biochim Biophys Acta 10:193

Kamiya M (1970) Haemagglutination test in rats infected with *Angiostrongylus cantonensis*. Southeast Asian J Trop Med and Publ Hlth 1(4):570–571

Kawamura A Jr (1969) Fluorescent antibody techniques and their applications. University of Tokyo, Tokyo, Japan, p 203

Ko R, Chiu M, Kum W, Chan S (1984) First report of human angiostrongyliasis in Hong Kong diagnosed by computerized axial topography (CAT) and enzyme linked immunosorbent assay. Trans Roy Soc Trop Med Hyg 78:354–355

McLaren DJ, Clegg JA, Smithers SR (1975) Acquisition of host antigens by young *Schistosoma mansoni* in mice: correlation with failure to bind antibody in vitro. Parasitology 70:67–75

McLaren DJ, Hockley DJ, Goldring OL, Hammond BJ (1978) A freeze fracture study of the developing tegumental outer membrane of *Schistosoma mansoni*. Parasitology 76:327–348

Rodola BJ (1973) Analytical and preparative isoelectric focusing in gel-stabilized layers. Ann NY Acad Sci 209:127–143

Suzuki T, Sato Y, Yamashita T, Sekikawa H, Otsuru M (1975) *Angiostrongylus cantonensis:* Preparation of a specific antigen using immunosorbent columns. Exp Parasitol 38:191–201

Svendson PJ (1973) Fused rocket immunoelectrophoresis. In: Axelsen NH, Kroll J, Weeke B (eds) A manual of quantitative immunoelectrophoresis: methods and application. Universitetsforlaget Oslo, Norway, p 69

Vesterberg O (1972) Isoelectric focusing of proteins in polyacrylamide gels. Biochim Biophys Acta 257:11–19

Voller A, Bartlett A, Bidwell DE (1976) Enzyme immunoassays for parasitic diseases. Trans Roy Soc Trop Med Hyg 70:98–106

Weber K, Osborn M (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 244 (16):4406–4412

Weeke B (1973) Crossed immunoelectrophoresis. In: Axelsen NH, Kroll J, Weeke B (eds) A manual of quantitative immunoelectrophoresis: methods and applications. Universitetforlaget Oslo Norway, p 47

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