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Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *Angiostrongylus cantonensis*

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Abstract
The enzyme-linked immunosorbent assay (ELISA) was employed for the sero-diagnosis of *Angiostrongylus cantonensis* infections in rats and man. Metabolic and 'purified' antigens of adult or juvenile *A. cantonensis* were evaluated by ELISA and compared against *Toxocara canis* antigen for sensitivity and specificity. Sera and cerebrospinal fluid (CSF) from both rats and man gave higher ELISA values against antigens of *A. cantonensis* than those obtained from negative control samples. The results showed that cross reaction occurred between *T. canis* and *A. cantonensis*. Of four *A. cantonensis* antigens tested, a Sephacryl S-300 "purified' fraction was shown to be superior in the ELISA to determine angiostrongyliasis.

Introduction

Angiostrongylus cantonensis (Chen, 1933) is a causative agent of human eosinophilic meningitis in South-east Asia and the Pacific Islands. The characteristic symptoms of patients with this disease are often confused with other central nervous disorders and as a result few cases of angiostrongyliasis would be diagnosed. Proven cases of human infection showing worms from cerebrospinal fluid (CSF) are rare (CHEN, 1979) and a reliable diagnostic assay, specific for angiostrongyliasis, is urgently needed to confirm diagnosis.

Recently, THARAVANIJ, (1979) reported that ELISA using an *A. cantonensis* antigen prepared from larvae recovered from rat brain, was a most promising technique for the sero-diagnosis of angiostrongyliasis. WELCH *et al.* (1980) reported the successful development of both serum and cellular immunity assays to determine infection with *A. cantonensis* in animal and human hosts.

This study is an assessment of the sensitivity and specificity of ELISA in the diagnosis of *A. cantonensis* infections in rat and man by using crude and purified antigens prepared from adult and juvenile *A. cantonensis*.

Materials and Methods

Antigen preparation

Infective larvae of *A. cantonensis* were collected as described previously (CHEN *et al.*, 1981). Albino rats were orally infected with the larvae using a small rubber tube. Juvenile and adult worms of *A. cantonenis* were obtained from the subarachnoid space of brain and pulmonary artery of rats at 22 or 80 days after infection, respectively. Adult *T. canis* were collected from intestines of infected dogs.

Motile *A. cantonensis* and *T. canis* were washed three times in normal saline and then homogenized in 0·05 M carbonic buffer solution (pH 9·6) using a glass microhomogenizer (Belco, Vineland, New Jersey, USA). Subsequently, the homogenized materials were centrifuged at 10,000 *g* (4°C) for 30 min. The supernatants were measured for the protein concentration by utilizing Folin reagent as described by LOWRY *et al.* (1951) and stored at −70°C.

Washed, live adult *A. cantonensis* were incubated in a medium containing normal saline plus 0·5% glucose at 37°C for 24 hours for the preparation of metabolic antigen. The culture medium was collected and the protein concentration in the medium was measured as described above.

Gel filtration

The crude antigen of adult *A. cantonensis* was fractionated on Sephacryl S-300. The gel was equilibrated with 0·05 M carbonic buffer, pH 9·6 and packed in a 2·6 × 36 cm column at 4°C. The antigen containing approximately 20 mg of total protein was mixed with 2 ml of 0·05 M carbonic buffer, applied to the column and eluted with the same buffered solution. Fractions of 2 ml/tube were collected at a flow rate of 11 ml cm^{-2} hr^{-1}. These were monitored photometrically at 280 nm by using a Gilford 250 Spectrophotometer. Each fraction was then used as an antigen in the ELISA.

Selected fractions were collected, concentrated using celluphane tubes and dialysed in 0·05 M carbonic buffer (pH 9·6). Protein concentrations were then measured on each fraction, called 'purified' antigen. These purified antigens were used in subsequent experiments.

Antisera

Sera from rats were obtained before, and then at weekly intervals, after oral infection (eight albino rats each infected with 100 third-stage larvae). Positive human sera and CSF were obtained from three patients with parasitologically proven *A. cantonensis* in the CSF. Sera and CSF from five confirmed Japanese encephalitis patients were employed as negative controls. The samples were obtained from the National Taiwan University Hospital.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was performed in polystyrene EIA microtitration plates containing 96 flat-bottomed wells (Linbro, Flow Laboratories, Inc., McLearn, Virginia, USA). The procedures used were modified after those described by VOLLER *et al.* (1974).

The optimal serum dilution was found to be 1:50 in phosphate buffered saline (PBS). Carbonic buffer (pH 9·6) was also suitable for the coating procedure and this system was employed for the detection of antibodies from infected rat and human sera and CSF.

For coating procedures, 0·1 ml of each crude antigen with a total protein concentration of 25 µg/ml was added to each well. The plates were then sealed, incubated at 4°C overnight and washed three times with PBS containing 0·05% Tween 20 (PBST). Serial dilutions of test sera were then added immediately to the wells. The plates were incubated at 37°C for three hours and then washed with PBST. After shaking dry, 0·1 ml peroxidase-labelled rabbit anti-human (1:200 dilution) or anti-rat immunoglobulin conjugate in PBS (1:50 dilution) was added and incubated for four hours at 37°C. Anti-human and anti-rat rabbit sera were purchased from US Biochemical Corporation and the substrate from Sigma Chemical Co., St. Louis, Missouri, USA. Excess conjugate

was removed by washing five times with PBST. Bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, Missouri, USA) was used as a control.

The detection of antibody against *A. cantonensis* in human sera and CSF was done by coating each well with 0·1 ml of 'purified' antigen at a protein concentration of 25 μg/ml. Fresh substrate solution was prepared by dissolving 10 mg of 0-phenylenediamine in 0·1 M PBS containing 1 μl 30% H_2O_2 and then added into each well. After incubation at room temperature for 50 min, the colour reaction was stopped by the addition of 0·05 ml 1N NaOH. The absorbance in each well was read at 450 nm using a Titertek Multiskan (Flow Laboratories).

Values are expressed as two ratios (VOLLER *et al.*, 1979 and YOLKEN *et al.*, 1977) calculated from ELISA readings for both tested and referenced sera.

Results

Optimal buffered solutions for antigens and antibodies

EIA microplates were incubated with adult *A. cantonensis* antigen diluted in carbonic buffer and carbonic buffer plus 1% BSA, then reacted with anti-serum in various buffered solution including PBS, PBST and PBST + 1%BSA. The results of ELISA showed that there were no significant differences in coating efficiency and in the reactions between antigen and antibody by using different buffered solutions or supplementations. Therefore PBS and carbonic buffer were used for all further experiments.

Animal experiments

Serum from eight laboratory-infected rats showed that antibodies to *A. cantonensis* could be demonstrated from three weeks to eight weeks following the ingestion of the third-stage larvae. The sera from all infected animals showed ELISA values that increased with time after infection. Significant decrease in ELISA value was observed with the increase of serial dilutions ranged from 2^{-2} to 2^{-11}.

Significantly higher ELISA values were detected in sera from infected rats (three weeks and up to 26 weeks after infection) than in non-infected control rat sera (Figs. 1 and 2).

Of the three *A. cantonensis* crude antigens used, the highest ELISA values were observed with the adult worm antigen. High ELISA values were determined with *A. cantonensis* positive sera against *T. canis* antigen which suggested considerable cross reaction between these two nematodes (Fig. 2).

Table I gives the ratios calculated from the ELISA values obtained from sera of normal and infected animals. The results show that positive sera gave higher ratios than normal sera. In addition these ratios indicated that adult worm antigen was superior to the other crude antigens.

The results obtained after fractionation of adult worm antigen using Sephacryl S-300 showed five protein peaks detected at 280 nm. When individual fractions of 2 ml were tested against the infected rat sera, the higher ELISA value was obtained in the second high molecular weight. In comparison, low ELISA values were obtained with the same fraction reacted with the normal rat sera. The fractions with lower molecular weight revealed little specificity against *A. cantonensis* infected rat sera (Fig. 3).

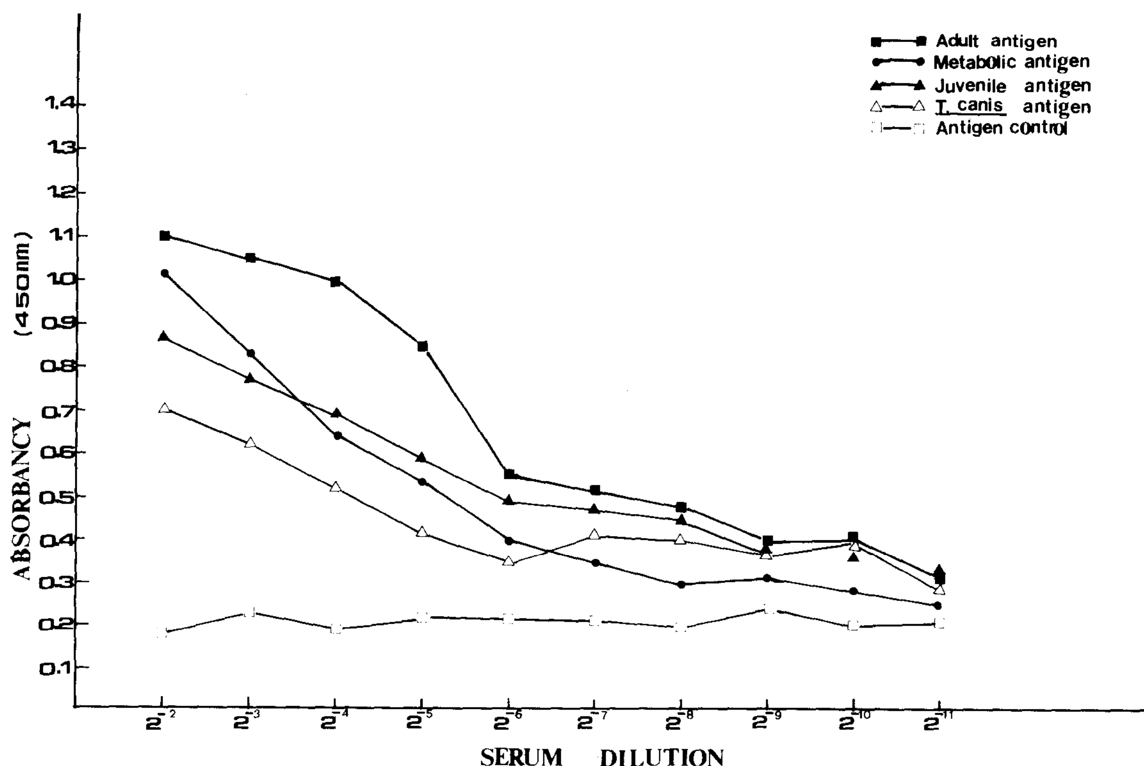

Fig. 1. The ELISA values of sera obtained from 8 rats infected with 100 *Angiostrongylus cantonensis* infective larvae at 8 weeks after infection by using adult worm, juvenile worm or metabolic antigen of *Angiostrongylus cantonensis* and *Toxocara canis* antigen.

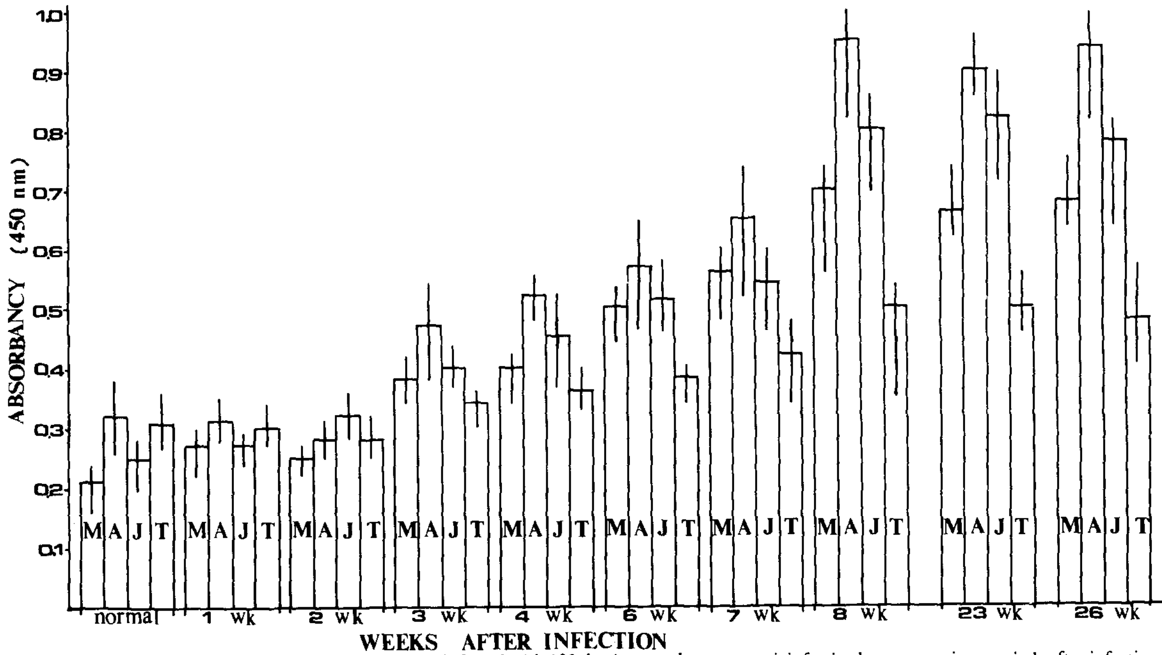

Fig. 2. The ELISA values of sera obtained from 8 rats infected with 100 *Angiostrongylus cantonensis* infective larvae at various periods after infection by using adult worm, juvenile worm or metabolic antigen of *Angiostrongylus cantonensis* and *Toxocara canis* antigen. M: Metabolic antigen of *A. cantonensis* A: Adult worm antigen of *A. cantonensis* J: Juvenile worm antigen of *A. cantonensis* T: Adult worm antigen of *Toxocara canis*.

ELISA Value for Positive Serum
ELISA Value for Negative Serum
Elution Pattern of Adult Worm Antigen

O. D. 280

ABSORBANCY AT 450nm

FRACTION NO.

Fig. 3. The gel filtration of adult worm antigen of *Angiostrongylus cantonensis* on Sephacryl S-300 column (2·6 × 36 cm, in carbonic buffer, pH 9·6) and the ELISA value for each eluate against normal and infected rat sera. The eluates were collected at a volume of 2 ml per tube in a flow rate of 11 ml cm^{-2} hr^{-1} and monitored at 280 nm. Each ELISA value was obtained from 8 rats before and after infection of 100 *Angiostrongylus cantonensis* infective larvae. Each eluate was coated on microplate without measuring the protein concentration.

Table I—The enzyme-linked immunosorbent assay (ELISA) results of sera from *Angiostrongylus cantonensis* infected and uninfected rats using four different antigens

Antigens	Adult *Toxocara canis*			*Angiostrongylus cantonensis* Adult			*Angiostrongylus cantonensis* Juvenile			*Angiostrongylus cantonensis* Metabolite		
Antisera	E_{450}	Voller's Ratio	Yolken's Ratio	E_{450}	Voller's Ratio	Yolken's Ratio	E_{450}	Voller's Ratio	Yolken's Ratio	E_{450}	Voller's Ratio	Yolken's Ratio
Negative	0·358			0·284			0·256			0·202		
1 week	0·330	0·92	0·87	0·292	1·23	1·61	0·255	1·00	0·99	0·221	1·09	1·37
2 week	0·332	0·93	0·88	0·330	1·39	2·05	0·254	1·00	0·98	0·228	1·13	1·50
3 week	0·355	0·99	0·99	0·432	1·82	3·20	0·344	1·34	1·83	0·370	1·83	4·23
4 week	0·373	1·04	1·07	0·448	1·88	3·39	0·386	1·51	2·23	0·377	1·87	4·37
6 week	0·425	1·19	1·32	0·497	2·09	3·94	0·490	1·91	3·20	0·408	2·02	4·96
7 week	0·387	1·08	1·14	0·540	2·27	4·43	0·550	2·14	3·77	0·463	2·30	6·02
8 week	0·395	1·10	1·18	0·940	3·95	8·98	0·167	2·40	4·40	0·492	2·44	6·58
23 week	0·514	1·44	1·75	0·877	3·68	8·26	0·656	2·56	4·77	0·516	2·55	7·04
26 week	0·599	1·67	2·16	0·912	3·83	8·66	0·673	2·63	4·93	0·593	2·94	8·52

$$\text{Voller's Ratio} = \frac{\text{absorbance value of sample}}{\text{absorbance value of reference negative sample}}$$

Yolken's Ratio: $P/N = (E_{sample}\text{-}E_{blank}) \div (E_{negative}\text{-}E_{blank})$
The average of E_{blank}: 0·150

Antisera were collected from rats following different periods (as described in the table) of infection by the ingestion of *Angiostrongylus cantonensis* infective larvae and tested for the ELISA value in a dilution of 1:50 in PBS.

Negative sera were collected from rats before infection of *Angiostrongylus cantonensis* and tested for the ELISA value in a dilution of 1:50 in PBS.

Each value was obtained from 8 experimental rats

Table II—The enzyme-linked immunosorbent assay (ELISA) results of sera from *Angiostrongylus cantonensis* infected and uninfected rats using four different antigens

Antigens	Adult *Toxocara canis*			*Angiostrongylus cantonensis*								
				Adult			Juvenile			Metabolite		
Antisera	E_{450}	Voller's Ratio	Yolken's Ratio	E_{450}	Voller's Ratio	Yolken's Ratio	E_{450}	Voller's Ratio	Yolken's Ratio	E_{450}	Voller's Ratio	Yolken's Ratio
Negative	0·358			0·284			0·256			0·202		
1 week	0·330	0·92	0·87	0·292	1·23	1·61	0·255	1·00	0·99	0·221	1·09	1·37
2 week	0·332	0·93	0·88	0·330	1·39	2·05	0·254	1·00	0·98	0·228	1·13	1·50
3 week	0·355	0·99	0·99	0·432	1·82	3·20	0·344	1·34	1·83	0·370	1·83	4·23
4 week	0·373	1·04	1·07	0·448	1·88	3·39	0·386	1·51	2·23	0·377	1·87	4·37
6 week	0·425	1·19	1·32	0·497	2·09	3·94	0·490	1·91	3·20	0·408	2·02	4·96
7 week	0·387	1·08	1·14	0·540	2·27	4·43	0·550	2·14	3·77	0·463	2·30	6·02
8 week	0·395	1·10	1·18	0·940	3·95	8·98	0·167	2·40	4·40	0·492	2·44	6·58
23 week	0·514	1·44	1·75	0·877	3·68	8·26	0·656	2·56	4·77	0·516	2·55	7·04
26 week	0·599	1·67	2·16	0·912	3·83	8·66	0·673	2·63	4·93	0·593	2·94	8·52

$$\text{Voller's Ratio} = \frac{\text{absorbance value of sample}}{\text{absorbance value of reference negative sample}}$$

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The average of E_{blank}: 0·150

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Negative sera were collected from rats before infection of *Angiostrongylus cantonensis* and tested for the ELISA value in a dilution of 1:50 in PBS.

Each value was obtained from 8 experimental rats.

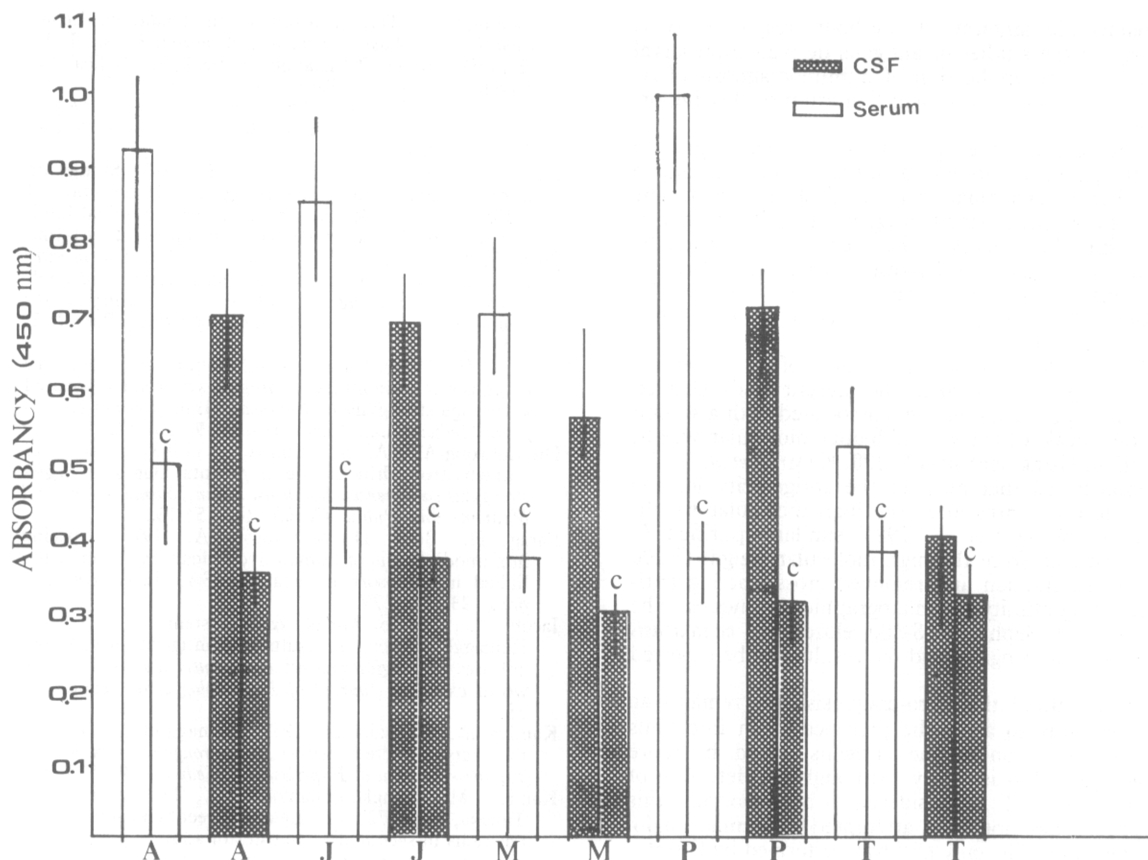

Fig. 4. The ELISA values of normal and *Angiostrongylus cantonensis* infected sera and cerebrospinal fluids, determined using adult worm, juvenile worm, metabolic or 'purified' antigen of *A. cantonensis* and adult *Toxocara canis* antigen. The 'purified' antigen was obtained by the filtration on Sephacryl S-300 using carbonic buffer, pH 9·6 and coated on the microplate at a total protein concentration of 25 μg per well. A: adult worm antigen of *A. cantonensis*, J: Juvenile worm antigen of *A. cantonensis*, M: Metabolic antigen of *A. cantonensis*, P: 'purified' antigen of *A. cantonensis*, T: Adult *T. canis* antigen, C: Negative control. The values were obtained from 3 proven cases of *A. cantonensis* infection and 5 normal individuals.

Human sera and cerebrospinal fluid (CSF)

The ELISA values of sera and CSF from three *A. cantonensis* infected patients at 1:50 dilution were compared with those obtained from five control individuals. Five antigen preparations (*A. cantonensis*; adult and juvenile crude worm antigen, a metabolic antigen and a purified antigen, and *T. canis* crude worm antigen) were employed in this study. The sera exhibited higher ELISA values than the corresponding CSF sample from each individual tested. The highest values recorded for both sera and CSF were obtained with 'purified' antigen. However, the values obtained using 'purified' antigen against control sera were lower than those obtained with adult or juvenile antigen (Fig. 4). The patients with angiostrongyliasis also gave higher ELISA values (CSF and serum) against *T. canis* antigen than the negative controls.

Discussion

In studies of laboratory diagnosis of eosinophilic meningitis associated with angiostrongyliasis, CROSS (1978) reported that in all parasitologically confirmed and presumptive cases, ELISA determinations on sera from these individuals were significantly higher than for the control sera. Similarly, in the present study positive and negative sera and CSF of man and experimentally infected rats showed clear differences in ELISA value. These results confirmed that ELISA can be used to detect and measure antibody in the determination of *A. cantonensis* infections.

In comparison with juvenile worm antigen of *A. cantonensis*, adult worm antigen exhibited a better sensitivity for detection of antibodies in the infected cases. The higher ELISA values in infected rat sera were maintained up to 26 weeks after infection. This suggested that antigenic stimulation was mainly a result of material released by adult worms. A similar suggestion was made by KAMIYA & TANAKA (1969) who found that the appearance of detectable antibody coincided with appearance of larvae in the faeces. Other workers (JACOBS *et al.*, 1965; KAMIYA *et al.*, 1972) demonstrated that antigenic factors are associated with the adult female, but not adult male, worms. using polyacrylamide gel and radioimmunoprecipitation techniques DHARMKRONG-AT & SIRISINHA (1983) demonstrated that polypeptides with molecular weights of 80,000, 39,500 and 22,000 were present in more than one developmental stage and were antigenically related. The 15,500 dalton protein was present primarily in L3s and stimulated a

considerable amount of antibody response. It is therefore the studies of antigens derived from larval stages on the application of immunodiagnosis of *A. cantonensis* infection in man that are probably important.

The present investigation showed that Sephacryl S-300 'purified' antigen was superior to crude antigen in ELISA determination for angiostrongyliasis. The need for highly purified antigens for ELISA diagnosis of helminthic infections has been demonstrated by other investigators for hydatid disease, onchocerciasis and schistosomiasis in man (FARAG *et al.*, 1975; BARTLETT *et al.*, 1975; BARAKAT *et al.*, 1983; VOLLER *et al.*, 1976).

The purification of adult antigen of *A. cantonensis* using Sephacryl S-300 in the present study showed that the specific antigen was associated with a second elution peak which was of higher molecular weight fraction. Using Sephadex G-200, KAMIYA *et al.* (1973) demonstrated that most of the antigenicity for the IHA test was present in the high molecular weight fraction. WELCH *et al.* (1983) similarly purified *T. canis* antigen from the high molecular weight G-200 Sephadex fraction and removed cross-reacting antigens using affinity chromatographic techniques. The analysis of Sephacryl S-300 eluted *A. cantonensis* antigen is in progress and the results will be reported elsewhere.

It is realized that sero-diagnosis only remains an indirect way to assay the presence of an infectious agent. Detection of the antigens would be more practical and satisfactory. Although the detection of antigens in CSF is considered to be important, this approach has not been attempted in human angiostrongyliasis. The only trial was performed by CHEN *et al.* (1972) who used IHA in the experimental monkeys. Unfortunately, the antigen titres recorded in CSF were relatively low and the results obtained from four experimental animals were variable. Monoclonal antibody or specific rabbit antiserum against *A. cantonensis* may also contribute to the discovery of antigens in infected animals.

A reliable technique is needed for the direct and early diagnosis of angiostrongyliasis. The versatility, sensitivity and simplicity of ELISA and its suitability for use in assay to detect circulating antigen suggests the possibility of its application in the detection of antigens in blood or CSF of patients infected by *A. cantonensis*.

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