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Antigens of Adult Female Worm of *Angiostrongylus cantonensis* Recognized by Infected Humans

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Abstract

Humoral immune responses of humans to antigens of adult female worms of *Angiostrongylus cantonensis* were evaluated using an immunoblot technique. Four sera from infected humans were obtained from angiostrongyliasis patients in Kaohsiung, Taiwan, The Republic of China, and from 23 sera from clinically suspected patients in the Fiji Islands. To identify any cross reactivity of the antigens, we used serum samples from 9 healthy individuals in a hyperendemic area in Kaohsiung and 14 Japanese patients with other helminthic or protozoan infections. The serum samples of angiostrongyliasis patients strongly recognized polypeptides with molecular weights of about 31 kDa and 29 kDa. Neither antigen reacted with the sera from normal individuals and patients with other helminthic or protozoan infections. Both proteins responded to WGA lectin and the antigenicity was lost following treatment by 0.2% pronase, but not by 50 mM periodic acid. These results suggest the usefulness of these two antigenic proteins in the immunodiagnosis of human angiostrongyliasis.

Key words: *Angiostrongylus cantonensis,* angiostrongyliasis, Western blot, serodiagnosis, humans

Introduction

Angiostrongyliasis is a major zoonotic infection not only in the tropics and subtropics, but also in temperate regions, such as Japan. The infection is diagnosed by its specific neurological manifestations, laboratory findings and historic antecedents. Additionally, serology is highly informative in diagnosing this infection (Kliks, *et al.*, 1988).

Serum antibodies to *Angiostrongylus cantonensis* have been detected in both permissive and non-permissive hosts (Young and Dobson, 1982; Bhopale, *et al.*, 1984; Jaroonvesama *et al.*, 1985; Chen, 1986; Kum and Ko, 1986; Ko *et al.*, 1987; Fujii, 1987; Fujii, 1989; Perez *et al.*, 1989). In humans, micro-ELISA is the most useful tool for serological diagnosis of the disease (Yen and Chen, 1991). However, little is known about the characteristics of the antigens that elicit immune responses in man. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of *A. cantonensis* has identified 2 components that are recognized by Western blot using monoclonal antibodies; one is a 204-kDa protein of young-adult worms (Chang *et al.*, 1990) and the other is a 91-kDa antigen of infective third-stage larvae of the worm (Shin and Chen, 1991). We describe here the immunoglobulin G response of infected humans to adult female worm of *A. cantonensis* and the cross reactivity of the antigens against the sera of other helminthic and protozoan infections.

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Materials and Methods

Preparation of antigens. Phosphate buffered saline extracts of adult female worms were prepared by the slightly modified method of

Yoshimura and Soulsby (1976). Briefly, the worms were recovered from lungs of infected rats at 35 days post-infection (PI). They were then extensively washed three times in distilled water and 0.1 M phosphate buffered saline containing 0.03% NaN_3 (PBS; pH 7.2). After removal of excessive PBS on paper filter, the worms were frozen at −80°C and dried by vacuum-drying equipment (Yamato Freeze-dryer, Model DC-35, Yamato Scientific Co., Ltd., Tokyo, Japan). The worms were cut into small pieces and macerated with a mortar-driven pestle. The frozen powder was sonicated in ten volumes of PBS using an ultrasound disrupter (Tomy Seiko Co., Ltd., Tokyo, Japan) at 160 W for 5 min on ice. The product was centrifuged at 10,000 × g for 1 hr. The supernatant was collected and dialyzed against distilled water at 4°C for 48 hr, and centrifuged at 10,000 × g for 1 hr. Finally, the supernatant was sterilized by membrane filtration through a 0.22-μm-pore membrane (Millipore) and lyophilized. Protein concentration was measured by a dye-binding assay (Bradford, 1976).

Serum samples. Serum samples from four infected humans and 9 normal individuals were obtained from a hyperendemic area in Kaohsiung, Taiwan, ROC. The four infected patients were parasitologically confirmed to be angiostrongyliasis cases, and the 9 normal individuals inhabited the same area, but did not have a past history of neurological disorder. Sera from 23 clinically suspected cases of angiostrongyliasis were kindly provided by J. U. Mataika (The Wellcome Virus Laboratory, Tamavua Hospital, Suva, Fiji). Sera from 14 Japanese patients with parasitic infections (see Table 1) were tested for cross-reactions. Pooled sera from healthy Japanese subjects without a history of parasitic infection was used as a control.

Immunoblot. SDS-PAGE, transfer of electrophoretically separated proteins to nitrocellulose, and subsequent blotting were performed according to previously described methods (Akao *et al.*, 1991). Briefly, approximately 40 μg total protein of the antigen was applied to 15% acrylamide gels. The discontinuous buffer system of Laemmli was used (Laemmli, 1970). After separation by SDS electrophoresis, the proteins were transferred onto a GVHP sheet (Millipore, Nihon Millipore Kogyo, Japan) by a horizontal electrophoretic blotting apparatus (Atto, Tokyo, Japan). The sheet was then cut into strips. Guide strips were stained with Indian ink (Hancock and Tsang, 1983). The GVHP strips were soaked for 1 hr at 37°C in PBS (50 mM, pH 7.2) supplemented with 0.05% Tween 20 (PBS-T) and 10% bovine serum albumin. After brief washing, the strip was reacted with test serum diluted 1:100 in PBS-T for 1 hr at 37°C. The strip was then incubated for an additional 1 hr in horseradish peroxidase conjugated anti-human IgG (Fc specific; Cappel, U.S.A.) diluted 1:200 in PBS-T. Binding of the enzyme-labeled antibody was detected by adding 40 mg of 3,3'-diaminobenzidine 4 HCl (Dotite, Tokyo) dissolved in 5 ml of 0.1M Tris-HCl (pH 7.2) containing 25 μl of 8% $NiCl_2$ and 15 μl of 30% H_2O_2.

To detect glycoconjugates among the antigens, biotinylated lectins (kit from Vector, CA, U.S.A.) were used. For removal of the sugars or proteins from the glycoproteins, the GVHP strips were treated with 50 mM periodic acid for 3 hr or 0.2% pronase for 2 hr at 37°C at room temperature. The strips were washed thoroughly with PBS-T, and then reacted with a serum from a patient with *A. cantonensis* infection.

Table 1 Sera used for investigating cross reactivity of adult female antigens of *Angiostrongylus cantonensis*

Sera from patients with	No. of sera examined
Vivax malaria	1
Amebiasis	2
Dirofilariasis	3
Ascariasis	1
Strongyliasis	1
Sparganosis	4
Cysticercosis	1
Gnathostomiasis	1

Results and Discussion

Figure 1 shows the results of our immunoblot analysis of the sera of angiostrongyliasis patients in Taiwan, ROC. Four sera of angiostrongyliasis patients responded strongly to the antigens at the molecular weights of 31 kDa and 29 kDa. In contrast, none of bands was seen in the sera from subjects in hyperendemic area in Taiwan who had no history of angiostrongyliasis or in the control serum.

Antigen recognition of the Fujian sera against *Angiostrongylus* antigens is shown in Figure 2. These sera were from clinically suspected patients experiencing severe headache, fever and eosinophilia. Two of 23 serum samples strongly recognized the antigens with a molecular mass of 29–31 kDa region (Fig. 2 lane b and n), and 3 were slightly reactive with the antigens (Fig. 2 lane j, w and x). These data suggested that antibody production seemed to be varied in each patient in the course of the infection. Therefore, a follow-up study using paired sera is needed to confirm the production of the antibodies in the sera of the remaining 18 samples and in the weakly reacted sera.

In experimental infections, rats and mice produced antibodies against molecular masses ranging from 100 kDa to 200 kDa of the worm extracts, especially of reproductive organs (Fujii,

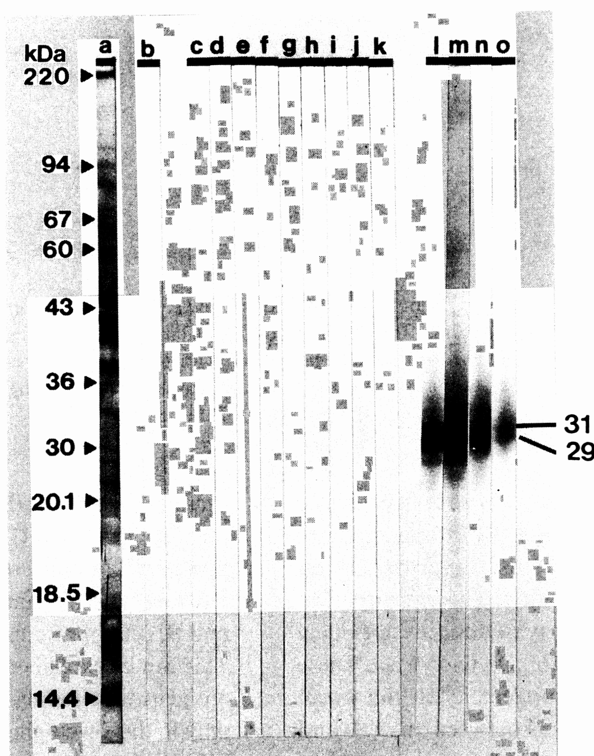

Fig. 1 Immunoblot of *Angiostrongylus cantonensis* antigens with sera from patients of hyperendemic area in Kaohsiung, Taiwan, ROC. Lane (a): Protein profiles of the antigens stained with India ink; Lane (b): a pooled serum of Japanese; Lanes (c) to (k): serum collected from hyperendemic area in Kaohsiung with no history of angiostrongyliasis; Lanes (l) to (o): sera of confirmed patients collected from hyperendemic area in Kaohsiung.

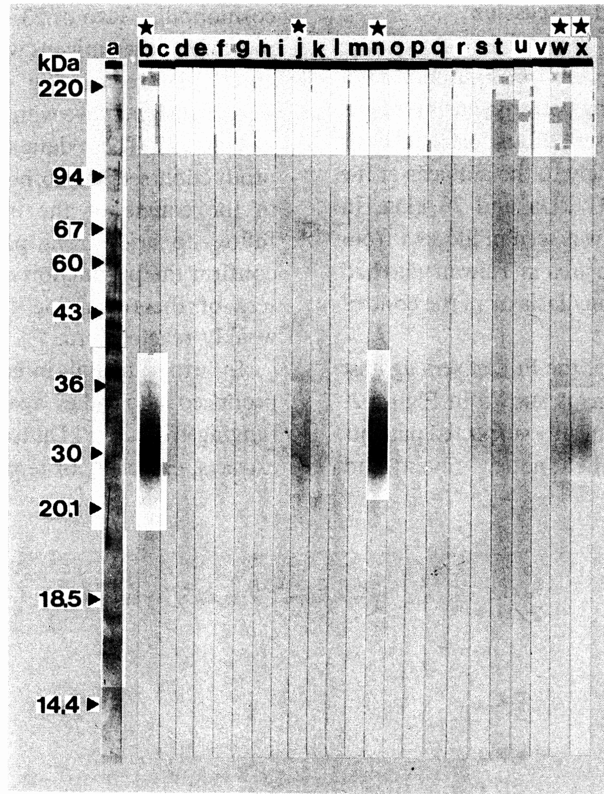

Fig. 2 Immunoblot of *Angiostrongylus cantonensis* with sera collecting from patients of Fiji Islands. Lane (a); protein profiles of the antigens stained with India ink; Lanes (b) to (x): serum collected from clinically suspected angiostrongyliasis in Fiji Islands. Positive serum is indicated by *.

1987; Fujii, 1989). However, those antigens failed to be recognized serologically by infected humans in our study. Fujii (1987) also reported that rat serum obtained 150 days after infection responded to a wide variety of antigens, including molecular masses from 26 kDa to 55 kDa. These data suggest that immunogenicity of the worm extract components differs between animals and humans, and indicate that there may be immunodominant epitopes to humans within the low molecular weight masses of the antigens. A recent study demonstrated that a molecular mass of 204-kDa protein was recognized by four monoclonal antibodies to young-adult worms of *A. cantonensis* (Chang *et al.*, 1990). Furthermore, monoclonal antibody to the 91-kDa antigen from excretory-secretory (ES) products of the infective third-stage larvae (L3) reacted with sera from infected humans (Shih and Chen, 1991). However, no immune responses to the 204-kDa and 91-kDa proteins were found in this study. This can be explained by assuming that these two antigens were highly stage-specific and expressed only in the developing stage of the worm, but not in the adult worm. Therefore, the sera used in this study might not respond to the two antigens.

To clarify the cross reactivity of the antigenic bands, 14 serum samples from patients with eight different parasite infections were used (Fig. 3). Among these infections, gnathostomiasis and cysticercosis are the most important diseases to be distinguished from angiostrongyliasis in

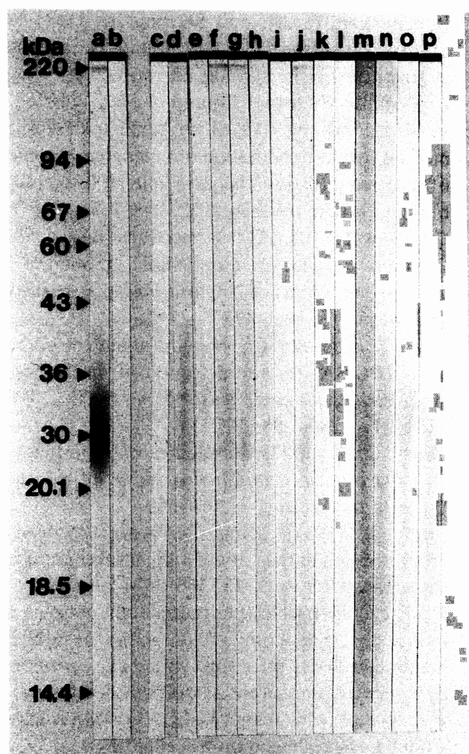

Fig. 3 Cross reactivity of *Angiostrongylus cantonensis* antigens. Lane (a): a patient serum of angiostrongyliasis; Lane (b): a pooled serum of Japanese; Lane (c): serum of vivax malaria patient; Lanes (d) and (e): amebiasis; Lanes (f) to (h): dirofilariasis; Lane (i): ascariasis; Lane (j): strongyliasis; Lanes (k) to (n): sparganosis; Lane (o): cysticercosis; Lane (p): gnathostomiasis.

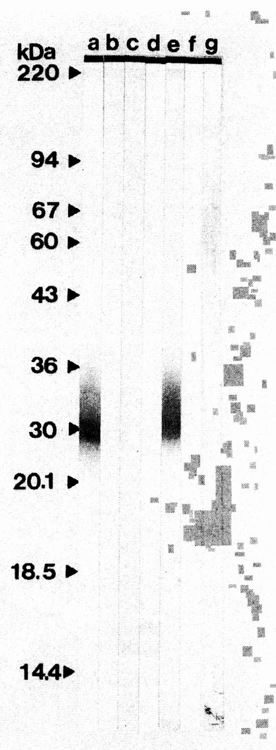

Fig. 4 Immunoblot analysis of glycoconjugates of *Angiostrongylus cantonensis* antigens using six lectins after SDS-PAGE and transfer to GVHP paper. Lane (a): a serum of angiostrongyliasis; Lane (b): biotinylated PNA; Lane (c): biotinylated SBA; Lane (d): biotinylated DBA; Lane (e): biotinylated WGA; Lane (f): biotinylated RCA1; Lane (g): biotinylated ConA.

endemic areas (Cross, 1987). Figure 3 clearly demonstrates that no bands reacted with the sera from other helminthic and protozoan infections. On the basis of these results, we believe that these two bands represent the specific antigens of the adult female worm of *A. cantonensis* recognized by infected humans. The yield of antigen from the adult worm is larger than that from the young-adult worm. A recent study also demonstrated that the sensitivity and specificity in the detection of antibodies using adult worm antigens were similar to those with young adult worm antigens (Yen and Chen, 1991). In addition, it is difficult to harvest enough ES from the infective larvae for serodiagnosis. Thus, Western blotting using the adult worm antigens can also provide information on antibody response to *A. cantonensis* infection as well as the young-adult worm or L3 antigens.

Lectin binding assays revealed that the antigenic bands possessed glycoconjugates, which reacted strongly with only WGA lectin, but not with the other lectins (Fig. 4). Antigenicity of the bands was completely lost following treatment with 0.2% pronase, but not with 50 mM periodic acid (Fig. 5). These findings suggest that the sugar residues of the specific antigens are not responsible for the antigenicity. Lectin affinity chromatography in conjunction with WGA lectin

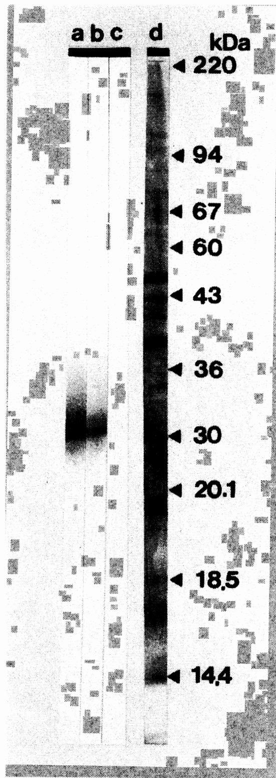

Fig. 5 Effects of pronase and periodic acid against antigenicity of *Angiostrongylus cantonensis* antigens. IgG response of a patient of angiostrongyliasis without treatment (lane a), after treatment with 50 mM periodic acid (lane b), and after treatment of 0.2% pronase (lane c). Protein profiles of the antigens were indicated on the right.

shows promise of yielding purified antigens to be recognized by infected humans.

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References

1) Akao, N., Kondo, K. and Fujita, K. (1991): Immunoblot analysis of *Dirofilaria immitis* recognized by infected humans. Ann. Trop. Med. Parasitol., 85, 455–460.
2) Bhopale, M. K., Limaye, L. S., Pradhan, V. R., Renapurkar, D. M. and Sharma, K. D. (1984): Studies on suspected clinical and experimental angiostrongyliasis: serological responses. J. Hyg. Epidemiol. Microbiol. Immunol., 29, 283–288.
3) Bradford, M. M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248–254.
4) Chang, J. H., Yen, C. M. and Chen, E. R. (1990): Characterization of monoclonal antibodies to young-adult worms of *Angiostrongylus cantonensis*. Hybridoma, 9, 465–471.
5) Chen, S. N. (1986): Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *Angiostrongylus cantonensis*. Trans. R. Soc. Trop. Med. Hyg., 80, 398–405.
6) Cross, J. H. (1987): Public health importance of *Angiostrongylus cantonensis* and its relatives. Parasitology Today, 3, 367–369.
7) Fujii, T. (1987): *Angiostrongylus cantonensis*: immunoblot analysis of the antigens recognized by rats. Parasitol. Res., 73, 366–374.
8) Fujii, T. (1989): Characterization of the antigens that induce the production of antibodies in mice immediately after infection with *Angiostrongylus cantonensis*. Parasitol. Res., 75, 636–640.
9) Hancock, K. and Tsang, V. C. W. (1983): India ink staining of proteins on nitrocellulose paper. Anal. Biochem., 133, 157–162.
10) Jaroonvesama, N., Charoenlarp, K., Buranasin, P., Zaraspe, G. G. and Cross, J. H. (1985): ELISA testing in cases of clinical angiostrongyliasis in Thailand. Southeast Asian J. Trop. Med. Public Health, 16, 110–112.
11) Kliks, M. M., Lau, W. K. K. and Palumbo, N. E. (1988): Neurologic angiostrongyliasis: parasitic eosinophilic meningioncephalitis. In laboratory diagnosis of infectious disease: principles and practices, Balows, A., Hausler, W. J., Ohashi, M., Turna, A., eds., Springer-Verlag, New York, 754–767.
12) Ko, R. C., Chan, S. W., Chan, K. W., Lam, K., Farrington, M., Wong, H. W. and Yuen, P. (1987): Four documented cases of eosinophilic meningioencephalitis due to *Angiostrongylus cantonensis* in Hong Kong. Trans. R. Soc. Trop. Med. Hyg., 81, 807–810.
13) Kum, W. W. and Ko, R. C. (1986): Surface antigens of *Angiostrongylus cantonensis* developing in permissive and non-permissive hosts. Z. Parasitenkd., 72, 517–524.
14) Laemmli, U. K. (1970): Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature, 227, 680–685.
15) Perez, O., Lastre, M., Capron, M., Neyrinck, J. L., Jouault, T., Bazin, H. and Capron, A. (1989): Total and specific IgE in serum and cerebrospinal fluid

of rats and guinea pig infected with *Angiostrongylus cantonensis*. Parasitol. Res., 75, 476–481.
16) Shih, H. H. and Chen, S. N. (1991): Immunodiagnosis of angiostrongyliasis with monoclonal antibodies recognizing a circulating antigen of mol. wt 91,000 from *Angiostrongylus cantonensis*. Int. J. Parasitol., 21, 171–177.
17) Yen, C. M. and Chen, E. R. (1991): Detection of antibodies to *Angiostrongylus cantonensis* in serum and cerebrospinal fluid patients with eosinophilic meningitis. Int. J. Parasitol., 21, 17–21.
18) Young, W. K. and Dobson, C. (1982): Antibody responses in rats infected with *Angiostrongylus cantonensis* and the passive transfer of protective immunity with immune serum. Z. Parasitenkd., 67, 329–336.
19) Yoshimura, K. and Soulsby, E. J. L. (1976): *Angiostrongylus cantonensis*: Lymphoid cell responsiveness and antibody production in rats. Am. J. Trop. Med. Hyg., 25, 99–107.