COMMON ANTIGENICITIES BETWEEN *ANGIOSTRONGYLUS CANTONENSIS* AND VARIOUS SPECIES OF SNAILS

Yuzuru Iwanaga

Department of Immunology and Parasitology, Division of Molecular Science, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan

Abstract. Studies on common antigenicities were carried out by using rabbit sera immunized with *Angiostrongylus cantonensis* adult worms or the third stage larvae and antigens of various species of snails and *vice versa* by the immunoblotting technique. The results obtained are summarized as follows: 1) Common antigenicities between *A. cantonensis* adult worms and snails susceptible to *A. cantonensis* were observed in a range of molecular weights of 14.3 to 200 kDa. In Puerto Rican pigmented *Biomphalaria glabrata* and *Achatina fulca*, which had high infection rates with *A. cantonensis*, we recognized 15 to 16 bands against the adult worm, especially the band with a molecular weight of 29 kDa, which had a more intense reaction. 2) Common antigenicities between *A. cantonensis* third stage larvae and snails susceptible to *A. cantonensis*, were observed in a range of molecular weights of 14.3 to 97.4 kDa, especially *A. fulica* and *B. glabrata*, where we detected many bands in molecular weight range of 18.4 to 43 kDa. Based on the common antigenicities between *A. cantonensis* and snails susceptible to *A. cantonensis*, it is possible that the common antigenicities are one of the factors defining the different susceptibilities of various species of snails to *A. cantonensis*, and more bands are seen with increasing infection rates with *A. cantonensis*. Of those bands, the protein with the molecular weight of 29 kDa may be the main common antigen between the *A. cantonensis* adult worm, the third stage larvae and the snails susceptible to *A. cantonensis*.

INTRODUCTION

Since the first report of angiostrongylosis cantonensis by Nomura and Lin (1945), *Angiostrongylus cantonensis* has been recognized as a causative agent of eosinophilic meningoencephalitis in humans in South Asian and Pacific areas (Roux *et al*, 1987; Purohit *et al*, 1991; Re and Gluckman, 2001). It is well known that various molluscan species can serve as intermediate hosts for *A. cantonensis* (Liat *et al*, 1965; Hori *et al*, 1976; Iwanaga *et al*, 1983; 1995). However, the third stage larvae of *A. cantonensis* shows different infectivities to various experimental intermediate hosts. These differences are probably due to a combination of physiological and biochemical differences between the snails (Newton and Brand, 1955; Tsuji *et al*, 1978; Iwanaga, 1992, 1997, 2002). Biochemical studies of hosts and parasites have been done for a long time (Capron *et al*, 1965; Tsuji *et al*, 1969; Tsuji and Yokogawa, 1972; Sugiyama *et al*, 1987; Santana *et al*, 1992; Iwanaga, 1994). Marrero and Hillyer (1985) reported that sera from humans infected with *Schistosoma mansoni* cross-reacted with the *Biomphalaria glabrata* antigen. It has been suggested that there are some common antigenicities between infected human sera and the *B. glabrata* antigen. Iwanaga *et al* (1988) found that more common antigenicities were seen between the Japanese strain of *Schistosoma japonicum* egg and *Oncomelania hupensis nosophora*, which was the most suitable host for the Japanese strain of *S. japonicum*. Although common antigenicities between *Schistosoma* spp and the intermediate hosts have been described (Jackson and Moor, 1976; Iwanaga *et al*, 1992), few detailed observations have been made of the common antigenicities between *A. cantonensis* and snails.

This paper deals with common antigenicities between various species of snails and *A. cantonensis* adult worms or the third stage larvae by the Western blotting method.

Correspondence: Yuzuru Iwanaga, Department of Immunology and Parasitology, Division of Molecular Science, Graduate School of Biomedical Science, Hiroshima University, Hiroshima 734-8551, Japan.
Tel: +81-82-257-5176; Fax: +81-82-257-5179
E-mail:yiwanaga@hiroshima-u.ac.jp

MATERIALS AND METHODS

Strains of *Angiostrongylus cantonensis* and snails

The strain of *A. cantonensis* used in this study originated from Puerto Rico, and the cycle was maintained in our laboratory by passage through pigmented *B. glabrata*, Puerto Rico and albino rats, *Rattus norvegicus*. The snails employed in these experiments, pigmented and albino types of Puerto Rican strains of *B. glabrata*, were obtained from Puerto Rico via the NIH in the USA. The Brazilian strains of *B. glabrata* and *B. straminea* were from the following areas in Brazil: the Pigmented *B. glabrata* were from Jaboatao, the Pernambuco, albino *B. glabrata* were from Belo Horizonte and the pigmented *B. straminea* were from Sao Lourenco da Mata. *Oncomelania hupensis* snails were obtained as follows: the *Oncomelania hupensis nosophora* were from Yamanashi, Japan, the *O. h. hupensis* were from Shanghai, China, and the *O. h. quadrasi* were from Leyte, Philippines. These *Biomphalaria* and *Oncomelania hupensis* snails were reared in our laboratory by the method modified from Iwanaga and Tsuji (1972). The *Achatina fulica* were from Okinawa, Japan, and the *Succinea lauta, Euhadra awaensis idzumonis* and *Semisulcospira bensoni* were from Hiroshima, Japan.

Preparation of antigens and antisera

The *A. cantonensis* adult worms (ACAW) and adult snails were prepared with 0.15 M phosphate buffered saline (PBS) extracts, according to the methods of Tsuji (1974, 1975) and Iwanaga and Tsuji (1985). The preparation of the third stage larval antigen of *A. cantonensis* (ACL3) was as follows: the complete bodies of the snails infected *A. cantonensis* were minced and digested with a 1% HCl-pepsin solution for 2 hours. After digestion, the solution was centrifuged at 2,000 rpm for 5 minutes and the sediment was washed twice in 0.15 M PBS and then the third stage larvae were collected. The larvae were homoginated with a Teflon homoginizer. The homogenates were stirred to extract antigenic components in 0.15 M PBS for 12 hours at 4ºC. The supernatant fluid was collected by centrifugation at 10,000 rpm for 30 minutes and then was lyophilized. The dried material was used as ACL3 antigen. The protein concentration of antigens used in this study was approximately 6 mg/ml and stored at -80ºC until used.

Antisera (*ie*, immune sera) were prepared by the following method: Emulsion containing 2 -3 mg/ml (approximately 1,500 μg protein) of each antigen mixed in 1.5 ml of Freund's complete adjuvant (Difco Laboratory, Detroit, USA) was injected into the proximal limbs of rabbits intramuscularly ten times each week. Antisera were obtained from blood drawn from these rabbits ten days after the final injection (modification of Tsuji, 1974, 1975)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis

SDS-PAGE was carried out with 5-20% gradient gels according to the method of Laemmli (1970) using a DPE-2210 apparatus (Daiich Chemicals, Tokyo, Japan). Antigen samples for electrophoresis were mixed with an equal volume of sample buffer (2.5% SDS, 25% glycerol, 2.5% 2-mercaptol ethanol, 0.001% bromophenol blue in 0.125 M Tris-HCl buffer, pH 6.8). The solutions were treated at 100ºC for 5 minutes, and a 20 μl sample/well was then loaded onto the gel, *ie*, approximately 60 μl of the antigen protein was applied to a well of acrylamide gel. After separation by SDS electrophoresis, the proteins were electrophoretically blotted (Towbin *et al*, 1979) onto a PVDF membrane (Bio-Rad Laboratories, USA) by a horizontal electrophoretic blotting apparatus (Atto, Tokyo, Japan). The membranes were first blocked with 5% nonfat milk in PBS (pH 7.2) at 4ºC, overnight. After washing with PBS, the membranes were incubated with antisera diluted 1:1,000 for 2.5 hours at room temparature, and were then washed with PBS containing 0.1% Tween 20 (PBS-T) 4 times at 5-minute intervals, and subsequently incubated with peroxidase conjugated goat anti-rabbit IgG (Medical and Biological Laboratories, Japan) diluted 1:1,000 in PBS containing 0.5% nonfat milk for 2.5 hours at room temperature. After incubation, the membranes were again washed with PBS-T as described above. The membranes were incubated with substrate solution containing 0.025% DAB and 0.03% H_2O_2 in Tris-HCl buffer (pH 7.5)

for 10-20 minutes until the protein bands were visualized. Then, the membranes were sufficiently washed with distilled water to stop the reaction.

RESULTS

Common antigenicities between ACAW and snails

Fig 1A shows the results of an immunoblot analysis between anti-ACAW serum and snail antigens. Anti-ACAW serum recognized 15 bands in the molecular weight (mw) regions from 14.3 to 200 kDa against each of the Puerto Rican pigmented *B. glabrata* and *A. fulica* antigens. Similarly, 12, 12, 10, and 8 bands were seen in the range of 14.3 to 97.4 kDa against Brazilian pigmented *B. glabrata*, Puerto Rican albino *B. glabrata*, Brazilian albino *B. glabrata* and pigmented *B. straminea* antigens, respectively. The 29 kDa band was intensely stained for *A. fulica* and *B. glabrata* against ACAW. Although in the *O. h. nosophora*, *O. h. hupensis* and *O. h. quadrasi* antigens, we recognized 6, 6, and 4 bands, respectively, ranging from 14.3 to 68 kDa with anti-ACAW sera, the *S. lauta* and *E. a. idzumonis* antigens had only one band at 68 kDa. S. *bensoni* had none. On the reversal experiments, using anti-snail sera against the ACAW antigen (Fig 1B), the ACAW antigen recognized 16 bands each for anti-Puerto Rican pigmented *B. glabrata* and *A. fulica* sera.Twelve bands were seen with anti-Brazilian pigmented *B. glabrata* serum, 11 bands with anti-Puerto Rican albino *B. glabrata* serum, 9 bands each with anti-Brazilian albino *B. glabrata* and pigmented *B. straminea* sera, 6 bands each with anti-*O. h. nosophora* and *O. h. hupensis* sera, 4 bands with anti-*O. h. quadrasi* serum, 3 bands with anti-*S. lauta* serum and only one band with anti-*E. a. idzumonis* serum. With the anti-*S. bensoni* serum no bands were detected against the ACAW antigen. The range of molecular weights for the bands observed was in almost the same regions (except for *S. lauta* and *E. a. idzumonis*) as those of the anti-ACAW serum and snail antigens in the above mentioned experiments. The bands for *O. hupensis* snails had very weak reactions compared to those of *A. fulica* and *B. glabrata*. Anti-*S. lauta* and *E. a. idzumonis* sera detected a few bands in the 29 to 68 kDa range with the ACAW antigen.

Common antigenicities between ACL3 and snails

Fig 2A shows the results of immunoblot analysis between anti-ACL3 serum and snail antigens. Anti-ACL3 serum recognized 10 bands against both the Puerto Rican pigmented *B. glabrata* and *A. fulica* antigens, 9 bands against the Brazilian pigmented *B. glabrata* antigen and 8 bands each against the Puerto Rican and Brazilian albino *B. glabrata* and pigmented *B. straminea* antigens. *O. h. hupensis*, *O. h. nosophora*, *O. h. quadrasi*, *S. lauta* and *E. a. idzumonis* antigens recognized 7, 6, 5, 3 and 1 bands, respectively, with anti-ACL3 sera. The *S. bensoni* antigen did not detect any bands. Those bands observed were in the range of 14.3 to 97.4 kDa. *A. fulica* and *B. glabrata* detected many bands ranging fron 18.4 to 43 kDa. In the reversal experiments, using anti-snail sera against the ACL3 antigen (Fig 2B), the ACL3 antigen recognized 10 bands with the anti-*A. fulica* serum, 9 bands each with the anti-Puerto Rican and Brazilian pigmented *B. glabrata* sera, 7 bands each for both strains of anti-albino *B. glabrata* sera, 6 bands with the anti-pigmented *B. straminea*, anti-*O. h. nosophora* and *O. h. hupensis* sera, 5 bands each with the anti-*O. h. quadrasi* and *S. lauta* sera and 2 bands with the anti-*E. a. idzumonis* serum. None was detected with the anti-*S. bensoni* serum. Though the range of molecular weights of those bands recognized was almost same as with the anti-ACL3 serum and snail antigens in the above mentioned experiments, the bands detected with the *O. hupensis* snails were much weaker than those detected with the anti-ACL3 serum.

DISCUSSION

In this study, common antigenicities between *A. cantonensis* and various species of snails were evaluated using the immunoblotting technique. It was found that *A. fulica* and *B. glabrata* produced more common bands than those of other snails to *A. cantonensis* adult worms. Of these, *A. fulica* and Puerto Rican pigmented *B. glabrata* recognized 15 to16 common antigenic components to ACAW. Although 4 to 6 bands were detected between *O. hupensis* snails and ACAW, *S. lauta* and *E. a. idzumonis* had only 1 to 3 tiny bands. Those bands were mainly observed in the

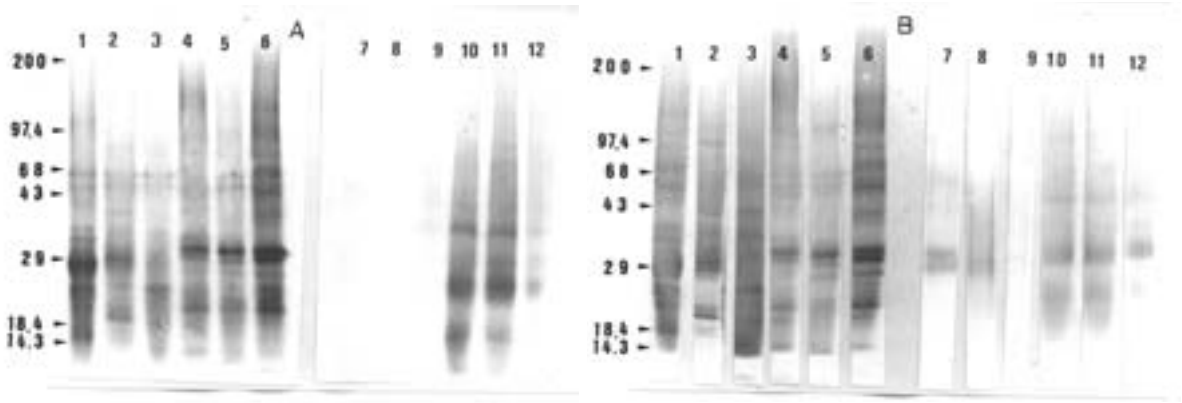

Fig 1–Immunoblot analysis between the *A. cantonensis* adult worm and various species of snails. A. Cross reactions of anti-*A. cantonensis* adult worm sera against snail antigens. B. Cross reactions of anti-snail sera against *A. cantonensis* adult worm antigen.

Lane 1: Pigmented *B. glabrata*, Puerto Rico
Lane 2: Pigmented *B. glabrata*, Brazil
Lane 3: Pigmented *B. straminea*, Brazil
Lane 4: Albino *B. glabrata*, Puerto Rico
Lane 5: Albino *B. glabrata*, Brazil
Lane 6: *A. fulica*
Lane 7: *S. lauta*
Lane 8: *E. a. idzumonis*
Lane 9: *S. bensoni*
Lane 10: *O. h. nosophora*
Lane 11: *O. h. hupensis*
Lane 12: *O. h. quadrasi*

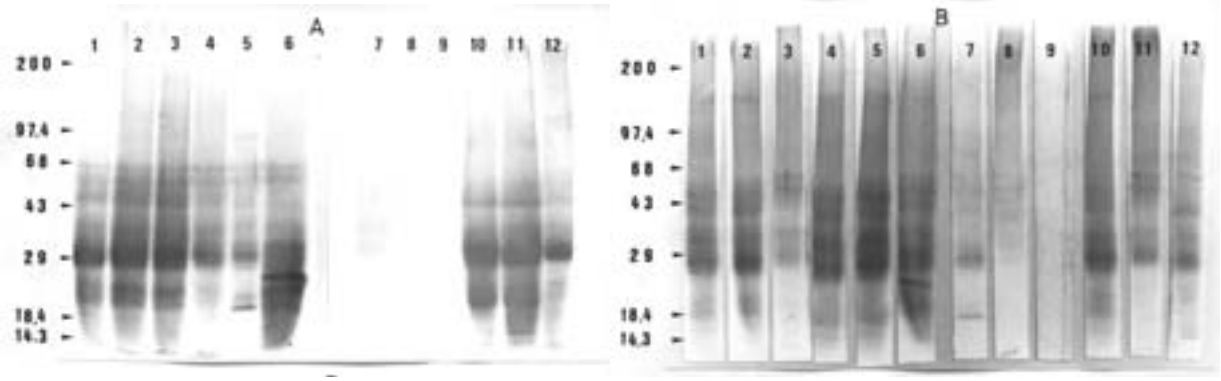

Fig 2–Immunoblot analysis between *A. cantonensis* third stage larvae and various species of snails. A.Cross reactions of anti-*A. cantonensis* third stage larvae serum against snail antigens. B. Cross reactions of anti-snail sera against *A. cantonensis* third stage larvae antigen.

Lane 1: Pigmented *B. glabrata*, Puerto Rico
Lane 2: Pigmented *B. glabrata*, Brazil
Lane 3: Pigmented *B. straminea*, Brazil
Lane 4: Albino *B. glabrata*, Puerto Rico
Lane 5: Albino *B. glabrata*, Brazil
Lane 6: *A. fulica*
Lane 7: *S. lauta*
Lane 8: *E. a. idzumonis*
Lane 9: *S. bensoni*
Lane 10: *O. h. nosophora*
Lane 11: *O. h. hupensis*
Lane 12: *O. h. quadrasi*

region of 18.4 to 43 kDa. The band at 29kDa was strongly stained. Common antigenicities between ACL3 and snails were mainly observed in the range of 14.3 to 68 kDa, but the bands resulting from ACL3 to snails were less than those with ACAW. With regard to the main proteins of ACAW, a few researchers have used the Western blot using the monoclonal antibodies of humans and rats, which had bands from 29 to 31 kDa (Akao *et al*, 1992) and from 26 to 55 kDa (Fujii, 1987). For ACL3, Shih and Chen (1991) reported that the 91 kDa antigen was the main band in third stage larvae by Western blot using monoclonal antibodies. Although the main protein of ACAW, by their report was observed to have common bands with snails susceptible to *A. cantonensis*

Table 1
Infection rates of the third stage larvae of *Angiostrongylus cantonensis* in various species of snails.

Snails	Locality	Infection rate (%)
Pigmented *B. glabrata*, Puerto Rico	Puerto Rico	77.1
Pigmented *B. glabrata*, Brazil	Brazil	62.0
Pigmented *B. straminea*, Brazil	Brazil	39.8
Albino *B. glabrata*, Puerto Rico	Puerto Rico	59.3
Albino *B. glabrata*, Brazil	Brazil	51.5
A. fulica	Japan	75.0
S. lauta	Japan	10.7
E. a. idzumonis	Japan	6.7
S. bensoni	Japan	0
O. h. nosophora	Japan	40.0
O. h. hupensis	China	40.0
O. h. quadrasi	Philippines	38.0

The data are extrapolated from reports of Hori *et al*, 1976; Iwanaga *et al*, 1983; Iwanaga, 1995, 2002.

and ACAW, the 91kDa protein was not found in the common antigens between the snails and ACL3 in this study. This suggests that the immunogenicities of the ACAW and ACL3 extract components differ between humans, rats, and snails. The common antigenicities observed between snails and ACL3 were partly detected in the components resulting from the ACAW. It seem that some snail proteins were incorporated into the larvae during their development in the snail to surve as common antigenicities until the adult worm stage is reached. The origins of these common antigenicities between hosts and parasites, however, have not yet been ascertained.

As shown in Table 1, infection rates of *A. fulica* and Puerto Rican pigmented *B. glabrata* due to *A. cantonensis* were 75.0 and 77.1%, respectively. Infection rates for Puerto Rican albino *B. glabrata*, and Brazilian pigmented and albino *B. glabrata* were 59.3, 62.0, and 51.5%, respectively. *B. glabrata*, the pigmented types showed a slightly higher susceptibility to infection than the albino types. The infection rate for *B. straminea* was only 39.8%. On the other hand, infection rates for *O. hupensis* snails ranged from 38.0-40.0%, 10.7% for *S. lauta* and 6.7% for *E. a idzumonis*. *S. bensoni* were not found to be susceptible to infection with *A. cantonensis*.

With regard to the relation of common antigenicities and the experimental infection rates of *A. cantonensis* in snails, no differnces in infection rates were seen among *B. straminea* and *O. hupensis* snails. *B. straminea* produced more bands than *O. hupensis* snails with ACAW and ACL3. This problem needs further investigation. This showed the tendency that more bands were seen with increasing infection rates with *A. cantonensis*. These results agree with the findings that *B. glabrata*, which showed high infection rates with the Brazilian strain of *Schistosoma mansoni*, produced many common antigenicities against *S. mansoni* adult worms and eggs (Santana *et al*, 1992; Iwanaga *et al*, 1992; Iwanaga, 1994). These findings support the hypothesis that common antigenicities are one of the factors defining the different susceptibilities of various species of snails to *A. cantonensis*. Finally, these data suggest that the 29 kDa compoment detected in ACAW, ACL3 and the snails may be a necessary protein for developing to the third stage larvae of *A. cantonensis*.

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