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Analysis of Soluble Egg Antigen of *Schistosoma japonicum*: Comparison of Seroreactivities Among Schistosomiasis Patients of Different Infection Phases

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Abstract

Seroreactivities to fractions of soluble egg antigen (SEA) of *Schistosoma japonicum* were investigated in ELISA in the Chinese and Japanese patients with schistosomiasis japonica. SEA was separated with high performance liquid chromatography. When we subdivided patients into two groups, "fresh" and "previous", according to the time course after medical care, sera from the two groups recognized different SEA fractions. Although both two groups' sera had strong responses to high molecular weight fractions, sera from "fresh" patients showed significantly higher responses to fractions of retention time 8–9 min, which were estimated to be around 400kD, compared with "previous" patients' sera ($p<0.05$). Strong responses to high molecular weight fractions were observed not only in schistosomiasis patients, but also in cases of other trematode infections or even in healthy subjects. The diagnostic 8–9 min fractions were heat-sensitive, however, were not affected by lectin treatment. Our observation suggests that IgG response to the 400kD fraction(s) might be informative for estimating infection phases of patients, and that high molecular weight fractions of SEA were less diagnostic because of cross-reactions and/or nonspecific responses.

Key words: Schistosomiasis japonica, ELISA, HPLC, Crossreaction

Introduction

ELISA is one of the best immunological methods to diagnose schistosomiasis (McLaren *et al.*, 1979; Tanaka *et al.*, 1979; Lunde and Ottesen 1980). It is, however, well known that tight crossreactions among trematode infections often result in difficulties in making accurate clinical diagnosis and getting detailed information in seroepidemiological survey (Pelley and Hillyer 1978). The crossreactivity has been observed among different genus and species of trematodes or different stages in parasite life cycle. It has been controversial whether it is possible to assess efficacy of chemotherapy by immunological determination, however, high titers of anti-parasite antibodies can be observed in the host for long period even after patients were satisfactorily treated (Hagi *et al.*, 1990). In seroepidemiological study, it is not easy to evaluate whether the studied population are in active parasitic infection or infection has been eradicated.

The present study was planned to seek a simple method of immunodiagnosis that has high sensitivity to schistosomiasis with no or low reactivities to other trematode antigens. We also intended to seek a method to distinguish population of new and old infection. We performed

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ELISA using soluble egg antigen (SEA) fractionated by high performance liquid chromatography (HPLC). Sera of patients with schistosomiasis, fascioliasis or paragonimiasis were tested, and the OD patterns of IgG response in ELISA were compared. We discuss serological characteristics of SEA in immunodiagnosis of human schistosomiasis japonica.

Materials and Methods

Sera: We tested sera obtained from patients with schistosomiasis japonica, fascioliasis and paragonimiasis. Of 40 schistosomiasis patients, 32 were Chinese and 8 Japanese. Chinese patients were tentatively subdivided into two groups; 25 "fresh" patients who were treated within 3 years, and 7 "previous" patients whose treatment was more than 10 years before. We obtained sera of 25 "fresh" patients from two different areas; 6 were from area A and 19 from area B. No patient has shown evidence of re-infection. Ten Japanese patients with paragonimiasis and 7 fascioliasis were diagnosed by stool examination and/or serological methods. As controls, sera from 26 healthy Japanese volunteers were tested. All sera were cryopreserved at –20°C before testing.

SEA fractionation in HPLC: SEA was prepared from eggs of *S. japonicum* Yamanashi strain (Ishii and Owhashi, 1982a) and was adjusted at 2.5mg/ml, and 10 μl of the solution was applied on HPLC system (Hitachi, Tokyo, Japan). HPLC was carried out under the condition of pressure 37–39kg, flow rate 1 ml/min, eluent with 0.01M phosphate buffered saline (PBS) (pH 7.2). The gel column used was Tosoh G-3000 SWXL (Tosoh, Tokyo, Japan). Elution containing fractionated SEA of every 0.25 min was consecutively collected and directly poured into 96-well microtiter plate (Nunc, Rockilde, Denmark) during retention time between 2 and 22 min.

ELISA: ELISA to detect specific IgG antibodies was carried out by the method described elsewhere (Min *et al.*, 1990). In brief, 250 μl/ml of crude SEA (10 μg/ml) or HPLC-fractionated SEA was coated in ELISA plate overnight at 4°C. After washing and blocking each well, the first antibodies (patients' sera) were added at the dilution of 1:150. An anti-human IgG goat antibody conjugated with horse radish peroxidase (MBL Co., Nagoya, Japan) was added at 1:1500 dilution as the second antibody. As substrates, 2,2'-azinobis (3-ethylbenz-thiazolinesulfonic acid) (ABTS, Sigma, St. Louis, USA) and H_2O_2 were used. OD values at 410 nm was assessed. The samples of which mean OD values of duplicate testing were more than 2.1 folds of those given by infection-free sera were considered as positive responses.

Inhibition of ELISA reactivities by heat or lectin treatment of antigen: Effects of heat or lectin treatment of SEA on ELISA reactivities were tested. Fractionated SEA were autoclaved at 121°C for 20 min. Lectins used here were phytohaemagglutinin (PHA), poak weed mitogen (PWM), concanavalin A (Con A), wheat germ agglutinin (WGA) and peanut agglutinin (PNA). Antigen-coated wells were pretreated with lectin of various concentrations; 0.005–0.5% for PHA, 6–240 μg/ml for Con A, PWM and PNA, and 3–120 μg/ml for WGA. After treatment for 60 min at 37°C, residual lectins were washed away. Treated SEA fractions were used in ELISA, and OD values were compared.

Statistical analysis: Statistical analysis were done by the Student's t-test (Swinscow 1976) or χ^2 test.

Results

IgG responses of "fresh" and "previous" schistosomiasis patients to fractionated SEA. SEA was separated in HPLC and UV monitoring at 280 nm detected several protein molecules. There were two major peaks as was reported previously (Ishii and Owhashi, 1982a). The first one was located at around 6 min fractions, and the other peaks were in fractions of 13 min and later (Fig. 1). ELISA using fractionated SEA was reproducible, and mean r values were more than 0.9 (data not shown). We tested sera from 7

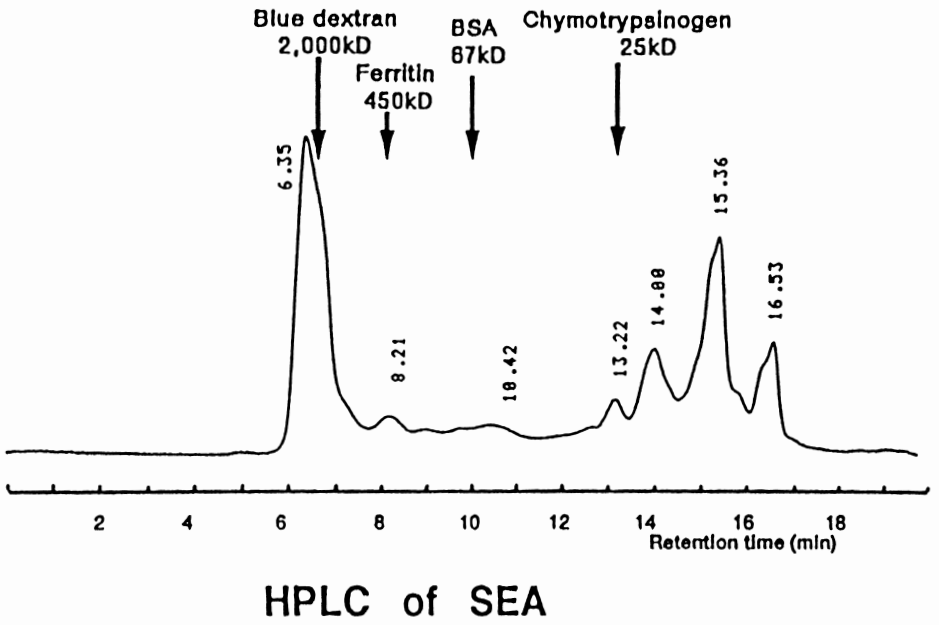

Fig. 1 Fractionation of SEA by HPLC. Absorbance at 280nm was used for monitoring eluate and four molecular weight markers.

"previous" and 6 "fresh" Chinese patients both of which were obtained in area A. Both two groups showed high OD values to crude SEA as well as to fractions of 6–7 min (>1600kD), and only faint responses to fractions of 15 min and later (<10kD) in spite of the existence of enough protein contents. Significant differences were observed in reactivities to fractions of 7.5 min (800kD) and later ($p<0.05$). Only "fresh" patients showed high OD values. When we tested other 19 Chinese "fresh" patients in area B, we observed the same reactive patterns as the "fresh" group in area A. We combined data of the two "fresh" patient groups, and results are shown in Fig. 2.

Confirmation of the characteristic responding profiles of sera in two ethnically different populations. Since there has been no newly infected schistosomiasis patient in these decades in Japan, all Japanese patients were thought to be "previous" cases. Similar reactive profiles of the "previous" group were observed for Japanese patients. We observed the most apparent difference at the 8 min fractions ($p<0.05$), which were assumed to be around 400kD, and no "previous" serum showed positive reactivity to the fractions (Fig. 2).

Reactivities of sera from fascioliasis, paragonimiasis and healthy subjects to fractionated SEA. Positive IgG responses to crude SEA were observed for 5 of 7 fascioliasis patients (71.4%), 3 of 10 paragonimiasis (30%), and 5 of 26 healthy subjects (19.2%) at the condition of our ELISA. When we tested sera showing positive response to crude SEA, all crossreactive sera responded to fractions around 6–7 min (>1600kD). Several sera showed positive responses at the 7.5 min (800kD) fraction, however, no detectable response was observed for fractions of 8 min and later (<400kD) (Fig. 3).

Inhibitory effects on ELISA by heat or lectin treatment of SEA. Each SEA fraction was autoclaved and used in ELISA. Responses to high molecular weight fractions were partially inhibited, but significant responses were still

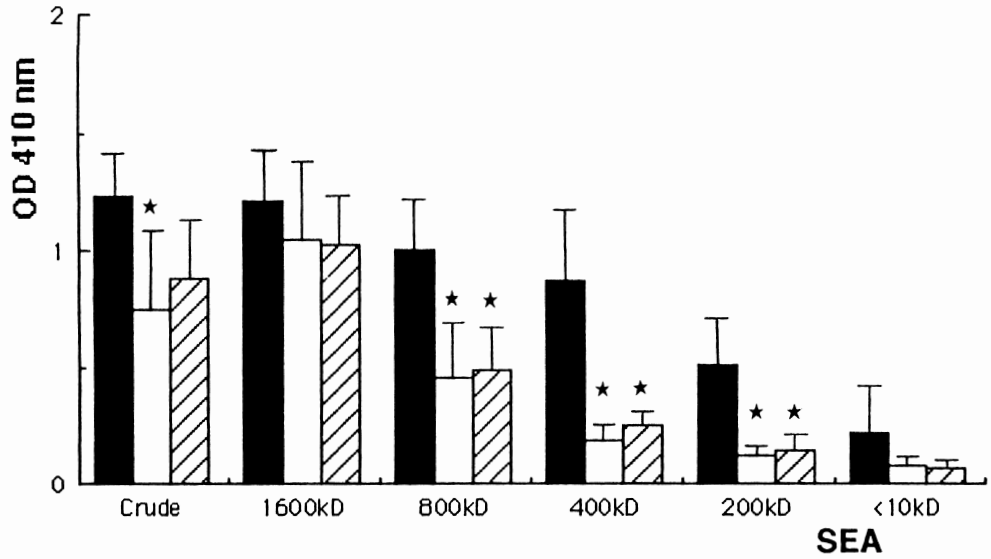

Fig. 2 Comparison of seroreactivities to fractionated SEA among three groups of schistosomiasis japonica patients. The 400kD fraction gave the most discriminatory difference. ■: Chinese "fresh" patients (n=25), □: Chinese "previous" patients (n=7) and ▨: Japanese patients (n=8). ★: Statistically significant difference in comparison with "fresh" patients ($p<0.05$).

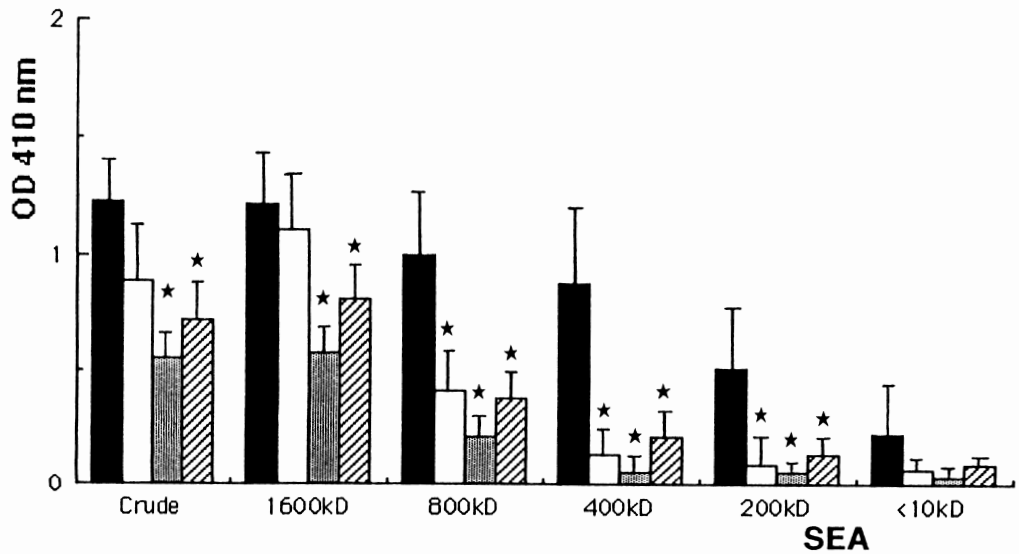

Fig. 3 Mean OD values of ELISA to crude or fractionated SEA in patients with fascioliasis, paragonimiasis, or healthy subjects. The 1600kD fraction seems to be responsible for crossreaction. ■: "fresh" schistosomiasis patients (n=25), □: Fascioliasis (n=7), ▩: Paragonimiasis (n=10), and ▨: Healthy subjects (n=26). ★: Statistically significant difference in comparison with "fresh" schistosomiasis patient ($p<0.05$).

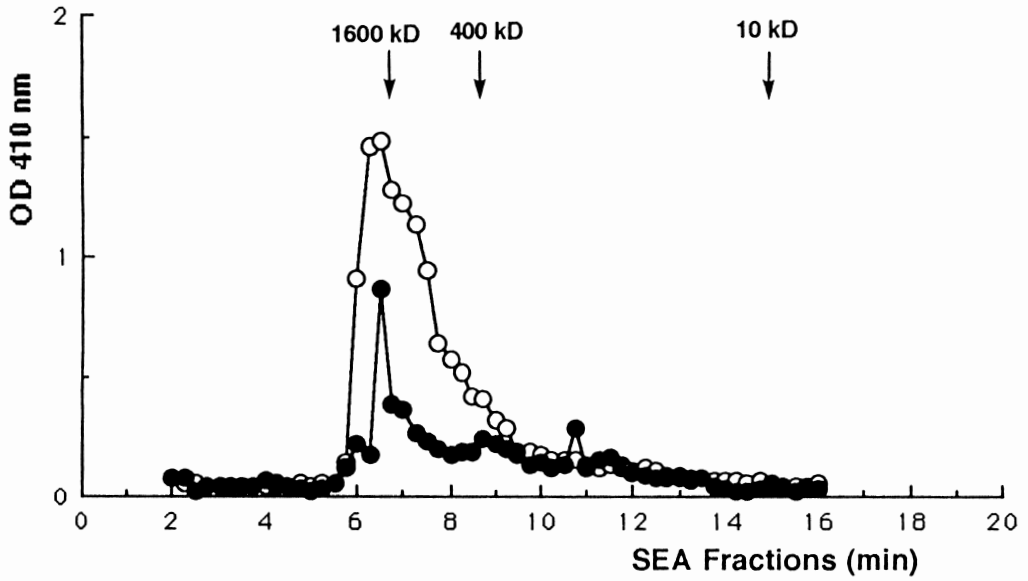

Fig. 4 Effects of heat treatment of SEA on seroreactivities of anti-SEA IgG. Inhibition to reactivity to the 6.5 min fraction was not complete, while complete inhibition was observed for reactivities to around 8 min fractions. ○—: SEA without heat treatment. ●—: SEA with heat treatment.

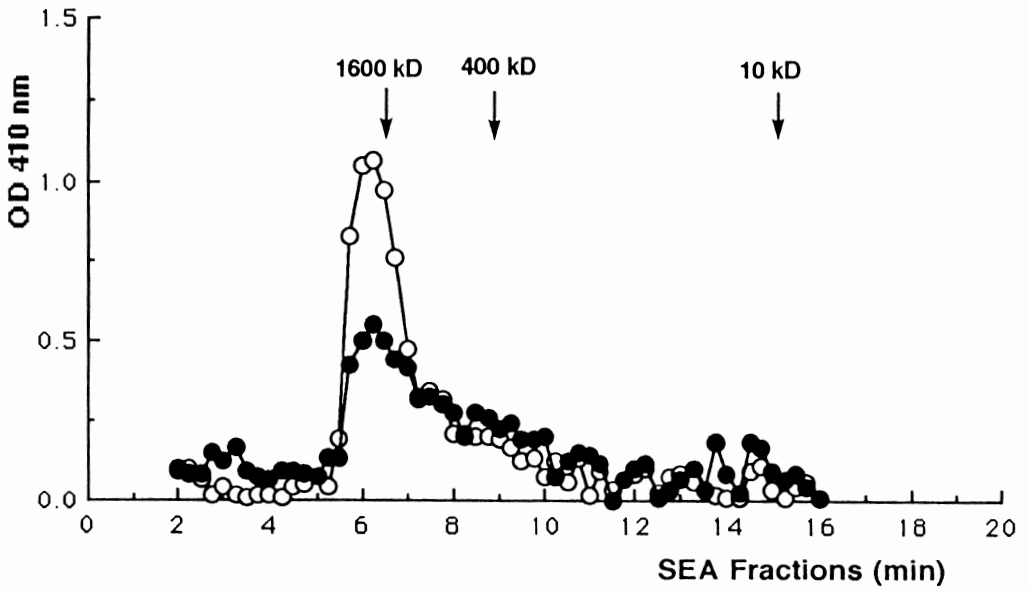

Fig. 5 Effects of lectin treatment on seroreactivity to SEA. WGA showed partial inhibition to reactivities to the 6–7 min fractions. ○—: SEA without WGA treatment, ●—: SEA with WGA treatment.

observed. OD values against 8–9 min fractions became background level. Representative result is shown in Fig. 4. We tested various lectins for their competitive inhibition in ELISA. Among them, WGA and PNA showed significant inhibition only against responses to 6–7 min fractions (Fig. 5). Other lectins tested showed no detectable effect at any concentrations tested (data not shown).

Discussion

In the present study we observed that reactivity to a particular SEA fraction(s) seems to segregate the new and old schistosomiasis, although the number of patients tested were still not enough. In the light of our results, specific reaction to SEA was molecular weight-dependent. The sera from "previous" patients responded only to high molecular weight fraction(s) (>1600kD). Sera of "fresh" patients responded to additional fractions less than 800kD, suggesting that the reactivity to those SEA fractions fell down quickly after worm removal from the host. The fraction(s) around 400kD gave the most discriminatory results to which all but 2 "fresh" patients showed positive responses, while no "previous" serum showed positive response ($\chi^2 = 32.5$, $p < 0.001$). Although we investigated two ethnically different populations by using the schistosome antigen of Japan-origin, Chinese "previous" patients showed the same ELISA profiles as Japanese "previous" group. This suggests that the SEA used was not likely to have big influence. A previous report by Ishii and Owhashi (1982b) showed a similar response pattern in "fresh" Filipino patients with schistosomiasis japonica as was observed in the Chinese "fresh" patients.

Immunological mechanisms why two patient groups recognized different fractions are not clear. There have been several reports describing antibody responses to schistosome antigens which were characteristic for a particular infection stage(s) (Owhashi *et al.*, 1986; Evengard *et al.*, 1990). It is known that host responses to parasite antigens are somehow regulated by the existence of adult worms, eggs, and both of them (Pearce *et al.*, 1991). Considering the presence or absence of adult worms, we determined "fresh" and "previous" patients. One of the possibilities is that the SEA-400kD fractions contain a determinant(s) shared with adult worms or their metabolic products, and continuous antigenic stimulation has been kept in "fresh" patients.

Although it is a big problem that non-specific reactions are common in serodiagnosis of schistosomiasis, recent researches have revealed that some fraction(s) of schistosome antigens had high diagnostic value without crossreactions (Moser *et al.*, 1990). Several molecules of such diagnostic antigens were demonstrated in adult worm antigen of *S. mansoni* in western blotting experiments, however, details are still not fully understood in case of egg antigens of *S. japonicum*. It was reported that specific IgG to *S. mansoni* SEA were rather homologous among different infection phases in western blotting (Harn *et al.*, 1989; Montesano *et al.*, 1989). Our data were inconsistent with those results, and the difference might have come from the method used. Comparing with western blotting, our method enabled us to analyze native antigen molecules in crude antigen preparation. Although molecular basis is not fully understood, we would like to demonstrate that the 8–9 min (400kD) fractions of *S. japonicum* SEA have a highly diagnostic value for schistosomiasis. These fractions contain only less than 2% of whole protein contents of SEA (Fig. 1), and are likely to express high immunogenicity for "fresh" patients. On the other hand, high molecular weight fractions of >1600kD were of less diagnostic because of strong cross-reactions and/or non-specific reactions. We confirmed that sera obtained from patients with fascioliasis are much more crossreactive to schistosome antigen compared with the case of paragonimiasis (Aronstein *et al.*, 1985).

As for a possibility if we could apply our data for clinical or epidemiological studies, there are several problems. We observed almost same responding patterns to fractionated SEA between "previous" schistosomiasis and fascioliasis. We have to find SEA molecules recognized only by *S. japonicum*-infected sera. Another problem is the existence of diversity in reactivities to fractionated SEA. Some "fresh" patients gave only

faint reactivities to the 8–9 min fractions. We think that the present information can be applicable only for population study, but not for individual clinical evaluation.

Physicochemical characteristics of the diagnostic or crossreactive SEA fractions are elusive. IgG reactivities to crossreactive high molecular weight fractions were still partially preserved even after vigorous heat treatment. The findings that WGA and PNA inhibited the reactivity suggest the involvement of carbohydrate chains, especially mucinous sugars in the high molecular weight fractions. On the other hand, the heat-sensitive diagnostic 400kD fraction seems to be a protein antigen. It is required to purify the responsible molecule(s) in the fraction.

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