

IMMUNOBLOT ANALYSIS OF *SCHISTOSOMA JAPONICUM* EGG ANTIGENS WITH SERA FROM PATIENTS WITH ACUTE AND CHRONIC SCHISTOSOMIASIS JAPONICA

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Abstract. Humoral immune responses of IgG, IgM, IgA, IgE and IgG subclass antibodies to *Schistosoma japonicum* egg antigens were determined by immunoblotting with serum samples from individuals in China with acute (n=24) or chronic (n=35) schistosomiasis. In general, IgM, IgA, and IgE in sera from acute patients exhibited strong binding to antigens but binding was much weaker in chronic cases. Reaction of IgG4 of chronic cases was stronger than that of IgG4 of acute cases. The recognition profile of each antibody isotype in sera was analyzed for 11 major antigen molecules (antigens with apparent molecular weights of 82, 76, 61, 57, 53, 46, 40, 32, 27, 10 and less than 6.5 kDa). Except for the 10 kDa molecule, they were well-recognized by IgA and IgE in sera of acute cases. In other combinations of antibody class and clinical phase, recognition patterns against these molecules differed among individuals. Notably, the 10 kDa molecule was specifically recognized by total IgG and IgG4 in sera from most of the chronic patients, but in sera from only one acute case. This result suggests that the 10 kDa molecule is one of the major target antigens of IgG4 and may be useful as a marker antigen to characterize the clinical phases of *S. japonicum* infection.

INTRODUCTION

The occurrence and development of schistosomiasis is strongly related to human immunity to schistosomes. Therefore, analysis of schistosome antigens is useful not only for understanding of immunoprophylaxis and of the immunological pathogenesis of this disease but also for providing antigens for establishing a specific, sensitive diagnostic technique. There have been many studies of the profile of immunoglobulin class and IgG subclass antibody responses to schistosomiasis. Changes in reactivity of IgM and/or IgG subclass antibodies in sera collected from schistosome-infected mice were investigated with ELISA (Mountford *et al*, 1994; Yokoi *et al*, 1996). For human schistosomiasis patients, Jassim *et al* (1987) reported the avidity of IgG, IgM, IgA and four IgG subclass antibodies against larval, adult and egg stage antigens of *Schistosoma mansoni*,

using ELISA. In our previous study (Hu *et al*, 1999), the responses of immunoglobulins in sera from Chinese schistosomiasis japonica patients against *S. japonicum* egg antigens were determined by ELISA, and the results for acute and chronic disease were compared. We found that the averages of ELISA values for IgM, IgA and IgE in sera were lower in chronic than in acute disease. In contrast, the average in ELISA value for IgG4 in chronic disease was higher than that in acute disease. The biological basis of the development of particular subclasses of antibodies against parasite antigens during the various stages of infection is not fully understood.

Immunoblot analysis has been used by many authors to determine target antigens suitable for serodiagnosis. They found that antibodies against a 31 kDa of *S. mansoni* adult worm antigen (AWA) (Ruppel *et al*, 1985a,b) or a 32 kDa of *S. japonicum* AWA (Itoh *et al*, 1988) were detected in the early phase of schistosome infection. Webster *et al* (1997) reported that 22 kDa molecules of *S. mansoni* AWA and *S. japonicum* AWA were predominant antigens reactive with IgE. Langley *et al* (1994) investigated the responses of IgG subclass antibodies to *S. mansoni* egg an-

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tigens with ELISA and immunoblotting, although they provided no description of particular antigens.

In this study, we attempted to identify immune responses related to clinical manifestations of schistosomiasis japonica by using immunoblotting with *S. japonicum* egg antigens to dissect the anti-schistosome immunoglobulin isotype and IgG subclass antibody responses in individuals infected with *S. japonicum*.

MATERIALS AND METHODS

Human sera

The human sera used in this report were of the same lot as those used by Hu *et al* (1999). Acute case sera: 24 Chinese patients with *S. japonicum* eggs in their stool were obtained from the clinic of the Hubei Institute of Schistosomiasis Control (People's Republic of China). About one month after contact with water contaminated with cercariae of *S. japonicum*, subjects exhibited fever, diarrhea and liver enlargement.

Chronic case sera: 35 Chinese patients aged 10-57 years with *S. japonicum* eggs in their stool were obtained from a highly endemic area in Gonggan County, Hubei Province, People's Republic of China. Most of the patients had minimal or no hepatosplenomegaly. All of the patients had a high level of IgG anti-soluble egg antigen (SEA).

Parasite antigen

S. japonicum Yamanashi strain was maintained in *Oncomelania nosophora* snails and ICR strain mice. *Schistosoma japonicum* eggs were isolated from the intestine of infected mice by digestion with pronase and collagenase, lyophilized and frozen at -70°C until use (Matsuda *et al*, 1984).

Immunoblot analysis

Individual patients were screened to identify reactivity of each class of immunoglobulin (IgG, IgM, IgA and IgE) and subclass of IgG (IgG1, IgG2, IgG3 and IgG4) to *S. japonicum* egg antigen by Western blotting. Egg antigens extracted by the same method as used by Yokoi *et al* (1997) were separated in a 4~20% SDS polyacrylamide gel (TEFCO, Japan) and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore). PVDF strips were blocked with a blocking solution (5% skim milk, 0.3% Tween20 in PBS) at 4°C overnight and then washed three times for 10 min-

utes with a solution (0.5% skim milk, 0.3% Tween20 in PBS) before incubation with serum. For IgG, IgM, IgA, and IgE assay, the egg antigen-coated PVDF sheets were incubated with 500 µl of 1:200 (IgG, IgM assay) or 1:10 (IgA, IgE assay) dilutions of individual human sera for 2 hours at room temperature. They were then washed 3 times in a washing solution (0.3% Tween20 in PBS) and incubated with goat anti-human IgG (γ-chain) conjugated with horseradish peroxidase (HRP) (TAGO Inc No. 2390, Japan), goat HRP-conjugated anti-human IgM, goat HRP-conjugated anti-human IgA, goat HRP-conjugated anti-human IgE (Miles Scientific No.61-132, No. 61-131, No. 61-133, Israel) at room temperature for 60 minutes. After 4 more washes, the PVDF strips were developed using 0.02% (W/V) DAB (3,3'-diaminobenzidine) in PBS. For IgG subclass assay, the egg antigen-coated PVDF sheets were incubated with 500 µl of 1:50 dilutions of individual human sera for 2 hours at room temperature, and were then washed 3 times in the washing solution and incubated with mouse anti-human IgG1, IgG2, IgG3 and IgG4 monoclonal antibodies (COSMO BIO, Japan. No.0280, No.0281, No.0282, No.0283), the dilutions of which were 1:250, 1:1,000, 1:250, and 1:500, respectively, for 2 hours at room temperature. After 4 more washes, the bound human antibody was detected with a 1:1,000 dilution of goat HRP-conjugated anti-mouse IgG1 (ZYMED Japan No. 61-0120) and developed with DAB substrate solution.

After drying, immunoblotted sheets were photographed. For Figs 2 and 3, lanes of the photographs were cut out and arranged by class and subclass of immunoglobulins.

The apparent molecular weight of each band was determined using molecular weight markers (DAIICHI II, DAIICHI PURE CHEMICALS, Japan, and molecular weight marker II, TEFCO, Japan).

RESULTS

Twenty-four acute patients and 35 chronic patients were studied for IgG, IgM, IgA, IgE, and IgG subclass (IgG1, IgG2, IgG3 and IgG4) responses to *S. japonicum* egg antigens. There was great heterogeneity in the recognition profile of the egg antigens amongst infected individuals and antibody isotypes. Immunoblotting patterns with various classes of antibodies in serum from one acutely infected patient are presented in Fig 1 to

show typical bands.

In general, immunoblotting with IgA and IgE from acute patients revealed strong binding to antigens (Fig 2) but much less binding in chronic cases. Recognition of the antigens by IgM was also strong in acute cases but not in chronic cases (Table 1). On the other hand, IgG4 in chronic cases exhibited stronger reactions than those in acute cases (Fig 3). These results agree with those of a comparative study using ELISA (Hu *et al*, 1999).

Of the various bands, 11 recognized by many patients were selected (Fig 1) and were investigated. We determined the presence/absence of the 11 bands on immunoblotting patterns and counted the number of patients recognizing these bands by each class and subclass of antibodies. The ratios of individuals recognizing each band by immunoglobulin class (Table 1) and IgG subclass (Table 2) are shown. On comparison of immunoblotting patterns between acute and chronic cases, both total IgG and IgG4 responses were found to a band with molecular weight of 10 kDa in most chronic cases (Fig 3). Responses of IgG1 and IgG3 to the 10 kDa antigen were weaker and lower in frequency (48.6% and 37.1%, respectively) than those of total IgG and IgG4 (94.3% and 82.9%, respectively) (Tables 1, 2). The 10 kDa antigen was found in only one acute case (Fig 1).

DISCUSSION

Choice of antigen preparation is important in designing an immunodiagnostic system. Col-

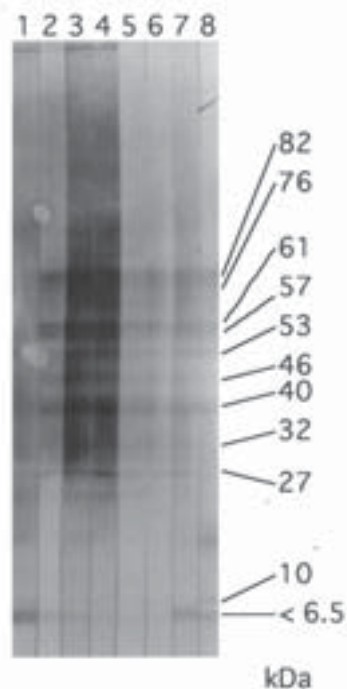


Fig 1—Immunoblot analysis of a *Schistosoma japonicum* egg extract with serum from one acutely *S. japonicum*-infected patient. Blot developed for the presence of total IgG (lane 1), IgM (lane 2), IgA (lane 3), IgE (lane 4), IgG1 (lane 5), IgG2 (lane 6), IgG3 (lane 7), and IgG4 (lane 8). Bars on the far right indicate the positions of 11 predominant bands with apparent molecular weight of 82, 76, 61, 57, 53, 46, 40, 32, 27, 10, and less than 6.5 kDa.

Table 1

Rate of recognition of each band by immunoglobulin class (%). Immunoblots were performed with sera of 24 acute patients and 35 chronic patients.

Band (kDa)	IgG		IgM		IgA		IgE	
	Acute	Chronic	Acute	Chronic	Acute	Chronic	Acute	Chronic
82	50.0	20.6	50.0	0.0	79.2	32.4	79.2	23.5
76	12.5	14.7	29.2	0.0	50.0	14.7	41.7	8.8
61	66.7	64.7	54.2	0.0	79.2	55.9	87.5	50.0
57	37.5	50.0	54.2	0.0	83.3	38.2	79.2	32.4
53	62.5	61.8	45.8	0.0	79.2	44.1	79.2	35.3
46	50.0	50.0	29.2	0.0	87.5	32.4	91.7	29.4
40	70.8	52.9	58.3	0.0	87.5	47.1	87.5	41.2
32	29.2	55.9	20.8	0.0	66.7	20.6	66.7	20.6
27	50.0	85.7	33.3	2.9	87.5	61.8	66.7	52.9
10	4.2	94.3	0.0	0.0	0.0	2.9	0.0	2.9
<6.5	58.3	100.0	50.0	2.9	50.0	28.6	33.3	2.9

Table 2

Rate of recognition of each band by IgG subclass (%). Immunoblots were performed with sera of 24 acute patients and 35 chronic patients.

Band (kDa)	IgG1		IgG2		IgG3		IgG4	
	Acute	Chronic	Acute	Chronic	Acute	Chronic	Acute	Chronic
82	79.2	54.3	50.0	2.9	79.2	37.1	62.5	22.9
76	25.0	8.6	20.8	0.0	25.0	17.1	29.2	14.3
61	70.8	77.1	41.7	5.7	75.0	37.1	54.2	34.3
57	62.5	48.6	37.5	0.0	50.0	28.6	45.8	40.0
53	50.0	65.7	29.2	2.9	45.8	31.4	33.3	51.4
46	58.3	54.3	33.3	0.0	62.5	34.3	54.2	68.6
40	75.0	77.1	50.0	2.9	70.8	48.6	62.5	68.6
32	8.3	34.3	4.2	0.0	12.5	17.1	12.5	80.0
27	62.5	62.9	50.0	0.0	62.5	31.4	62.5	77.1
10	0.0	48.6	0.0	2.9	0.0	37.1	4.2	82.9
<6.5	8.3	65.7	0.0	5.7	33.3	34.3	8.3	74.3

laborative studies on antigens for immunodiagnosis of both schistosomiasis mansoni and schistosomiasis japonica showed that crude schistosome egg extracts yielded higher levels of sensitivity and specificity than crude worm extracts (Mott and Dixon, 1982; Mott *et al*, 1987). Further, it was shown that the use of CEF6, which was partially purified *S. mansoni* egg antigen, may provide even higher specificity than crude egg extracts obtained by ELISA (Dunne *et al*, 1984, 1991; McLaren *et al*, 1981). Two cationic antigens, ω -1 and α -1, have been identified in CEF6 and exhibited high antigenicity for *S. mansoni*-infected patients on immunoblotting analysis (Hamilton *et al*, 1998). These results suggest the importance of information on the characteristics, including antigenicity, of particular antigen molecules in developing an immunodiagnostic system. This study was designed to identify *S. japonicum* egg antigen molecules that are related to different clinical stages of infection and to examine the development of particular subclasses of antibodies against specific parasite antigens.

In Chinese subjects with acute schistosomiasis, 82, 61, 57, 53, 46, 40, 32, and 27kDa molecules were detected by IgA and IgE in most cases (66.7~91.7%). This result suggests that these molecules are strong antigens inducing IgA and IgE antibody production during the early phase of infection. In chronic cases, however, the rates of recognition of these molecules by IgA and IgE were decreased (20.6~61.8%). Ottessen *et al*

(1981) reported that the egg serves as the most important antigen for IgE in acute cases of *S. mansoni* infection. These results suggest that serodiagnosis by detection of anti-egg antigen IgA and IgE should be possible for cases of acute schistosomiasis.

The most striking difference between acute and chronic cases seen in this study was the 10 kDa egg antigen recognized only by IgG and IgG4 in sera from cases of chronic schistosomiasis japonica. This difference was so pronounced that we could find 10 kDa antigen present in only one acute case (Table 1). Comparing IgG4 response bands in acute and chronic cases, it was found that the patterns differed between the two groups, with chronic cases having stronger bands than acute cases (Fig 3). The 10 kDa molecule was also detected by IgG1 and IgG3 in sera from chronic cases, but the ratios of positive individuals (48.6% and 37.1%) were significantly lower than that for IgG4 (82.9%). This result suggests that the 10 kDa antigen should be useful as a target molecule for immunodiagnosis with IgG or IgG4 to indicate chronic schistosomiasis japonica infection.

Relatively high rates of recognition of the 10 kDa antigen with IgG4 from a chronic schistosomiasis patient living in an endemic area suggests another interesting characteristic of the 10 kDa molecule, that is, its immunological relation to reinfection by the parasite. Seroepidemiological studies have suggested that IgG4/IgE ratios may

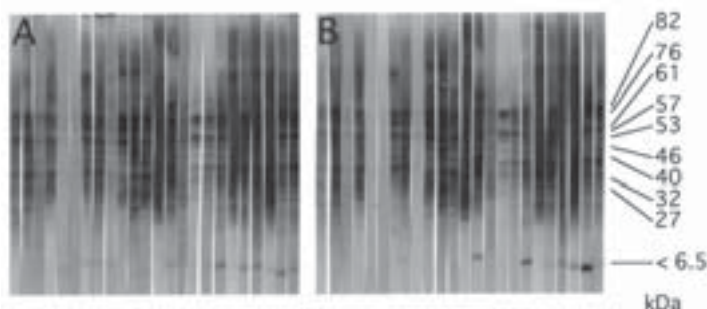


Fig 2—Immunoblot analysis of a *Schistosoma japonicum* egg extract with individual sera from patients acutely infected with *S. japonicum*. Blot developed for the presence of total IgA (A) and IgE (B). Proteins were separated by SDS-PAGE using a 4-20% acrylamide gradient gel. Bars on the far right indicate the positions of 10 bands depicted in Fig 1, other than that of 10 kDa.

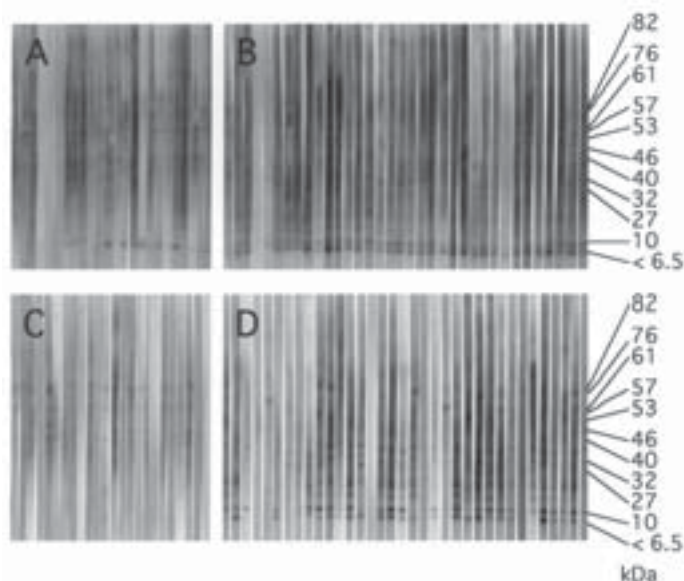


Fig 3—Immunoblot analysis of a *Schistosoma japonicum* egg extract with individual sera from patients acutely (A and C) and chronic (B and D) infected with *S. japonicum*. Blot developed for the presence of total IgG (A and B) and IgG4 (C and D). Proteins were separated by SDS-PAGE using a 4-20% acrylamide gradient gel. Bars on the far right indicate the positions of 11 predominant bands with apparent molecular weight of 82, 76, 61, 57, 53, 46, 40, 32, 27, 10, and less than 6.5 kDa.

be related to human resistance to schistosome infection (Demeure *et al*, 1993; Caldas *et al*, 2000; Li *et al*, 2001). Briefly, IgG4 excess has been linked to reinfection and IgE excess to lack of reinfection. More studies are required to determine whether the 10 kDa molecule affects reinfection by inducing IgG4 production.

Studies on immunodiagnosis to find differences in immunoreaction between acute and chronic cases of schistosome infection have been reported by many authors (Valli *et al*, 1997; Hu *et al*, 1999; de Gouvea Viana *et al*, 2001). They showed that immunoreaction of each immunoglobulin class and subclass may indicate the clinical phase of schistosome infection. Li *et al* (1994) studied utilization of keyhole limpet hemocyanin (KLH) as a target antigen for ELISA. KLH has a carbohydrate epitope which is shared with the surface of schistosome larvae. They showed that antibodies (both IgG and IgM) that recognize KLH were present at higher levels in sera of patients with acute schistosomiasis japonica infection than in chronic cases, using ELISA. In contrast, the 10 kDa molecule found in this study was specific to sera from individuals with chronic schistosomiasis, with a high rate of recognition. Development of diagnostic systems to detect immunoreaction by class and subclass of antibodies to particular antigens, such as KLH or the 10 kDa molecule, which have different antigenicities depending on the clinical phase of schistosomiasis, may yield more information on the stage of infection.

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