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Monoclonal Antibodies Against UV Attenuated Cercariae of *Schistosoma japonicum*. 2. Analysis of Progressive Changes of Surface Antigens along Developmental Stages

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Abstract

Monoclonal antibodies (McAb) that bound to the surface antigens of *Schistosoma japonicum* schistosomula were produced by using spleen cells from BALB/c mice vaccinated with UV attenuated live cercariae. Thirteen McAb were differentiated by the results of immunofluorescence to the egg and developmental stages, and by reactions in ELISA. Responses were also examined for 11 McAb to cercaria, schistosomula at 3 to 72 hr cultures, lung schistosomula and juvenile worms in the liver. Eight McAb, which did not bind to cercariae bound to 3 hr-schistosomula. From this observation, the sudden occurrence of a drastic change in the surface antigenicity was proven. Nevertheless, changes of surface antigenicity of schistosomula were not observed during cultivation. A remarkable difference was also realized in surface antigenicities between the schistosomula at the early stage and lung schistosomula, since 4 McAb out of the above 8 did not react to the lung schistosomula. The difference was also noticed between lung schistosomula and juvenile worms in the liver. Thus, the progressive changes of surface antigen were clarified for the first time along the developmental stages of *S. japonicum* using McAb of varied characteristics.

Key words: *Schistosoma japonicum,* monoclonal antibody, developmental stages, surface antigen

Introduction

In our previous report (Nakamura *et al.*, 1990), monoclonal antibodies (McAb) were produced by using spleen cells obtained from BALB/c mice immunized with live cercariae attenuated by UV irradiation, and 2 McAb among them had protective activities in killing schistosomula of *Schistosoma japonicum, in vivo* and *in vitro*.

The present study describes antigenic characteristics of different stages of *S. japonicum, i.e.* from eggs to adults, by using 13 McAb, including the above two.

It has been reported that the target antigens or stages of the worm in protective immunity are schistosomulum transformed from cercaria (Smithers *et al.*, 1977). Especially, in the protective immunity induced by cercariae attenuated by irradiation, the target stage has been suggested to be the schistosomula in the course of migration immediately after skin penetration, through the lung, up to the liver (Miller and Smithers, 1982; von Lichtenberg *et al.*, 1985; Dean and Mangold, 1984; Mangold and Dean, 1986). Special attention was paid in the present study to observe the changes on surface antigens recognized by varied McAb on hand for the stages from cercariae to lung schistosomula. During analysis of surface antigens, the different characteristics of McAb were also demonstrated.

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Materials and methods

Schistosoma japonicum and experimental animals:

The Yamanashi strain of *S. japonicum* successively maintained in our laboratory in female DDD mice at age 5 to 8 weeks and in *Oncomelania nosophora* was used. BALB/c mice at age 7 to 8 weeks were used for immunization and maintaining McAb in ascites.

Parasite and antigens:

1. Cercariae. Cercariae floating on the surface of the water were collected by the same procedures as in our previous paper (Nakamura *et al.*, 1990).

2. Schistosomula and juvenile worms. Schistosomula were prepared from collected cercariae by the mechanical transformation method (Colley and Wikel, 1974) and maintained in sterile RPMI 1640 medium at 5% CO_2 gas phase at 37°C.

Mice were infected with 2,000 cercariae percutaneously on the abdominal wall by the cover slip method (Yasuraoka *et al.*, 1978). For collecting lung schistosomula, mice were sacrificed 4 days after infection at which time the lungs were removed. Collecting juvenile worms, entailed the removal of the liver 10 days after infection. The removed tissues were dissected into pieces with scissors, maintained in RPMI 1640 medium under the same culture condition overnight and schistosomula or worms coming out of the tissues were collected.

3. Adult worms. Mice were infected with 60 to 80 cercariae subcutaneously with a 23G needle. After 7 to 8 weeks, mice under anesthesia were bled by heart puncture, and adult worms in the portal system and mesenteric veins were collected. After they were kept in PBS for 1 to 2 hrs, they were placed on a sheet of filter paper for blotting water, lyophilized and kept at −70°C.

4. Eggs. Isolation and purification of eggs from the intestine of infected mice was performed by digesting the dissected intestine using 0.01% pronase and 0.05% collagenase following the method of Matsuda *et al.* (1981). Some adult worms and eggs were embedded in Tissue Tek II® and kept frozen at −70°C as the antigens for immunofluorescence (IF).

5. Antigens for ELISA. Antigens for ELISA was prepared by extracting from adults and eggs in 0.05M carbonate buffer at pH 9.6 by the method of Matsuda *et al.* (1981) and kept at −70°C.

6. Antigens for IF. Adults or eggs embedded in Tissue-Tek II® were sectioned at 5 μm by Cryostats, and the sectioned antigens on a glass slide were kept at −20°C after fixing them with cold acetone for 10 minutes.

Production of McAb:

Production of McAb was followed by the descriptions in our previous paper (Nakamura *et al.*, 1990). BALB/c mice were immunized by two different schedules with about 500 cercariae attenuated by UV irradiation either at a dose of 300 mW•sec/cm^2 or 500 mW•sec/cm^2.

Spleen cells collected from immunized mice were fused with the mouse myeloma cells, P3xAg8.653, in the presence of polyethylenglycol. Hybridoma cells in a well, producing active antibodies bound to surface antigens of live schistosomula detected by IF, were selected. Cloning was conducted twice by the single cell manipulation method in the same manner as our previous study (Nakamura *et al.*, 1990).

Immunofluorescence (IF):

As the antigens for IF, sectioned adult worms or eggs mounted on a glass slide were used, and indirect IF was performed by the same procedures as our previous paper (Kawamura and Aoyama, 1983; Nakamura *et al.*, 1990), using the supernatant of culture medium or ascites diluted at 1:100 as the primary antibody.

After the reactions, antigen specimens were mounted with buffered glycerin solution and observed under a fluorescent microscope (Tiyoda, FM200B). The strength of fluorescence observed was coded with the marks from − to + + +.

When cercariae and schistosomula were used for the antigen for IF, 30 larvae in solution were put into each of the 96 wells on a Millititer Plate (SV; Millipore) with a membrane filter bottom. The water in the wells was taken out through the membrane by a suction apparatus and larvae in

the wells were washed 4 times by pouring 0.15M PBS containing 1% BSA and 0.05% Tween 20 into wells and sucking through the membrane. Whole procedures of reactions for IF were performed against larvae in wells by sucking solutions through the membrane at the end of each reaction. After all reactions for IF, larval antigens were transferred on a glass slide, mounted and observed in the routine method.

Enzyme-linked immunosorbent assay (ELISA):

Flat bottom wells of micro-ELISA plates (Dynatech, M129A) were sensitized with 100 μl of antigen solution each with egg or adult worm extract at a concentration of 10 μg/ml. After sensitization at 37°C for 2 hrs, plates with antigen solution were kept at 4°C overnight. Antibodies in supernatant of culture media and ascites diluted at 1:100 were examined by the method of Matsuda *et al.* (1984) in which peroxidase labeled goat anti-mouse immunoglobulins (Cappel) and ABTS (2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid) were used for the secondary antibody and substrate, respectively. Absorbance was measured by a micro-plate photometer (Corona MTP-12, Hitachi) at a wave length of 405 nm.

Results

Characterization of produced McAb by IF and ELISA

Among the McAb produced, those which bound to the surface of schistosomula were 13 (Table 1). By the determination of their class and subclass, 4 were IgG_1 and 9 were IgM. Their reactivities to the different locations of the adult worm and egg in frozen sections as well as the whole schistosomulum was studied by IF for characterization of each McAb. ELISA using egg and adult antigens was also performed as a reference parameter.

Of the four IgG_1, a McAb coded as 6-10D was indicated to be specific to schistosomula by the reactions i.e., the McAb did not bind to adults and eggs but it did bind only to schistosomula by IF (Fig. 1A). Absorbance in ELISA against adult and egg antigens was also low with this McAb.

A McAb, 6-5E reacted to schistosomula and the surface of adult worms (Fig. 1B, H). 3-9A did not bind to eggs but to the parenchyma of adults except the intestine. 2-5C bound to eggs, especially to the miracidium in eggs.

One of 9 McAb of IgM, (14-12H) bound to a substance located in the space between the vitelline membrane and miracidium, and to the membrane but the McAb did not bind to the adult worm. The other 8 McAb recognized the antigens common to adults and eggs. Among the above 8, 11-11A bound to the subtegumental tissues spottedly (Fig. 1G) and to the testes. Among these 8 IgM, 3-12F bound to the surface of the anterior part of the cercarial body (data not shown) but were not identical to 4-2F. Thus these 13 prepared McAb which recognized the surface of the schistosomulum, all antibodies were differentiated by their difference of recognizing sites of tissues in the egg, adult worm and lung schistosomulum, and by the difference of ELISA.

Reactions of McAb to adult worms and eggs were not always identical in IF and ELISA. 1-11C was strongly positive to egg antigen in IF, while it was negative for ELISA. Inversely, 14-12H which was negative to adult worms by IF showed high absorbance in ELISA (OD 0.826).

Reactivity of McAb to different stages of S. japonicum by IF

Reactivity of 11 McAb was examined by IF to the surface antigen of larval stages along their development for studying progressive changes of antigenicity. The larval stages examined were cercariae, schistosomula cultured in RPMI 1640 for 3, 24, 48 and 72 hrs after transformation, those recovered from the lungs of mice 4 days after infection, and juvenile worms from the liver 10 days after infection.

The results of reactivity are shown in Table 2. Of the 11, 8 McAb did not react to cercariae, but to the surface of cultured schistosomula. From these results, a remarkable change was observed in the surface antigenicity between cercaria and schistosomulum. Among 8, 5-6F showed positive reactions to schistosomula from

Table 1 Reactivity of monoclonal antibodies to different stages of *S. japonicum* examined by IF and ELISA

			Immunofluorescence*							ELISA (OD)†	
			Schistosomula‡	Adult			Egg				
No.	Clone code	Ig	Surface	Teg	Parc	Gut	Vm	Vs	Mir	Adult	Egg
1	6-10D	G1	+++	–	–	–	–	–	–	0.114	0.086
2	6-5E	G1	++	+++	–	–	–	–	–	0.243	0.072
3	3-9A	G1	+++	–	+++	–	–	–	–	0.321	0.047
4	2-5C	G1	++	–	–	–	–	–	++	0.301	0.274
5	1-11C	M	+++	–	+++	–	+++	+++	+++	0.800	0.081
6	14-5B	M	+++	–	+++	++	+++	+++	+++	0.537	0.988
7	4-2F	M	+++	++	++	++	+++	+++	+++	>2.000	0.342
8	5-6F	M	++	+++	+++	+++	+++	+++	+++	0.356	0.184
9	14-12H	M	++	–	–	–	+++	+++	–	0.826	0.531
10	5-9C	M	+++	–	++	–	–	+++	–	0.452	0.122
11	11-11A	M	+++	–	+++	–	+++	+++	+++	0.387	>2.000
12	16-10D	M	+++	–	+++	–	+++	+++	+++	0.386	0.692
13	3-12F	M	++	++	++	+++	+++	+++	+++	0.954	1.301
	NMS		–	–	–	–	–	–	–	0.116	0.086
	IMS		+++	+++	+++	+++	+++	+++	+++	1.405	1.597

* Intact schistosomula and cryosections of adult worms and eggs were reacted with ascites diluted at 1:100. NMS and IMS were diluted at 1:20 and 1:50, respectively.

† CBS soluble adult and eggs antigen were used at the concentration of 10 μ/ml. Ascites was diluted at 1:100. NMS and IMS were diluted at 1:500 and 1:800, respectively.

‡ 24h-mechanical schistosomula.

Abbreviations: NMS; Normal mouse serum, IMS; Infected mouse serum, McAb; Monoclonal antibody, Teg; Tegument, Parc; Parenchyma, Vm; Vitelline membrane, Vs; Vitelline space, Mir; Miracidium

24 hrs cultivation onward, and the other 7 were positive from 3 hrs. Three clones of McAb 6-10D, 14-5B and 16-10D recognized antigens common to both cercariae and schistosomula.

There was no big difference in reactivity among 11 McAb to the surface antigens of schistosomula at different cultivation period from 3 hrs to 72 hrs.

The different reactivity of the antigens to these McAb demonstrated that the characteristics of surface antigens varied according to developmental stages of schistosome (Table 2).

To surface antigens of lung schistosomula, 7 McAb showed positive reactivity. McAb, 6-10D, 11-11A and 16-10D, which reacted to the surface of 3 hr-schistosomula, did not recognize the surface of lung schistosomula. McAb, 5-6F, 11-11A and 16-10D, which bound to the surface of juvenile worms in the liver did not react to the lung schistosomula.

Only one McAb, 14-5B IgM, bound to an antigen common to all developmental stages from cercariae to juvenile worms. Nevertheless, it did not react to the surface of adult worm. An IgG_1 McAb 6-5E was the only one among McAb present to be characteristic in binding widely to the stage of schistosomulum, lung schistosomulum (Fig. 1D), surface of juvenile worms and adult worms. 6-10D IgG_1 McAb was unique in binding to cercariae and schistosomula, although the McAb bound selectively to the cercarial body but not to the tail (Fig. 1E).

Table 2 Reactivity of monoclonal antibodies to the surface of developing stages of *S. japonicum* by immunofluorescence

Clone code	Ig	Cercariae	Schistosomula*				Lung Schisto-somula	Liver worms
			3h†	24h	48h	72h	4 day‡	10 day
6-10D	G1	+++	+++	+++	+++	++	–	–
6-5E	G1	–	+	+	++	++	++	++
2-5C	G1	–	+	+	+	ND	+	+
1-11C	M	–	++	+	++	ND	++	+
14-5B	M	+	+++	++	+++	++	+	++
4-2F	M	–	++	+	+	+	+	+
5-6F	M	–	–	+	+	ND	–	+
14-12H	M	–	+++	++	++	ND	++	+
5-9C	M	–	++	+++	+	+++	++	+
11-11A	M	–	++	+++	++	++	–	++
16-10D	M	+++	+++	+++	+++	++	–	++
NMS§		–	–	–	–	–	–	–
IMS‖		++	+++	+++	+++	+++	++	++

* Mechanically transformed schistosomula
† Hours post-transformation
‡ Days after infection
§ Normal mouse serum diluted at 1:20
‖ Infected mouse serum diluted at 1:50

Discussion

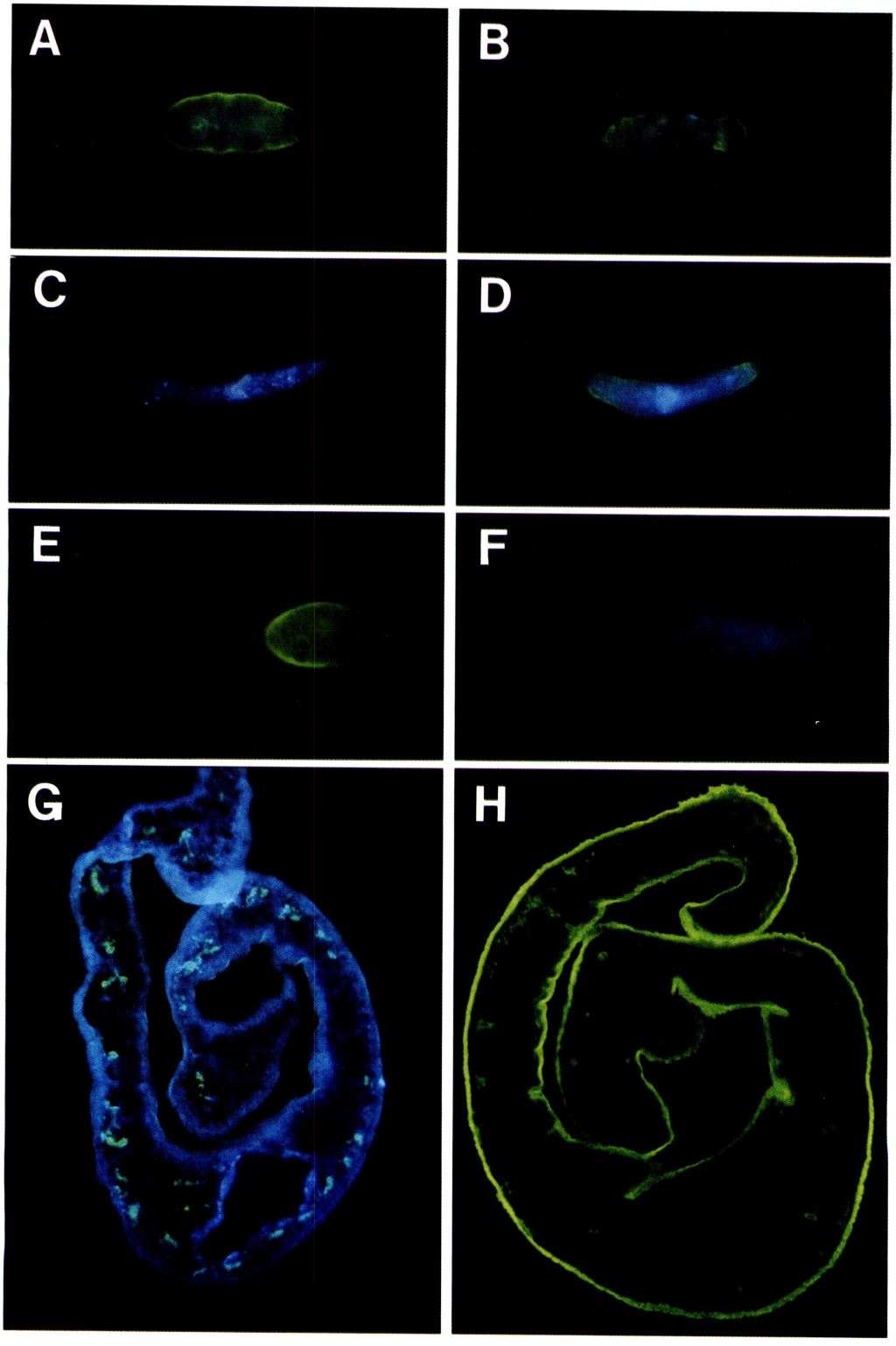
In the present study, 13 McAb recognizing the surface of schistosomula were prepared from spleen cells from mice immunized with UV attenuated cercariae. These McAb were differentiated by their characteristics of affinity to various stages of *S. japonicum* by IF.

Riengrojpitak *et al.* (1989) produced 16 McAb by using surface antigens of *S. mansoni* adults and classified them into 9 groups according to their reactivity to cercaria, mechanically transformed 3 hr-schistosomula and other developmental stages to adults by IF. Their McAb, however, were not reactive to lung schistosomula or juvenile worms in the liver but reactive to the antigens in certain tissues, commonly to all the developmental stages, including portions of the tegument.

The results in the present study, differing greatly to the above results, demonstrated that each McAb was differentiated by its special characteristics in binding to the different antigens along the developmental stage by analysis of reactions in IF, and in ELISA, using egg and adults antigens.

Out of 13 McAb produced, 9 were IgM class, 8 of which reacted strongly and similarly to parenchymal antigens of adults and eggs. The epitopes of IgM McAb against schistosomes previously reported were mostly polysaccharides. An IgM McAb, 11-11A, showed a peculiar

Fig. 1 Indirect immunofluorescence patterns with monoclonal antibodies of 6-10D (A, C and E), 6-5E (B, D, F and H) and 11-11A (G) against different developmental stages of *S. japonicum*. Surface of live cercariae (E and F), mechanically transformed 24 hr schistosomula (A and B), 4 day lung schistosomula (C and D), and cryostat sections of adult worms (G and H) of *S. japonicum*.


A
B
C
D
E
F
G
H

picture, closely resembling that of binding PNA lectin (Beisler *et al.*, 1984) and those by IF with IgG_2 and IgM McAb by de Water *et al.* (1987). Referring to the previous works, our McAb 11-11A was also considered to be the one recognizing the antigenic determinants related to those polysaccharides.

It was noticed that there was disagreement between the results of IF and ELISA (Table 1). It could be interpreted that when IF reactive antigen was hardly eluted by the carbonate buffer in the procedures of antigen preparation for ELISA or difficult to adsorb to the solid phase, antigenicity for ELISA was weakened. On the contrary, when determinants of ELISA antigens in adults or eggs were denatured or eluted out in the procedures of fixation for IF, reactivity in IF was reduced enormously.

It has been known that the surface of schistosome was changed by repeated desquamation and reconstruction along the larval development (Hockley and McLaren, 1973; Mitchell and Cruise, 1985). In accordance with that, it was observed in the present study that there was a big difference in reactivity of McAb to cercariae and schistosomula by IF.

The antigenic structures on the surface of the cercariae have been reported to fall out in 24 hrs after transformation to schistosomula (Samuelson *et al.*, 1980). Occurrence of new antigens has been also reported on the surface of schistosomula of *S. mansoni,* mechanically transformed and cultured *in vitro* (Simpson *et al.*, 1984). These antigenic components were almost the same as skin recovered schistosomula (Payares *et al.*, 1985) and the surface antigenicity has been reported to be stable for a period from 3 to 24 hrs after transformation (Dissous *et al.*, 1982; Payares *et al.*, 1985). The stable antigenicity was also observed in the present study in schistosomula in a period from 3 to 72 hrs after transformation.

There are a few reports on the McAb that can recognize lung schistosomula of *S. mansoni* (Gregoire *et al.*, 1987; Harn *et al.*, 1985; Aronstein and Strand, 1983). In the present study the antigenic change of the schistosomula *in vivo* was unknown, but at least 7 McAb were found to bind themselves to lung schistosomula. Thus, the present paper is the first report on McAb to *S. japonicum* with such a character.

Contrary to the above results, out of 4 McAb which did not bind to lung schistosomula, this seems to indicate that some antigenic determinants on the surface disappeared at the phase of lung schistosomula. Three of the McAb (6-10D and 5-6F, 16-10D) which did not recognize the lung schistosomula even though they were treated to the method of Aronstein and Strand (1983), removed the host antigens from the surface of the lung schistosomula (unpublished observation). Similar results have been reported for *S. mansoni,* in which certain antigenic determinants become undetectable in schistosomula 5 days after infection (Simpson *et al.*, 1984) despite the fact that they retained the host derived antigens (Payares *et al.*, 1985).

Thus the present study demonstrated for the first time the change of distribution of antigenic determinants on the surface of worms along the developmental stages of *S. japonicum.*

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