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Monoclonal Antibodies Against UV Attenuated Cercariae of *Schistosoma japonicum* 1. Production and Their Effect on Protective Immunity

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Abstract

Monoclonal antibodies (McAb) that bound to surface antigens of *Schistosoma japonicum* schistosomula were generated from BALB/c mice vaccinated with ultraviolet (UV) attenuated live cercariae. The effective UV dose for attenuating cercariae was from 300 to 500 mW•sec/cm^2. The attenuated cercariae induced significant protective immunity (33.9 to 47.3%) against challenge infection with *S. japonicum*. A total of 13 hybridoma cell lines producing McAb was selected. Their isotypes were 4 IgG_1 and 9 IgM. Two IgG_1 McAb (coded as 6-10D and 6-5E) killed *in vitro* up to 72.3% of schistosomula in the presence of complement. These 2 McAb confer protective immunity to naive BALB/c mice resulting in significant reduction of worm burdens.

Key words: *Schistosoma japonicum*, Monoclonal antibody, UV attenuation, Protective immunity

Introduction

It is known that the host animals acquire resistance to challenge infection with schistosomes when they are inoculated with live cercariae or schistosomula attenuated by irradiation with X ray. The effect of vaccination with attenuated larval schistosomes was proven by the laboratory experiments (Stek *et al.*, 1981, Webb *et al.*, 1982) and also by the field trials (Hsü *et al.*, 1984, McCauley *et al.*, 1984) even though the protective effect was not complete. Since this protective immunity was introduced to the naive mice by parabiotic operation (Dean *et al.*, 1981) or by transfer of the immune serum, it was suggested that antibodies were the effective molecules of protection against schistosome infection (Mangold and Dean, 1986). The target of protective antibodies was known to be schistosomula (Smithers *et al.*, 1977). The use of ultraviolet (UV) ray for attenuation is safer than that of gamma irradiation, and was reported to be as effective as the latter (Dean, 1983, Dean *et al.*, 1983, Moloney *et al.*, 1985).

Recently, monoclonal antibodies (McAb) against surface antigens of schistosomula were produced, and an attempt was made to induce the protective immunity *in vivo* by these McAb with certain degree of success (Bickle *et al.*, 1986, Smith *et al.*, 1982, Zodda and Philips, 1982), and the mechanism of protective immunity induced by gamma attenuated worms was studied *in vitro* using schistosome specific McAb.

In the present study, the McAb which recognized surface antigens of schistosomula was produced by immunizing BALB/c mice with UV attenuated cercariae of *Schistosoma japonicum*. The main objective of this study was to select the McAb with effective protection against *S. japonicum* infection and to establish the effective

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cell lines.

MATERIALS AND METHODS

Schistosoma japonicum: Yamanashi strain maintained in the laboratory by serial passage in mice and *Oncomelania nosophora* was used.

Cercaria: The infected *O. nosophora* were crushed between 2 glass slides and put into aged tap water in a small test tube. Cercariae floated on the surface of the water were collected by a cover-slip method (Yasuraoka *et al.*, 1978) and used for infection or for preparation of schistosomula.

Preparation of Schistosomula: Schistosomula were prepared from pooled cercariae by mechanical transformation method (Colley and Wikel, 1984), and collected into RPMI 1640 medium by repeated centrifugation.

Attenuation by UV irradiation: Cercariae were concentrated and placed in aged tap water 4 mm deep in a glass petri dish of 55 mm diameter. UV irradiation was given by Minera Light Lamp, UVGL-25 (UVP, Inc., San Gabriel, Ca., USA) at a wave length of 254 nm. The intensity of UV irradiation was 2.5 mW/cm^2 when the lamp was set at a distance of 27 mm from the water surface.

Production of McAb:

1. Immunization procedures. BALB/c mice were immunized by two schedules. In one method, about 500 cercariae attenuated by UV irradiation at a dose of 300 mW•sec/cm^2 were inoculated into mice intraperitoneally (ip) twice at 4 week interval, and spleen cells were collected 8 weeks after the last immunization.

In another method, 500 cercariae attenuated by UV at a dose of 500 mW•sec/cm^2 were inoculated ip into mice twice at 2 week interval, 70 non-irradiated cercariae given ip 4 weeks after the 2nd inoculation, and then spleen cells were collected 16 weeks after the last inoculation.

2. Cell fusion and selection of antibody producing cells. Spleen cells were fused with the murine myeloma cells, P3xAg8.653, at the presence of 45% polyethylene glycol (M.W. 3,000, Merck). Hybridoma cells were selected on the HAT (hypoxanthine, aminopterin, thymidine) medium. Cells producing antibodies were selected by immunofluorescence (IF) binding assay to the surface of live schistosomula.

3. Cloning method. Cloning was conducted twice by the single cell manipulation method (Yoshida and Furuya, 1986).

4. Preparation of ascites. About 10^7 cloned cells were inoculated ip into a BALB/c mouse pretreated with 2,6,10,14-tetramethyl pentadecan (Pristan), and ascites fluid was collected 2 weeks later. The isotype of McAb was determined by an Ouchterlony's immunodiffusion method with anti-mouse IgM and IgG subclass antisera (Tago, Inc.).

5. IF. For the preparation of schistosomula to be used as the antigen of IF, freshly prepared schistosomula were cultured in RPMI 1640 at 37°C for 24 hr in 5% CO_2 gas phase. To each well of a Millititer Filtration Plate with 96 wells (SV; Millipore), 30 schistosomula were placed and washed 4 times with RPMI by sucking through the filter membrane at the bottom by negative pressure.

For dilution of antibodies and for rinsing antigen materials, 0.15M phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 was used. For IF, undiluted supernatant of the culture medium or the ascites diluted at 1/100 was used for the primary antibody and FITC-labeled goat anti-mouse Igs antibody (Cappel) diluted at 1/50 was used for the secondary antibody. The reactions with those antibodies were allowed to continue for 30 min each, and finally, schistosomula were transferred and mounted on a glass slide for fluorescent microscopic observation.

Schistosomula killing by McAb in vitro:

About 50 schistosomula in 180 μl sterile RPMI 1640 were placed into each of 2 or 4 flat bottom wells of a culture plate with 96 wells (Falcon) and incubated at 5% CO_2 gas phase at 37°C for 3 hr (3 hr schistosomula). To those wells, 20 μl McAb from ascites or antibody from mouse serum after inactivation at 56°C for 30 min were added and cultured for 30 min. Then, 20 μl of fresh guinea pig complement (Dunkin-Hartley strain provided by Shizuoka Experimental Animal Coop) were added. McAb or complement alone were used as controls.

Examination of each McAb was performed in 4 wells at one time or 2 wells at 2 experiments. Viability of schistosomula was determined after the cultivation for 18 hr by microscopic observation by the comprehensive results of motility, destruction of tegument, granulation of inner body, and trypan blue staining (Dean *et al.*, 1975), and % mortality rate was expressed as an average of 4 wells.

Protective effect by passive immunization:

Ascites containing McAb was diluted with saline at 1:2 and strained through filter paper at 0.45 μm porosity (Toyo Roshi). Inbred female BALB/c mice of 5 to 8 week old were injected ip with 1ml McAb solution. After 18 hr, 100 cercariae were inoculated by percutaneous infection by the coverslip method (Yasuraoka *et al.*, 1978). The same amount of diluted McAb was injected ip on the next day and 3 days after the infection. After 4 weeks, adult worms in the portal system were recovered by the perfusion method (Smithers and Terry, 1965) and remaining worms in the liver were allowed to go out by submersing the removed liver in PBS kept at 37°C overnight. The numbers of worms collected from mice were compared by Student's t-test between experimental and control mice.

Results

Optimal dose of UV irradiation to produce attenuated cercariae as vaccine for effective protection against re-infection:

Cercariae were attenuated with UV doses at 100, 200, 300, 500 and 1,000 mW•sec/cm^2. 300 cercariae attenuated with each dose of UV irradiation were inculated into mice (4–6 mice/group). To examine whether attenuated cercariae were capable to develop *in vivo*, mice were sacrificed 4 weeks after injection and worms were recovered by perfusion method (Table 1). Worm recovery from mice inoculated with cercariae attenuated with UV doses at 100 and

Table 1 Recovery of *S. japonicum* in BALB/c mice injected i.p. with UV irradiated cercariae

Irradiation dose (mW•sec/cm^2)	No. mice*	Mean worm recoveries ± SE**
Control	6	33.0 ± 4.3
100	5	10.4 ± 2.1
200	5	1.8 ± 0.6
300	5	0
500	4	0
1000	5	0

* Mice were injected i.p. with 300 cercariae.

** Worms were counted 4–5 weeks after injection.

Table 2 Protection against *S. japonicum* infection in BALB/c mice immunized with U.V. irradiated cercariae

			Worm recoveries**		
Exp. no.	No. mice*	Irradiation dose (mW•sec/cm^2)	Mean ± SE	Percent*** protection	P
1	6	Control	33.0 ± 4.3	–	
	5	300	21.8 ± 2.0	33.9	<0.05
	5	500	17.4 ± 3.8	47.3	<0.05
2	5	Control	23.4 ± 8.9	–	
	5	1000	20.8 ± 8.2	11.1	N.S.

*Mice were immunized i.p. with 300 irradiated cercariae and were challenged i.p. with 70 normal cercariae 4 weeks after immunization.

**Worms were counted 4–5 weeks after challenge infection.

***Calculated by (C-X) × 100/C, where C is the mean number of control mice and X is the mean number of immunized mice.

N.S. Not significant.

Table 3 Killing effect of monoclonal antibodies and complement on schistosomula of *S. japonicum in vitro*

McAb (Antibody)	Ig	Assay condition*	Mean percent of dead schistosomula ± SD***	P†
6-10D	G1	S + Ab	3.1 ± 4.6	
		S + Ab + C	72.3 ± 6.0	<0.01
		S + Ab + Ch	1.7 ± 3.3	
6-5E	G1	S + Ab	6.3 ± 0.9	
		S + Ab + C	41.0 ± 7.3	<0.01
		S + Ab + Ch	3.3 ± 2.5	
1-11C	M	S + Ab	2.5 ± 2.1	
		S + Ab + C	21.0 ± 4.1	N.S.
		S + Ab + Ch	1.7 ± 1.2	
14-5B	M	S + Ab	4.8 ± 3.3	
		S + Ab + C	16.1 ± 5.4	N.S.
		S + Ab + Ch	4.5 ± 4.3	
5-9C	M	S + Ab	8.0 ± 5.8	
		S + Ab + C	17.9 ± 11.4	N.S.
		S + Ab + Ch	5.8 ± 4.6	
14-8D**	G1	S + Ab	1.5 ± 2.1	
		S + Ab + C	22.8 ± 11.5	N.S.
		S + Ab + Ch	1.3 ± 1.6	
Normal mouse serum control		S + Ab	5.3 ± 4.4	
		S + Ab + C	23.0 ± 2.2	
		S + Ab + Ch	7.8 ± 4.3	
Infected mouse serum		S + Ab	3.3 ± 6.6	
		S + Ab + C	97.5 ± 3.4	<0.001
		S + Ab + Ch	2.6 ± 3.9	
Culture medium		S + C	13.4 ± 9.0	
		S + Ch	2.5 ± 2.3	
		S	3.9 ± 3.3	

* About 50 schistosomula were cultured at 37°C and at 5% CO_2 in air for 30 min before addition of ascites or serum. Complement was added 30 min after addition of ascites or serum. Observation was performed 18 hours after addition of the complement.

** McAb ascites unreactive with any stage of *S. japonicum* antigen detected by IF and ELISA.

*** Mean of two duplicate experiments ± SD.

† P value indicated by comparison with normal mouse serum control.

S; 3 hour-mechanically transformed schistosomula.

Ab; 10% ascites or serum inactivated at 56°C for 30 min.

C; 10% fresh guinea pig serum.

Ch; 10% guinea pig serum inactivated at 56°C for 30 min.

N.S.; Not significant.

200 mW•sec/cm^2, were 10.4 ± 2.1 (mean ± S.E.) and 1.8 ± 0.6, respectively. On the other hand, no worms were found in mice given with cercariae attenuated by UV dose of 300 mW•sec/cm^2 or higher. Pathological changes were not observed in mice inoculated with highly attenuated cercariae.

Percent protection against challenge infection was examined in mice vaccinated by the attenuated cercariae irradiated at 300 mW•sec/cm^2 or more. Vaccinated and non-vaccinated mice were injected ip with 70 normal cercariae 4 weeks after the vaccination. Worms were collected by the perfusion method 4 weeks after the challenge infection. The number of worms recovered from vaccinated mice was compared to that from control mice and the percent protection was calculated (Table 2). The maximal protection (47.3%) was induced by vaccination with cercariae irradiated with UV dose at 500 mW•sec/cm^2. On the other hand, protection was not significant at UV dose of 1,000

Table 4 Dose response of killing activity of 6-10D monoclonal antibody *in vitro*

McAb (Antibody)	Dose of Ab (%)	Assay condition*	Mean percent of dead schistosomula ± SD***
6-10D	0.5	S + Ab	1.2 ± 2.1
		S + Ab + C	48.6 ± 3.3
		A + Ab + Ch	3.8 ± 3.7
6-10D	2.5	S + Ab	1.4 ± 2.5
		S + Ab + C	58.6 ± 6.3
		A + Ab + Ch	0
6-10D	10.0	S + Ab	0
		S + Ab + C	68.0 ± 4.7
		A + Ab + Ch	0.6 ± 1.1
14-8D**	10.0	S + Ab	1.9 ± 3.3
		S + Ab + C	1.5 ± 2.5
		A + Ab + Ch	0
Infected mouse serum	10.0	S + Ab	1.3 ± 1.1
		S + Ab + C	90.1 ± 7.5
		A + Ab + Ch	N.D.
Culture medium	0	S + C	2.9 ± 1.3
		S + Ch	N.D.
		S	2.9 ± 2.4

* About 50 schistosomula were cultured at 37°C and at 5% CO_2 in air for 30 min before addition of ascites or serum. Complement was added 30 min after addition of ascites or serum. Observation was performed 18 hours after addition of the complement.

** McAb ascites unreactive with any stage of *S. japonicum* antigen detected by IF and ELISA.

*** Mean of triplicate experiments ± SD.

S; 3 hour-mechanically transformed schistosomula.
Ab; 10% ascites or serum inactivated at 56°C for 30 min.
C; 10% fresh guinea pig serum.
Ch; 10% guinea pig serum inactivated at 56°C for 30 min.
N.D.; Not done.

mW•sec/cm^2 where protection was only 11.1%. Therefore, cercariae attenuated by UV at 500 mW•sec/cm^2 was used for immunization for preparing McAb.

In vitro *killing effect of anti-schistosomulum McAb on schistosomula*:

Thirteen clones were selected by antibodies binding to the surface of schistosomula by IF. The isotype of McAb was both IgG_1 (4 clones) and IgM (9 clones). These 13 McAb have different reactivity to various stages of schistosome, *i.e.*, cercariae schistosomula, juveniles at lung and liver phases, adult worms and eggs. When the killing effect of these McAb to schistosomula was examined *in vitro* (Table 3). 2 McAb, 6-10D and 6-5E were IgG_1, showed 72.3% and 41.0% mortality, which was significantly higher than that by normal mouse serum.

Schistosomula killing effect *in vitro* was most prominent at 97.5% with serum from the BALB/c mice 8 weeks after infection with *S. japonicum*, whereas the effect of controls in a combination of normal serum and complement, and complement alone was as low as 23.0% and 13.4%, respectively. Except these two, other McAb did not have schistosomula killing effect.

Using 6-10D ascites, dose effective examinations were conducted at 0.5, 2.5 and 10% of ascites supplemented with 10% complement. The schistosomula killing increased from 48.6 to 58.6% and 68.0% dependent on the McAb dose (Table 4).

Protection by passive transfer with McAb:

Protective effect was examined by passive transfer of 6 McAb into BALB/c mice. As a control, a McAb 14-8D was used since this McAb had no reactivity by IF to sections of any developing stages of *S. Japonicum*. After inoculation with 14-8D, the number of worms recovered was 69.7 in BALB/c mice infected with 100 cercariae. The percent protection of other McAb in Table 5 was calculated based on the worm recovery with 14-8D. The significant protective effect was demonstrated in 2 McAb, 6-10D and 6-5E and their protective effect was 26.0% and 24.0%, respectively. They were the McAb which had the schistosomula killing effect *in vitro*.

A McAb 1-11C and another McAb 5-6F, both McAb were of IgM, had low protective effect at 17.4% and 16.1%, respectively. These values were not statistically significant. A McAb 5-9C and a McAb 16-10D had no protective immunity. They were also IgM McAb. Thus, some McAb such as 5-6F, 5-9C and 16-10D had no protective

Table 5 Protective effect of monoclonal antibodies against challenge infection in BALB/c mice

McAb*	Ig	No. of mice	Mean no. of worms ± SD†	Percent protection††	P
Control**	G1	4	69.7 ± 7.2	–	
6-10D	G1	5	51.6 ± 3.6	26.0	<0.01
6-5E	G1	5	53.0 ± 10.8	24.0	<0.05
1-11C	M	5	57.6 ± 13.7	17.4	N.S.
5-6F	M	5	58.5 ± 11.6	16.1	N.S.
5-9C	M	5	67.4 ± 5.4	3.3	N.S.
16-10D	M	5	66.6 ± 13.7	4.4	N.S.

* 1 ml of 2 fold diluted ascites was injected i.p. at 18 hours before and 1 day and 3 day after challenge.

** 14-8D ascites unreactive with any stage of *S. japonicum*.

† Mice were challenged percutaneously with 100 cercariae and worms were counted 4 weeks later.

†† Protection rate was calculated by following formula: (C-X) × 100/C, C is mean number of worms in control mice and X is mean number of worms in immunized mice.

effect against challenge infection although they recognize the surface of schistosomula by IF like protective McAb, 6-10D and 6-5E.

Discussion

The efficacy of protective immunity induced by X ray attenuated cercariae varied greatly among experiments and no definite evaluation has yet been attained generally (Murrel *et al.*, 1979). In the present study, high protective immunity at 47.3% was observed in BALB/c mice vaccinated with UV attenuated cercariae. This percent protection was comparable with 24.8–61.7% with *S. mansoni* (Dean *et al.*, 1983) and 16.8–74.8% with continental Chinese strain of *S. japonicum* (Moloney *et al.*, 1985).

Since protective immunity induced by UV attenuated cercariae was transferred to naive mice by parabiosis (Dean *et al.*, 1981). Protective immunity was also passively transferred by antibody from mice strongly immunized with X ray attenuated cercariae. Therefore, humoral immunity has been suggested to be an important component to induce such protective immunity (Mangold and Dean, 1986). In the present study, an attempt was made to produce McAb associated with protective immunity by immunizing mice with UV attenuated cercariae, and McAb which recognize the surface of schistosomula were selected by IF.

In a recent report (Maeda *et al.*, 1986), immunoglobulin subclasses, fractionated from serum of mice infected with *S. japonicum,* were examined for schistosomula killing effect *in vitro,* supplemented with complement, and IgG_1 was most effective than any other subclasses. In the present study, 2 effective McAb were IgG_1, though not all the IgG_1 McAb were effective. The similar studies were also reported with *S. mansoni* (Horrowitz *et al.*, 1983) in that schistosomula killing effect was closely related to the nature of surface epitopes of schistosomula recognized by a McAb. A difference in value about complement alone control between Table 3 and Table 4 is to be caused by the use of different lot of fresh guinea pig serum.

Two McAb showing *in vitro* killing were also effective to protect challenge infection *in vivo* in the present study. In general, the protective effect of passive immunization was incomplete. The effect was 40.0 to 58.5% with IgE McAb (Kojima *et al.*, 1987), 42.7% with IgM (Smith *et al.*, 1982), or 51.2% (Gregoire *et al.*, 1987) and 27 to 58% with IgG_{2a} McAb (Bickle *et al.*, 1986) produced by X ray attenuated cercariae. The protective effect was at about 25% in the present study.

An effective McAb 6-10D has a character to bind to only cercarial body and juvenile schistosomula and another McAb 6-5E strongly to the surface of developing stages and adults after cercarial phase (Nakamura *et al.*, unpublished data). McAb may affect different developing stages of schistosome. By this difference, if the time of inoculation with McAb fitted well to the reacted stage, the protective effect would be increased more.

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