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Immunobiological Studies on Schistosomiasis Japonica Using Hybridoma Technology (I) Characterization of Two Monoclonal Antibodies Obtained from Spleen Cells of *Schistosoma japonicum* Infected Mice, Reactive with a *Schistosoma japonicum* Egg Antigen or a Heterophil Antigen

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Abstract

Two monoclonal antibodies were obtained by fusion of P3-X63-Ag8-653 myeloma cells with spleen cells harvested from mice 8 wk after infection with *Schistosoma japonicum* (Sj). One monoclonal antibody (74D11, IgM) reacted specifically with an Sj egg antigen. Using an enzyme-linked immunosorbent assay, no cross reactivity was observed with 8 other parasite antigens. The molecular weight range of the egg antigen recognized by 74D11 was 14,000 to 92,000. In addition, the antigenic molecules, which were recognized with 74D11, were denatured by periodate oxidation in Ouchterlony analysis. These results show that 74D11 appears to recognize carbohydrate determinants on glycoprotein in Sj egg antigens. The other monoclonal antibody (84B3, IgG1), reacted strongly with red blood cells of sheep and goat origin, but showed no reaction with human, porcine or bovine erythrocytes, Sj egg antigens, nor 8 other parasite antigens.

Key words: monoclonal antibody, *Schistosoma japonicum,* heterophil antigen

Introduction

The adult Sj parasite resides in the host's mesenteric veins, thus escaping immunological attack and enabling it to cause severe infection. Many approaches have been undertaken to elucidate the mechanisms of immunological response induced by Sj infection. However, to date, these mechanisms have not been fully clarified. The various immunological phenomena which occur are thought to result from serial responses of the host to stimulation by antigens released from Sj. Therefore, purification of Sj specific antigens could play an important role in clarifying the mechanisms involved. In addition, isolation of purified Sj antigens could be useful in the diagnosis and therapy of human schistosomiasis japonica. Monoclonal antibodies (MoAb) are convenient tools for the analysis, characterization and isolation of parasite antigens. Moreover, they may provide a new approach to studying the complex immunologic mechanisms elicited during the course of parasitic infections. In view of these considerations, MoAb to adult worm and egg antigens of Sj have been obtained by immunization with crude or purified antigens (Mitchell *et al.*, 1983a, Cruise *et al.*, 1981, Cruise *et al.*, 1983, Nash and Deelder, 1985, Sidner *et al.*, 1987). These MoAb have been applied in diagnostic trials (Mitchell *et al.*, 1983b) or analysis of granuloma formation (Sidner *et al.*, 1987, Mitchell *et al.*, 1983c).

In order to produce MoAb to Sj, we used a strategy that differed from those employed by

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other researchers. For a source of MoAb producing cells, we used spleen cells from mice infected with Sj rather than from mice immunized with Sj antigens. The reason why we used this approach is that antigens recognized in Sj infected hosts may differ from those recognized in Sj immunized hosts. The antigenic determinants recognized in Sj infected hosts might have great relevance to the immunological study of the disease. In this paper, we report on two hybridomas which produce monoclonal antibodies specific for Sj egg antigen (SJEA) or sheep red blood cells (SRBC).

Materials and Methods

Production of hybridoma cell lines: BALB/c mice were intraperitoneally injected with approximately 20 cercariae of Sj (Yamanashi, Japanese isolate) which had been maintained in our laboratory by passage in *Oncomelania nosophora* and BALB/c mice. Eight weeks after infection, spleen cells (1×10^8 cells) were harvested from a mouse and fused with P3-X63-Ag8-653 myeloma cells (1×10^7 cells) in the presence of 50% polyethylene glycol. Fusion was carried out according to the methods of Oi and Herzenberg (1980). The resulting hybrids were dispensed into micro plates (Nunc, Rockilde, Denmark) at 200 μl per well in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (Filton, Altona, Australia). Hybridomas were selected by the addition of hypoxanthine, aminopterine and thymidine (Sigma, St. Louis, MO, U.S.A.). Three weeks after fusion, hybridomas that produced antibodies against SJEA were screened by enzyme linked immunosorbent assay (ELISA), and heterophil antibodies were screened by indirect radioimmune binding assay (RIA) with SRBC. In approximately 10 to 60% wells hybridoma cells grew, and 20 to 30% of them produced antibodies. Antibody producing cells were cloned by three times limiting dilution in the presence of 10^4/well BALB/c thymocytes. The wells containing the single cell at the beginning of the culture, as determined by microscope, were selected for monoclonal antibody. The cloning efficiency is approximately 80 to 90%. The established clones were i.p. injected into BALB/c mice which previously received an i.p. injection of 0.5 ml pristane. Ascites fluid was harvested 10 days after hybridoma inoculation. Isotyping of MoAb was determined by immunodiffusion using 5-fold concentrated culture supernatants and anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3 (Serotec Ltd., England).

SJEA: SJEA was prepared by the method described by Owhashi *et al.* (1982). Briefly, eggs were collected from the intestines of BALB/c mice infected with 50 cercariae of Sj (Yamanashi, Japanese strain) by enzymatic digestion using prune extract (0.01%, Sigma, St. Louis, MO, U.S.A.). The eggs were suspended in phosphate-buffered saline (pH 7.2) and sonicated at 100 watts for 20 min with Insonator 200M (Kubota, Co. Ltd. Tokyo). Unlysed eggs and debris were removed by centrifugation at 5,000g for 30 min. The supernatant was concentrated to 5 mg/ml protein, sterilized by filtration through a 0.22 μm filter, and stored at $-40°C$ until use.

Crude parasite antigens: Whole worm extracts of the adults of *S. japonicum, Fasciola* sp., *Paragonimus westermani, Dirofilaria immitis, Angiostrongylus cantonensis, Ascaris suum, Toxocara canis* and the larvae of *Anisakis simplex* (so called *Anisakis* Type I) were prepared following previously reported method (Sato *et al.*, 1974). Briefly, freshly collected worms were washed several times with phosphate buffered saline (0.05 M, pH 7.5) (PBS), chopped into short lengths, and homogenized thoroughly in PBS. The homogenate was centrifuged at 10,000 g for 30 min. The supernatant was dialyzed against PBS for 24 hr at 4°C, and stored at $-20°C$. The protein concentration of each parasite preparation was determined using a protein assay reagent (Bio-Rad, Laboratories, Richmond, CA, U.S.A.).

Treatment of SJEA: To characterize the nature of SJEA recognized by MoAb 74D11 the SJEA preparation was pretreated with heat, periodate, or pronase. Heat treatment was performed at 120°C for 15 min. Periodate oxidation was carried out with 20mM sodium metaperiodate (Wako Chemical Co., Osaka), and the reaction mixture was kept in a dark room for 48

hr at 4°C. The reaction was stopped by dialysis against PBS at 4°C for 48 hr using a membrane with an M.W. exclusion limit of approximately 8,000 daltons. Pronase treatment was carried out by addition of pronase (1 mg/ml) (Wako Chemical Co.) to the SJEA solution (10 mg/ml). The mixture was incubated at 37°C for 1 hr and the reaction was stopped by heat treatment at 100°C for 15 min.

ELISA for parasite antigens: Microelisa plates (Sumitomo Co. Ltd., Tokyo) were coated with parasite antigens (5 — 10 μg/ml, 100 μl/well) in 0.2 M carbonate coating buffer (pH 9.6). Coating with the parasite antigen was accomplished by incubation at 37°C for 2 hr and then 4°C for at least another 24 hr. Immediately before use, the plate was washed 3 times for 15 minutes with PBS containing 0.02% tween 20 (Tween-PBS). Residual protein binding sites on the plate were blocked by incubation with 3% bovine serum albumin (BSA) in PBS. Samples were added to the wells and incubated for 30 min at 37°C. After washing, 100 μl of a 1:300 dilution of peroxidase-conjugated anti-mouse immunoglobulin (DAKO, Co., CA, U.S.A.) were added to each well. The plate was incubated at 37°C for 1 hr. 0-phenylenediamine was used as a substrate for the enzyme. Optical density was measured at 492 nm with an automatic spectrophotometer (Titerteck, Flow Laboratories, Richmond, VA, U.S.A.). All assays were performed in duplicate.

ELISA detection of antibody to SRBC: We followed a method reported previously (Nakao *et al.*, 1981). Briefly, an SRBC suspension (2×10^8 cells) was treated with MoAb in a plastic tube at 37°C for 20 min. The tube was then washed three times in $GVBS^{2+}$ (Veronal buffered saline, 0.15 M, pH 7.4, containing 0.15 mM Ca^{2+}, 0.5mM Mg^{2+} and 0.1% gelatin) and centrifuged each time for 5 min at 300 g. Goat anti-mouse Ig-alkaline phosphate conjugate was added to the tube. After mixing, the tube was kept at room temperature for 2 hr. The cell pellet was then washed 4 times with VBS^{2+} and resuspended in VBS^{2+}. To this was added a solution of p-nitrophenyl phosphate (PNP) containing 0.137 M NaCl and 1 mM Mg^{2+}. After gentle mixing at room temperature for 1 hr, cells were pelleted by centrifugation at 2,500 rpm for 5 min and 3N NaOH was added to stop the enzyme reaction. The absorbance was read spectrophotometrically at 405 nm.

Indirect radio-immuno-binding assay: Red blood cells (RBC) suspended in PBS containing 1% BSA (1% BSA-PBS) were first incubated with hybridoma antibodies overnight at 4°C in a 96 well microplate. The RBC were washed three times in 1% BSA-PBS by centrifugation at 300 $\times$ g, and then incubated for 30 min at 37°C with ^{125}I-radiolabeled sheep anti-mouse whole immunoglobulin (50,000 cpm/well/100 μl, Radiochemical Centre, Amersham, Bucks, UK). After washing as described above, radioactivity was counted using a gamma counter (Aloca Auto Well Gamma System ARC-351).

Western blotting: SDS-polyacrylamide gel electrophoresis was performed on a 5% stacking and 10% running gel as described by Laemmli (1970). Low molecular weight standards, (Pharmacia, Uppsala, Sweden) were run on each gel. Samples (3 mg/ml) were treated with SDS alone (0.5M Tris-HCl, 10% glycerin, 0.05% bromophenol blue, 2% SDS), SDS plus 5% β-mercaptoethanol (2-ME) or SDS plus 4 M urea at 80°C for 5 min. The running buffer was 0.025 M Tris, 0.192 M Glycine and 0.1% SDS. Gels were run at constant voltage (100 V) at room temperature until the tracking dye reached the bottom of the gel. The proteins separated in the gel were subsequently transferred to a polyvinylidene difluoride membrane (Millipore Corp., MA, U.S.A.) by electrophoretic transfer blotting (Towbin *et al.*, 1979). After transfer, the membrane was immersed in 3% BSA-PBS at 37°C for 30 min to block unused protein binding sites. Before blocking, some of the membranes were immersed in 0.5% periodic acid and 0.05 M soidum metaperiodate for 30 min. These membranes were overlaid with the appropriate antiserum, and incubated in a moist chamber at 37°C for 60 min. After removal of the antiserum, the sheet was washed several times with Tween-PBS. The membrane was then incubated at 37°C for 60 min with peroxidase-labeled rabbit anti-mouse immunoglobulin diluted 1:300 in 1% BSA-PBS. The membranes were then washed five times with

Tween-PBS and soaked in a substrate solution of 0.01% DAB, 0.01% H_2O_2 in 50 mM Tris-HCl (pH 7.6) at room temperature. Color development was stopped by washing with distilled water. To determine whether proteins were transferred to the membrane, one of the membranes not blocked with BSA was stained with Coomassie Brilliant Blue R250 (CBB) (Merch, Darmstadt, FRG).

Double diffusion method: Thin agarose gels (1.2%) 1.5 mm in thickness were coated on a glass plate and wells were punched out. Each well was filled with antibody or antigen solution, and the plate was allowed to develop precipitin bands at 4°C for 48 hr. Following this, the plate was washed with veronal-HCl buffer for 3 days, dried with filter paper and stained with CBB. Immunoelectrophoresis was performed in the agarose gel-plate according to the micromethod of Ouchterlony and Nilsson (1978). SJEA (200 μg/10 μl/well) was added to the 4.0 mm diameter wells and a potential of 35 V was maintained across the slide for 4 hr. After electrophoresis, the troughs were filled with antibody solution, and precipitin bands were allowed to develop for 72 hr. Washing, drying and staining were carried out as described above.

Results

Isolation of hybridomas that produce antibodies reactive with SJEA and heterophil antigen. We isolated two hybridoma cell lines producing MoAb against SJEA or heterophile antigen (SRBC) from spleen cells of mice infected with Sj 8 wk previously. We were unable to obtain MoAb reactive with antigens of adult Sj worms. The MoAb which reacted with SJEA was designated 74D11, and the MoAb reacting with SRBC was designated 84B3. By immunodiffusion, 74D11 was an IgM antibody, while 84B3 was an IgG1 isotype. The antibody titers of 74D11 to SJEA are shown in Table 1. 74D11 showed high activity to SJEA with an ELISA titer of 0.44 at a dilution of 1:24,000.

For the detection of heterophil antibody, RIA, and ELISA were performed using SRBC as antigen. The antibody titer of 84B3 was closely related to the concentration of the ascites fluid (Table 2).

Table 1 ELISA titers of 74D11 to SJEA

Dilution	O.D. (492 nm)
1:2,000	1.88 ± 0.14
1:4,000	1.78 ± 0.09
1:8,000	1.48 ± 0.06
1:16,000	1.28 ± 0.05
1:32,000	1.17 ± 0.05
1:64,000	0.88 ± 0.06
1:120,000	0.65 ± 0.04
1:240,000	0.44 ± 0.03
1:480,000	0.21 ± 0.04
Ascites control 1:100	0.18 ± 0.04

ELISA titers were expressed as the optical density at 490 nm. Ascites controls were prepared from BALB/c mice i.p. injected with P3-X63-Ag8-653 myeloma cells. Results are expressed as mean ± standard deviation (S.D.) of four experiments.

Table 2 Reactivity of 84B3 to SRBC

Dilution	RIA (cpm)	ELISA (O.D. 405 nm)
1:50	3,835 ± 364	0.40 ± 0.01
1:100	2,152 ± 112	0.34 ± 0.02
1:500	2,328 ± 256	0.11 ± 0.01
1:1,000	1,690 ± 130	N.D.
1:5,000	660 ± 204	N.D.
1:10,000	453 ± 184	N.D.
1:50,000	308 ± 145	N.D.
Ascites control		
1:50	625 ± 168	0
1:50,000	310 ± 214	N.D.

Ascites controls were prepared from BALB/c mice i.p. injected with P3-X63-Ag8-653 myeloma cells. Results are expressed as mean ± S.D. of three experiments.

Specificity of MoAb, 74D11 and 84B3. The specificity of 74D11 and 84B3 was determined by ELISA and RIA respectively using nine different parasite antigens and RBC from six species (Tables 3 and 4). 74D11 exhibited a strong reaction with SJEA, but failed to react with any of

Table 3 Reactivity of 74 D11 and 84B3 to various parasite antigens

MoAb 1:50	O.D. at 492 nm								
	Antigens								
	SJEA	SJWE	Fa	Pw	Di	Ac	As	Tc	An
74D11	2.00	0.13	0.08	0.06	0.03	0.05	0.08	0.10	0.10
84B3	0.01	0.04	0.01	0.03	0.03	0.02	0.02	0.01	0.01
Ascites control	0.01	0.02	0.01	0.03	0.02	0.01	0.02	0.03	0.32

Whole worm antigen extracts of the adults of *S. japonicum* (SJWE), *Fasciola* sp. (Fa), *P. westermani* (Pw), *D. immitis* (Di), *A. cantonensis* (Ac), *A. suum* (As), *T. canis* (Tc) and the larvae of *A. simplex* (An). Ascites controls were prepared from BALB/c mice i.p. injected with P3-X63-Ag8-653 myeloma cells.

Table 4 Binding of 84B3 and 74D11 to RBC from various species

MoAb 1:50	Bound radioactivity (cpm)					
	Red blood cells					
	SRBC	GRBC	MRBC	PRBC	BRBC	HRBC
84B3	4,815	3,835	125	390	918	743
74D11	103	143	111	86	186	128
Ascites	113	157	117	142	176	89

Ascites controls were prepared from BALB/c mice i.p. injected with P3-X63-Ag8-653 myeloma cells. SRBC, sheep red blood cells; GRBC, goat red blood cells; MRBC, mouse red blood cells; PRBC, porcine red blood cells; BRBC, bovine red blood cells; HRBC, human red blood cells.

the other eight parasite antigens. 84B3 did not react with any of the parasite antigens examined (Table 3).

The binding of 84B3 or 74D11 to RBC from the six species examined was determined by RIA. As shown in Table 4, 84B3 reacted strongly with SRBC and goat RBC (GRBC), but very slightly with bovine RBC (BRBC) and human RBC (HRBC). To confirm the specificity of 84B3, ascites fluid containing 84B3 was absorbed with SRBC or mouse RBC (MRBC), and the residual antibody activity was determined by RIA. As shown in Table 5, antibody to SRBC was absorbed completely with SRBC, but not with MRBC which did not react with 84B3.

Immunoelectrophoretic and Western blot analysis of SJEA recognized with 74D11. The antigenic component of SJEA was analyzed by immunoelectrophoresis. 74D11 developed a single precipitin band against SJEA. On the other hand, 3 precipitin bands were shown between Sj infected BALB/c mouse serum and SJEA (Fig. 1). This indicated that 74D11 recognized one specific antigenic determinant of SJEA.

To determine the molecular weight of SJEA recognized by 74D11, Western blotting was employed. As shown in Fig. 2, when SJEA was treated with PBS or 2-ME (lane B, D), the com-

Table 5 Reactivity of 84B3 to SRBC after absorption with SRBC or MRBC

Dilution	Bound radioactivity (cpm)		
	Unabsorbed	Absorbed with SRBC	Absorbed with MRBC
1:50	5,529 ± 620	858 ± 128	5,029 ± 148
1:100	4,592 ± 340	525 ± 183	4,016 ± 284
1:1,000	2,267 ± 415	315 ± 109	1,263 ± 380
1:5,000	1,065 ± 218	237 ± 40	865 ± 204
Ascites control			
1:50	625 ± 108	794 ± 89	525 ± 125
1:5,000	318 ± 115	211 ± 10	415 ± 201

Absorption was carried out with 50% SRBC at 37°C for 60 min. Ascites controls were prepared from BALB/c mice i.p. injected with P3-X63-Ag8-653 myeloma cells. Results are expressed as mean ± S.D. of three experiments.

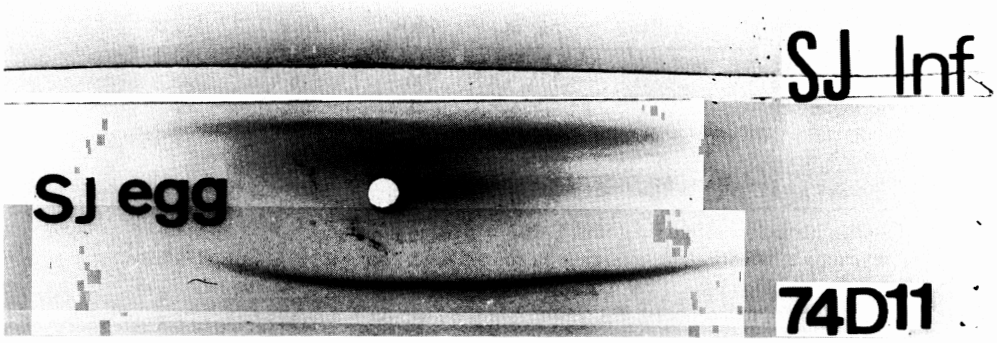

Fig. 1. Immunoelectrophoretic analysis of SJEA by MoAb. 74D11 ascitic fluid was used at a 1:5 dilution. Sj infected sera (Sj inf) were obtained from BALB/c mice infected with 50 Sj cercariae 8 wk previously. Sj inf was used at 3 fold dilution.

ponents recognized by 74D11 were spread over a major broad band ranging from 20,000 to 94,000 M.W. However, resolution slightly improved using 4 M urea (lane F). No band was found using ascites from mice inoculated with the parental myeloma.

The effect of various treatments of SJEA on its reactivity with 74D11. SJEA was treated with heat, pronase or periodate, and then tested by immunodiffusion. As shown in Fig. 3, antigenic determinant(s) detected by 74D11 were denatured by periodic acid treatment, but were unaffected by heat treatment at 120°C for 15 min. When SJEA was treated with pronase, the width of the precipitin line decreased, but a definitive line was still observed.

Discussion

In this paper, we report on two MoAb, obtained from spleen cells of Sj infected mice which react with either SJEA or a heterophil antigen.

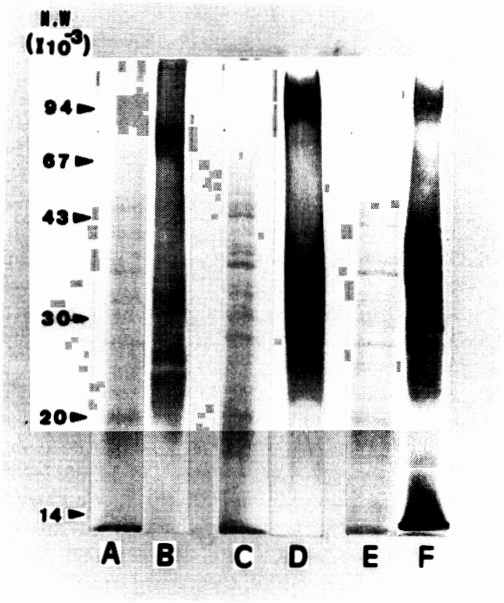

Fig. 2. Western blot analysis of SJEA recognized by 74D11. SJEA was treated with SDS alone (lane A, B), SDS plus 2-ME (lane C, D) or SDS plus urea (lane E, F). Lane A, C, and E were stained with CBB. Lane B, D, and F were reacted with 74D11 (1:100 dilution). Details of sample preparation and staining are shown in Materials and Methods.

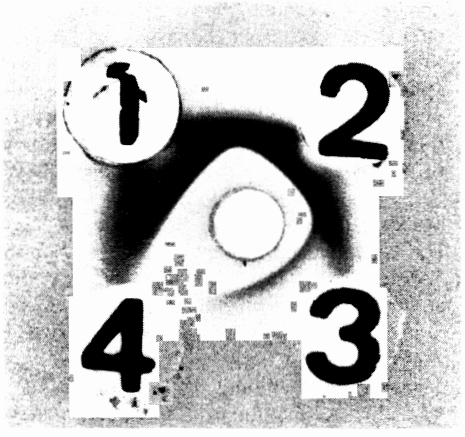

Fig. 3. Effect of heat, pronase or periodate treatment of SJEA on its reactivity with monoclonal antibody 74D11. Center well contains MoAb (74D11). The outer wells contain SJEA treated with PBS (1), heat (2), pronase (3), or periodic acid (4). Details of sample preparation are shown in Materials and Methods.

As to the SJEA epitope recognized by the MoAb, 74D11, complete removal of its antigenicity by periodate oxidation suggests that the antigenic determinant is carbohydrate in nature. Furthermore, the broad bands obtained in Western blotting as well as the absence of immunostaining when the transfer membrane is pretreated with periodic acid (Yamashita unpublished result), suggest that the SJEA recognized with 74D11 is a carbohydrate chain of various glycoproteins. Reduction in the width of the band in double immunodiffusion brought about by pronase treatment may strengthen the validity of this idea.

Partially purified glycoproteins have been demonstrated as SJEA which show circumoval precipitin inhibitory activity (Long *et al.*, 1981a, Long *et al.*, 1981b), or the ability to subcutaneously sensitize mice to lung granuloma formation (Tracy and Mahmoud, 1982). SJEA obtained from hatch fluid is also a glycoprotein (Kobayashi *et al.*, 1985). Furthermore, an eosinophil chemotactic factor obtained from extracts of Sj eggs is a glycoprotein (Owhashi and Ishii, 1981, Owhashi and Ishii, 1982). We are as yet unsuccessful in isolating SJEA reactive with 74D11, so further experiments need to be done before we can show the biological activities of 74D11 reactive antigen(s).

In this study, we also developed a heterophile MoAb. This monoclonal antibody, 84B3, reacted strongly with SRBC and GRBC only (Table 3 and 4), and not at all with SJEA. In various parasitic infections, including trypanosomiasis, filariasis, malaria, and schistosomiasis, it has been reported that production of anti-SRBC antibody increased after infection, (Houba *et al.*, 1969, Dammin and Weller, 1945). Kawabata *et al.* (1981) reported that IgM plaque-forming cells directed toward trinitrophenyl, polyvinylpyrolidon and SRBC increased in murine schistosomiasis japonica. Asahi *et al.* (1984) reported that anti-SRBC heterophile antibodies were detected in sera of infected mice, and that the specificity of this heterophile antibody(s) was distinct from that of Forssmann antibody, Paul-Bunnell antibody and heterophile agglutinins. The specificity of the heterophile MoAb, 84B3, is almost identical with that of heterophile antibodies in Sj infected mice. Thus,

this MoAb may be a tool for more sophisticated understanding of the role of heterophile antibodies in the pathogenesis of schistosomiasis japonica. The precise specificity of 84B3 and the characterization of its reactive antigen will be shown in the next paper.

In this study, we did not succeeded in developing MoAb from spleen cells of Sj infected mice which were reactive with Sj adult worm antigens. This may result from the fact that antibody producing clones in Sj infected mice are predominantly directed toward egg antigens because of the prevalence of Sj eggs in the infected hosts. This speculation may be supported by the previous report that type I hypersensitivity to Sj adult worm antigen was negative in Sj infected mice (Ishii and Owhashi, 1982). One way to develop MoAb reactive with Sj adult worm antigens may be to induce unisexual infection with Sj. Our upcoming paper will explore this possibility.

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