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Characterization of Two Monoclonal Antibodies Against Teguments of Adult and Schistosomula of *Schistosoma japonicum*

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SUMMARY **Two monoclonal antibodies (McAbs) were produced from BALB/c mice hyperimmunized with tegumental extract of *Schistosoma japonicum* (Chinese strain). The two McAbs were characterized with regard to antibody isotype, antigen binding specificity and parasite stage specificity. One McAb, 8G9-5, was identified as IgM, whereas the other McAb, 9E7, was determined to be IgG_{2a}. Immunoblotting assay indicated that McAb 8G9-5 binds strongly to the band of tegumental antigens of Mr 64 kDa but also binds weakly to other bands at 116, 105, 97, 54, 50, 47 and 45 kDa, whereas 9E7 McAb reacts specifically at Mr 54 kDa. Anatomical localization of the antigens in the adult worm by indirect immunofluorescence assay indicated that the target epitopes of McAb 8G9-5 are in the intra-tegumental structure, whereas the McAb 9E7 epitope is on the surface membrane. The two McAbs also react at similar sites within the teguments of schistosomula and lung worms.**

Schistosoma japonicum affects several million people in China and the Philippines. The latest estimation of the prevalence in China is more than five million cases [1] and over half a million cases in the Philippines. [2] Control of schistosomiasis by mass treatment or by snail control has proved to be too costly for these developing countries. Since the production of a schistosomiasis vaccine is still a distant goal, development of monoclonal antibodies (McAbs) against various stages of the parasite would contribute to more immediate applications in the areas of immunodiagnosis and antigen purification.

Studies on *S. mansoni* have indicated that schistosomula surface antigens are important targets of protective immunity whose specific antibodies can efficiently kill the parasite. [3,4] Payeres *et al,* [4] using anti-adult membrane antisera, have demonstrated that there are at least three different antigens of 32, 25 and 20 kDa sharing common epitopes with the schistosomular antigens. Several previous reports have also demonstrated cross-reaction between epitopes on the surfaces of the schistosomulum and the egg. [5-7]

It has been reported that immunity to schistosomes is species and strain specific. [8-11] Moloney *et al* [9,10] reported that animals immunized with irradiated cercariae were only resistant to homologous challenge but not to heterologous species or strains. Similarly, Bickle *et al* [8] demonstrated that the antigens of Mr 32 and 16 kDa obtained from extracts of *S.mansoni* schistosomula react only with the specific McAbs but not with sera from animals exposed to *S.japonicum*. Furthermore, the present genetic evidence indicates that Asian blood flukes belong to a super-species complex, especially Chinese and Philippine strains which have already diverged at a third of their structural gene loci. [12,13] Therefore, it has been concluded that common epitopes and species or strain specific epitopes are present on the surface membrane of schistosome parasites. [11] Thus, the tegument antigens of *S. japonicum* (Chinese strain) may differ substantially from those of *S.japonicum* (Philippines strain) and

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from those of *S.mansoni*. With respect to this complexity, it is of crucial importance to characterize the tegumental antigens by using the specific monoclonal antibodies.

In this paper, we describe the development of two specific monoclonal antibodies against the tegument of adult *S.japonicum* (Chinese strain), which recognize common epitopes residing in the adult and schistosomular surfaces.

MATERIALS AND METHODS

Parasites

The Chinese strain of *S.japonicum* was maintained routinely at the Center for Applied Malacology and Entomology, Department of Biology, Faculty of Science, Mahidol University in *Oncomelania hupensis* (from Anhui Province, China). Adult worms were collected from Swiss albino mice after eight weeks of infection by portal perfusion.[14] Schistosomula were prepared by mechanical transformation of the cercariae shed from the infected snails.[15] The schistosomula were washed three times in 10 ml of Earle's saline containing 0.5% lactalbumin hydrolysate, 100 μg/ml penicillin and 100 units/ml streptomycin (ELAC) at room temperature. The schistosomula were incubated at 37°C with 5% CO_2 for 3 hours in Eagles's Minimal Essential Medium (MEM, GIBCO) supplemented with 5% fetal calf serum (GIBCO), 10 μg/ml penicilin and 100 units/ml streptomycin before use.

Young worms were recovered from the lungs of infected mice on day five by puncturing the left ventricle of the heart, and the lung was perfused with normal saline containing 10 units/ml heparin via the right ventricle until all blood containing worms had been removed.

Preparation of the tegument from adult schistosomes

A pool of fresh 400-500 adult males of *S.japonicum* (Chinese strain), obtained from eight-week infected Swiss albino mice, was washed three times with MEM and another three times with 50 mM Tris-HCl pH 8 to remove any contamination by host blood. After the final wash, the worms were freeze-thawed on dry ice for 20 cycles, the shed pieces of surface tegument in the supernatant were aspirated into an Eppendorf tube and then centrifuged 100×g for 10 min at 4° C. The supernatant was collected into the sample glass tubes and stored at -72°C. The protein content of the tegument solution was determined by the method of Lowry *et al.*[16]

Preparation of McAbs

Six week old BALB/c strain mice were used for immunization with tegumental antigens of *S.japonicum* (Chinese strain). A group of 10 mice were used for each immunization schedule. Subcutaneous inoculation at 4 sites in each mouse with 200 μg of antigen mixed with an equal amount of complete Freund's adjuvant was carried out. The second and third immunization were given at two-week intervals by the same route with the same amount of antigen but with incomplete Freund's adjuvant. The fourth immunization was given intravenously two weeks after the third with 800 μg antigen. The antibody titers in each animal were determined by ELISA assay at 4-6 days after each immunization. Only mice with antibody titers higher than 1 in 400 were used for cell fusion.

P3Ul myeloma cells cultured in 25 cm^2 flasks in the exponential phase of growth were used for fusion with the spleen cells from 5-6 hyperimmune mice killed at four days after the last boosting. The hybrid cells that survived in HAT medium were tested for production of antibody by the ELISA assay. The antibody-producing cells were expanded in culture and cloning was performed by limiting dilution method. The cloned cells were expanded slowly *in vitro* by transferring the cells to 24-well plates and 25 cm^2 flasks, respectively, while the cell density was maintained between 10^5-10^6 cells/ml.

Determination of antibody class and isotype

The hybridomas found to produce sufficiently high antibody titers after 3-5 successive assays were recloned to ensure purity of the monoclonal antibody. The culture supernatants from these recloned cells were used for the determination of antibody class and isotype by ELISA assay using Hybridoma Subisotyping Kit for Mouse Monoclonal Antibodies (CALBIOCHEM).

Normal mouse sera and anti-schistosome antisera

Mouse antisera against *S.japonicum* (Chinese strain) (ISCH) were obtained by heart puncture from Swiss albino mice which were chronically infected with 30 cercariae for 8-10 weeks. Normal mouse sera (NMS) were obtained in a similar manner from uninfected mice.

Immunoblotting assay

The tegument obtained by freeze-thawing was solubilized in the SDS-PAGE sample buffer and applied onto 12.5% SDS-PAGE gels (50 μg/well). Electrophoresis was then performed at 100 volts for 4 hours and the proteins were blotted from the gel onto nitrocellulose paper using a blotting apparatus with a setting at 0.5 mA for 1 hour. Antigenic proteins on the nitrocellulose paper were detected by incubating nitrocellulose strips in either ISCH (dilution 1:50) or undiluted cell culture supernatants containing McAb, followed by 1:200 rabbit anti-mouse Ig labelled with horseradish peroxidase (HRP). The imunogenic proteins that bind with anti-mouse Ig-HRP were visualized by incubating the nitrocellulose strips

3,3 diaminobenzidine (DAB) and H_2O_2.

Indirect immunofluorescence assay

Freshly-obtained adult worms were fixed in 2% paraformaldehyde in 0.1 M PBS, pH 7.4, for 2 hours at 4°C. The fixed worms were washed with the same buffer, then they were frozen and cut into 4-6 μm thick sections in a cryostat. These frozen sections were picked up on gelatin coated slides and allowed to thaw at room temperature for 30 min. The sections were incubated with 10% normal sheep serum in 0.1 M PBS, pH 7.4, for 30 min and followed with the appropriate dilution of McAb for 2 hours at room temperature. After washing with PBS, the sections were incubated with secondary antibody (sheep anti-mouse immunoglobulin conjugated with FITC) for 1 hour at room temperature. At the end of the incubation period, the sections were washed with double distilled water three times for 10 min for each washing. The sections on slides were mounted in buffered glycerol and covered with glass coverslips. The completely stained sections were examined under UV light using incidence illumination with a Leitz Orthoplan microscope. Positive and negative controls were carried out simultaneously by replacing the monoclonal antibodies with ISCH and NMS, respectively. In addition, mechanically transformed schistosomula and 5-day old lung schistosomula were also used in the assay of experimental specimens and of positive and negative controls performed as for the adult parasites.

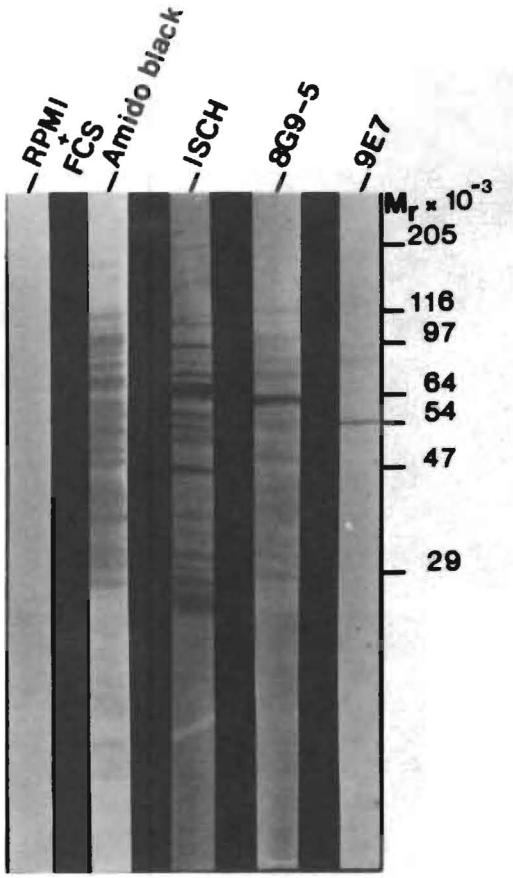

Fig. 1 Immunoblotting of the tegumental antigens of adult *S.japonicum* (Chinese strain) detected by McAbs 8G9-5 and 9E7, in comparison with anti-schistosomal anti-sera (ISCH). The protein profiles were stained with amido black and control supernatant culture fluid (RPMI-FCS).

RESULTS

Immunoblotting assay

There were 37 clones of hybridoma cells that reacted positively by ELISA assay. These clones were propagated further to obtain sizable volumes of culture fluid for immunoblotting and indirect immunofluorescence assays. Up to 6 consistent clones of McAbs could be identified by immunoblotting assay when probed against tegumental antigens. The Mr of antigenic bands reacting with McAb clones were estimated as 116, 105, 97, 86, 64, 62, 60, 56, 54, 52, 50, 47, 45 and 34 kDa. Most clones of McAb bound with two or more antigenic bands, except clone 8G9-5 and 9E7 which produced rather distinct characteristic patterns. The 8G9-5 McAb bound strongly to the band at Mr 64 kDa, but also showed a weak reaction with other bands between 29-116 kDa (Fig. 1). The 9E7 McAb reacted specifically with a single tegument antigen at the molecular mass of 54 kDa (Fig. 1). The isotype of the 8G9-5 McAb was determined to be IgM, whereas the 9E7 McAb was identified as IgG 2a.

Fig. 1 also shows that when immunoblotting assay was performed using ISCH to detect the electroblotted, tegumental antigens, at least 15 major bands of Mr 205, 116, 110, 97, 86, 68, 64, 56, 54, 47, 45, 38, 27 and 26 kDa were demonstrated. The tegumental antigens recognized by

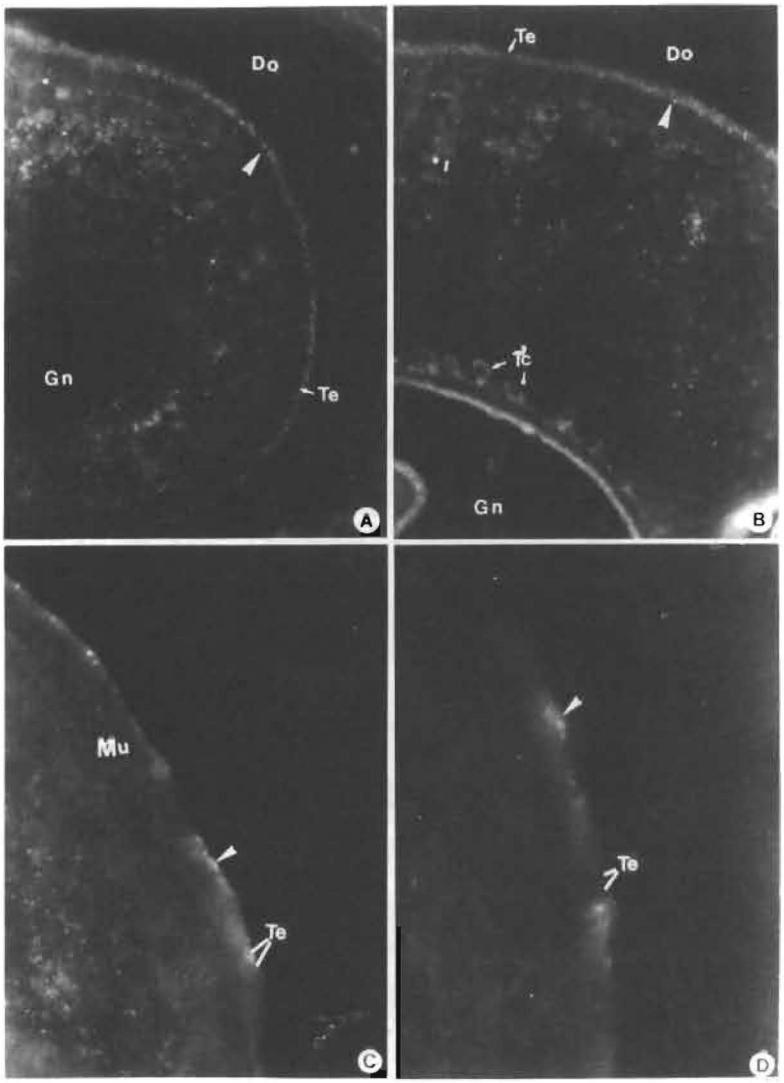

Fig. 2 Indirect immunofluorescence of adult *S.japonicum* (Chinese strain) detected by McAbs 8G9-5 and 9E7. (A, B) : sections react with McAb 8G9-5 : intense fluorescence appears over the tegument (Te) of the dorsolateral aspect (Do), the gynaecophoral canal (Gn), and in the cytoplasm of tegumental cells (Tc). Bright granules (arrows) are also numerous in the tegument (A, ×330; B, x660). (C, D) : section reacts with McAb 9E7 : intense fluorescence appears on the outer portion of the tegument (Te), and in some areas the fluorescence is brighter (arrows) than others and appears granulated (C, x660, D, x1320).

these two clones of McAbs fell within this range, and the bands at 64 and 54 kDa also appeared to be among major tegumental antigens. Although in immunoblotting bands that strongly reacted with ISCH were seen at 110, 97, 68, 66, and 47 kDa, no McAb was produced against these prominent tegumental antigens.

Indirect immunofluorescence assay

Culture supernatants containing 8G9-5 and 9E7 McAbs were tested by indirect immunofluorescence assay using frozen sections of adult *S.japonicum* (Chinese strain). McAb 8G9-5 which bound primarily to the antigen at 64 kDa exhibited even fluorescence over the whole thickness of the tegument and intense fluorescence on fine granules that were dispersed throughout the tegument. The fluorescence also appeared as intense staining over the tegumental cell cytoplasm that surrounded a dark round nucleus (Fig. 2, A and B). The 9E7 McAb which detected a single band of the tegumental antigens at Mr 54 kDa appeared to bind to the periphery and the outermost part of the tegument, but with brighter fluorescence in some areas than in others (Fig. 2 C and D).

When tested against the whole mount living schistosomula, McAbs 8G9-5 and 9E7 McAbs were found to bind over the whole surface of schistosomula, but McAb 9E7 tended to show stronger and more even fluorescence (Fig. 3, A and B) than McAb 8G9-5 (Fig. 3, C and D). These experiments thus demonstrated that both McAbs 8G9-5 and 9E7 reacted with adult tegumental antigens as well as with similar antigens on the surface of schistosomula.

DISCUSSION

Numerous McAbs against the tegumental antigens of *S.mansoni* have been described, some of which have been found to recognize both the teguments of adult worm and schistosomulum.[4,17,18] In this report,

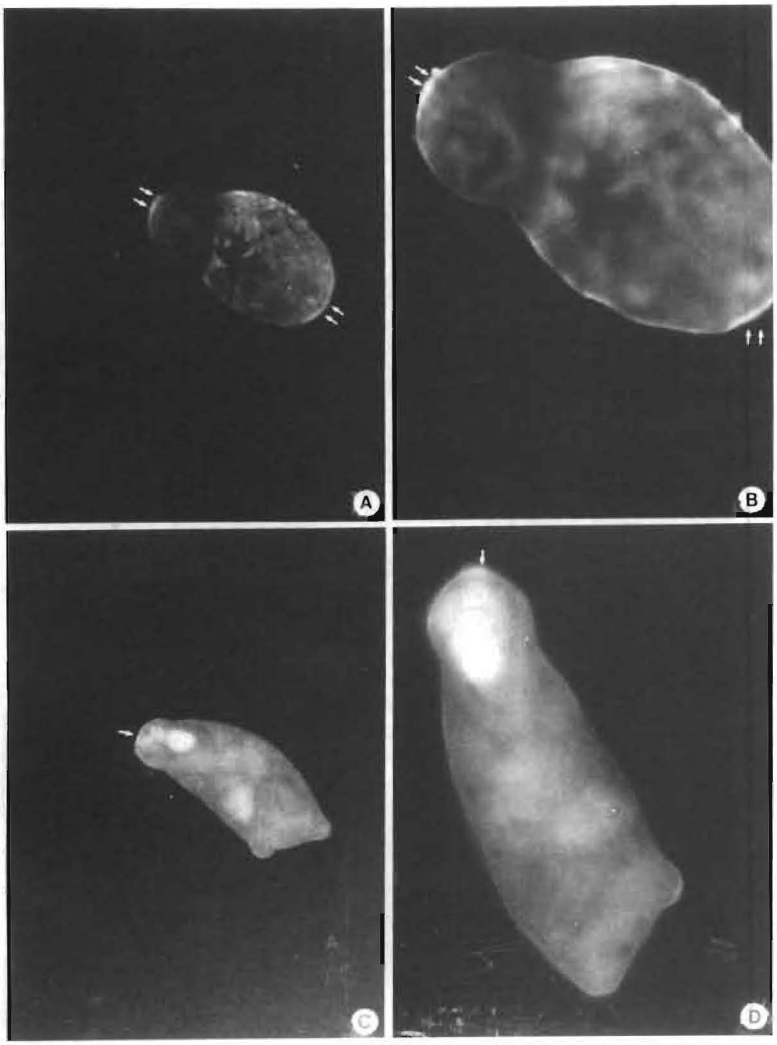

Fig. 3 Indirect immunofluorescence of live schistosomula of *S.japonicum* (Chinese strain) detected by McAbs 8E9-5 and 9E7. (A, B) : Schistosomula react with McAb 9E7, strong fluorescence occurs at the anterior and posterior ends (arrows)(A, ×330; B, ×1,320). (C, D) : Schistosomula react with McAb 8G9-5, strong fluorescence occurs at the anterior end (arrow) and in the histolytic gland (Gl).

we have produced two specific McAbs against the tegumental antigens of adult *S.japonicum* (Chinese strain) which recognized complexes of antigens at Mr 54 kDa and 64 kDa, respectively. These McAbs were also found to cross-react with the tegument of the schistosomulum.

Previously, some of the surface molecules of adult *S.japonicum* have been identified by probing with the specific monoclonal antibodies. The McAb IPH 134 produced from crude extract of the adult worm was found to bind specifically only with adult *S.japonicum* extract but not with egg extract or adult *S.mansoni* extract. [19] Later study revealed that the target epitope of IPH 134 was in the major immunogenic proteins of the adult worms of Mr 23 kDa (Sj 23). [20] Glutathione S-transferase isoenzyme (GSTs) of Mr 26 kDa (Sj 26) has also been demonstrated to be antigenic by using McAb 50-1 and McAb 250-7. Since Sj 26 could be detected in the host circulation, this target epitope has been considered to be an important marker for immunodiagnosis. [21]

We have been able to produce two specific monoclonal antibodies (8G9-5 and 9E7) which recognized target epitopes on the surface tegument of adult *S.japonicum* (Chinese strain). These target epitopes were different from those of Sj 23 and Sj 26 reported previously, since their molecular masses were found to be 64 kDa and 54 kDa, respectively. Anatomical localization in the adult worm indicated that the target molecule recognized by McAb 8G9-5 probably was associated with an intrategumental structure which could be tegumental granules that were present in abundance, thus giving a granulated form of fluorescence over the whole width of the tegument. The fluorescence due to McAb 9E7 has been found to be fairly intense on the outer rim of the tegument which could represent the staining of antigens present on the surface membrane. In some areas, the fluorescence was brighter than in others and appeared granulated. These areas may represent foci where there were an accumulation of membraneous granules which are known to coalesce, thus contributing the membranous antigenic content to the surface. In addition, the McAb 8G9-5 also bound to target molecules inside the tegumental cell cytoplasm, which indicated that the epitopes of this antigen are already available, even at the early stage of synthesis within the cell cytoplasm.

We have further demonstrated that the 64 kDa and 54 kDa antigens may be present on both adult worm and schistosomular surfaces. McAb

9E7 showed strong binding over the whole surface of the schistosomulum and tended to show stronger and more even fluorescence, whereas McAb 8G9-5 also showed even but weaker fluorescence. Cross-reactivity between adult worms and schistosomula has been reported previously in *S.mansoni*.[4] Our results also indicate that both mechanically transformed schistosomula and the lung worms give similar patterns of immunofluorescence with these McAbs.

These two McAbs may be relevant to the problem of immunodiagnosis of schistosomal infection, since the target antigens of these McAbs are expressed in both the tegument of adult worms and schistosomula. Cross reactivity of these two McAbs against *S.japonicum* (Philippines strain), *S.mansoni* and *S.mekongi* has been studied and will be reported separately.

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