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MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18  
FROM: AID/SCI, Victoria Ose *VO*  
SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. PR #4

Attachment

PH-FRE-105  
6.39  
PROGRESS REPORT NO. 4

IDENTIFICATION OF SURFACE ANTIGENS AND PRODUCTION OF  
MONOCLONAL ANTIBODIES IN *SCHISTOSOMA JAPONICUM*

A RESEARCH PROJECT

USAID/PSTC PROGRAM

GRANT NO. 936-5542-G-00-6076-00

SUBMITTED BY

PRASERT SOBHON, PH.D.

PROJECT LEADER

ANATOMY DEPARTMENT

FACULTY OF SCIENCE, MAHIDOL UNIVERSITY

PROJECT PROFILE

COUNTRY	Thailand
GRANT NO.	936-5542-G-00-6076-00
PROGRAM	Program on Science and Technology Cooperation
PROJECT TITLE	Identification of Surface Antigens and Production of Monoclonal Antibodies in <i>Schistosoma japonicum</i>
PROJECT LEADER	Dr. Prasert Sobhon
ORGANIZATION	Faculty of Science, Mahidol University, Bangkok 10400, Thailand
CO-INVESTIGATORS	Dr. Suchart Upatham Dr. Thanit Kusamran Dr. Vithoon Vaiyanant
PROJECT CONSULTANTS	-
AUTHORIZED OFFICER	Dr. Pairote Prempree, Dean, Faculty of Science, Mahidol University
TOTAL PROJECT BUDGET	US\$150,000.-
PROJECT DURATION	3 years
REPORTING PERIOD	April 1988 - September 1988
BUDGET ALLOCATION FOR THIS PERIOD	568,425 Bahts

## C O N T E N T

### 1. BACKGROUND

The grant was awarded on August 11, 1986. During the two year period we have finished the identification and characterization of major antigenic proteins from the surface of adult *S. japonicum* by using immunoprecipitation method. These proteins were also compared with another species of oriental schistosome, i.e., *S. mekongi* and *S. mansoni*. In this report we would like to present further work in progress on identification and characterization of antigenic surface proteins and lipids in adults of *S. japonicum* and other schistosome species using immunoblotting. Since this technique is more convenient to perform, we intend to use it as the principal method to detect specific monoclonal antibodies (MAB) to be raised against surface antigens. Therefore, first we have verified that the antigens (especially proteins) detected by this method did not differ greatly from that obtained from immunoprecipitation technique, which is more difficult to perform and not convenient for use in the screening of MAB(s). In this period we have also used immunoblotting to characterize the released protein antigens from adult parasites that had been incubated *in vitro* and compared this to the surface antigens that induced the host antibody production. There is also further progress on the characterization of lipids of the parasites' surface. Furthermore, we have obtained at least 10 clones of monoclonal antibodies which have been tested for activities by using ELISA, immunofluorescence and immunoblotting assays.

### 2. OBJECTIVES OF THE PROJECT

2.1 To identify and characterize antigens on the surface of the tegument of cercariae, schistosomula and adult *Schistosoma japonicum* (Chinese & Philippine), and to study their cross-reactions with surface antigens of other schistosome species.

2.2 To produce monoclonal antibodies (MAB) against parasite surface antigens and identify MAB that can react with specific surface antigens (and released antigens), which could be used for immuno diagnosis, and MAB that can kill schistosomes.

### 3. MATERIALS & METHODS

In this period we have performed 5 major experiments as follows :

#### 3.1 Identification and comparison of immunogenic surface protein antigens of adult *S. japonicum*, *S. mekongi* and *S. mansoni* by immunoblotting

In this experiment, the surface antigens of parasites were obtained as follows : the parasites' tegument were extracted by freeze-thawing on dried ice in tris-buffer for 20 cycles, and the shed pieces of tegument in the supernatant were collected after centrifugation of the mixture at 100 x g for 5 min. The supernatant was solubilized in the SDS-PAGE sample buffer and applied onto 12.5% SDS-PAGE gels. Then, electrophoresis was performed at 100 volts for 4 hrs, and the proteins on gel were blotted onto nitrocellulose (NC) paper using blotting apparatus with setting at 0.5 mA for 1 hr. Antigenic proteins on the nitrocellulose paper were detected by incubating NC strips in infected mouse serum and followed by rabbit anti-mouse Ig labelled with horseradish peroxidase (HRP). The immunogenic proteins that bind with anti-mouse Ig-HRP were visualized by incubating the nitrocellulose papers in 3,3-diaminobenzidine (DAB) and H<sub>2</sub>O<sub>2</sub>.

#### 3.2 Identification of lipids on the surface of *S. japonicum*

The presence of lipids as membrane surface components have been reported in various species of parasitic helminths, including *S. mansoni*. Some of the lipids have been demonstrated to be immunogenic in both animals or human infected by the parasites.

In this experiment, living adult *S. japonicum* (Chinese) were <sup>125</sup>I-labelled by a similar procedure as used in the protein labelling according to immunoprecipitation method. Freeze-thawed tegument

of the parasite was prepared and lyophilized into dry powder and lipid components were extracted by hexane-isopropanol. The solvent could reproducibly recover the majority of the radioactively labelled compounds, whereas the precipitate (presumably proteins) always represented less than 20%, suggesting that lipids are major components of the *S. japonicum* tegument. The lipid fraction was further extracted in an aqueous system to separate polar lipids (glycolipids/lipoproteins) from non-polar lipids; and it was found that there are polar glycolipids about 3-4 times higher than nonpolar glycolipids as determined from their radioactivities. The radioactively labelled lipids were observed to bind tightly to the Eppendorf plastic tubes leading to marked loss during the extraction. However,  $10^5$ - $10^6$  cpm of the  $^{125}\text{I}$ -glycolipids were usually obtained following the extraction. Analysis of labelled lipids were performed on thin layer chromatography, and revealed by autoradiography in comparison with standard lipids.

### 3.3 Identification of released antigens from adult male *S.japonicum* (Chinese) incubated *in vitro* in MEM by immunoblotting

Approximately 500 adult male *S. japonicum* (Chinese) worms, freshly perfused from mice, were incubated in 1 ml of minimal essential medium (MEM) at 37°C. The incubating medium was collected and 1 ml new MEM medium was added at 0, 1, 3, 9 and 20 h. Each collection was concentrated by using aquacide II, and protein fractions separated in 12.5% SDS-PAGE system using 100 V for 4 hours. After electrophoresis, the gel was electroblotted onto nitrocellulose paper (NCP) for 1 h at 0.5 A. The NCP was soaked in 1% BSA for 1 h at room temperature, and then reacted with *S. japonicum* (Chinese) infected mouse serum (1:50 dilution) for overnight at 4°C. The immune complexes on NCP were detected by reacting with peroxidase-conjugated rabbit anti-mouse antibody(1:200 dilution) for 2 hour at room temperature and further incubated in

0.3 mg/ml DAB + 0.01% H<sub>2</sub>O<sub>2</sub> for 10 min at room temperature to visualize the brown colored bands on the NCP strips.

### 3.4 Preparation of monoclonal antibodies (MAB) against surface antigens of *S. japonicum*

#### Immunization

Six weeks old BALB/c mice were used for immunization with tegument antigens of the Chinese and Philippines *S. japonicum*. Ten mice were used for each immunization schedule. Mice were first given 200 micrograms of antigens mixed with an equal amount of complete Freund's adjuvant inoculated at 4 sites subcutaneously. The second and third immunizations were given at two week interval on the same route with the same amount of antigens but with incomplete Freund's adjuvant. The fourth immunization was given 2 weeks after the third with 800 micrograms of the antigens intravenously. The antibody titers of each animal were checked by ELISA assay after 4-6 days of each immunization. Only mice found to have antibody titer of over 1 in 400 were used for cell fusion.

#### Preparation of monoclonal antibodies

P3U1 myeloma cells cultured in 25 cm<sup>2</sup> flasks in exponential phase of growth were used for fusion with the spleen cells from 5-6 hyperimmune mice killed at 4 days after the last boosting. The hybrid cells that survived in HAT medium were tested for production of antibody by ELISA assay. The antibody producing cells were expanded in culture and cloning was performed by limiting dilution method. The cloned cell was expanded slowly *in vitro* by transforming to 24 well plates, and cm<sup>2</sup> flasks, respectively, while the cell density was maintained between 10<sup>5</sup>-10<sup>6</sup> cell/ml.

### 3.5 Testing of monoclonal antibodies

#### 3.5.1 Immunoblotting assay

The adult parasites tegument was extracted by freeze-thawing and the protein fractions were separated by SDS-PAGE and blotted onto nitrocellular paper (NC) in the same manner as experiment 3.1. Instead of using immune sera, the NC strips were incubated in solutions of monoclonal antibodies (MAB) collected either from ascitic fluid or from cultures. The positive MAB that react with tegument proteins were detected by incubation with rabbit anti-mouse Ig-HRP and visualized by further incubation in DAB and  $H_2O_2$ .

#### 3.5.2 Indirect immunofluorescence assays

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  $\mu$ m thick sections in a cryostat. These frozen sections were picked up on gelatin coated slides and allowed to thaw at room temperature for 30 minutes. The sections were covered with 10% normal sheep serum in 0.1 M PBS, for 30 minutes and followed with the appropriate dilution of the monoclonal antibodies (MAB) for 2 hours at room temperature. After washing with PBS buffer, the sections were incubated with secondary antibody (sheep anti-mouse IgG conjugated with FITC) for 1 hour at room temperature. At the end of incubation period, the sections were washed with double distilled water 3 times for 10 minutes at each washing. The sections on slides were mounted in buffered glycerol and covered with glass coverslips. The completely stained sections were examined under the incidence illumination UV light in a Leitz Orthoplan microscope. The positive and negative controls were carried out simultaneously by replacing the monoclonal antibodies with an infected mouse serum and PBS, respectively.

#### 4. RESULTS & DISCUSSION

##### 4.1 Identification and comparison of immunogenic surface protein antigens of adult *S. japonicum* and other schistosoma by immunoblotting (Table 1, Figure 1)

It was found that in all species most bands that occur in immunoblotting correspond to those present in immunoprecipitation. However, there are slight shifts in MW of some bands in comparison to immunoprecipitation. After taking this into account, it appears that there are three regions with strong cross-reaction among various species. The first is at MW 86,000, which shows strong intensity in *S. japonicum* and *S. mekongi* using their specific antisera. Although this bands is detected in *S. mansoni* the intensity is much lower. This protein, therefore, appears to be more specific to oriental schistosomes. The second and most intense is the band at MW 68,000 which shows strong intensity for all species especially when using antisera of corresponding species. This protein is, therefore, probably the most common protein among all species that has relatively large quantity in comparison to other surface proteins. However, this protein may be slightly different in different species since it appears to be intense only when corresponding antibody is used. The third region that shows fairly strong cross reaction across species are at MW 38,000 and 34,000. Bands with MW at 30,000 and 28,000 are less intense than the bands mentioned above, but tend to show more intensity in oriental schistosomes, while band with MW at 26,000 appears to be more intense in *S. mansoni*. Immunoprecipitation experiment using *S. mansoni* antigen (MA) as a starting material shows that there is also consistent and strong cross reaction among bands 29,500 to 26,500, whereas the cross reaction at MW 68,000 and 86,000 are much less pronounced (Table 1, Figure 1). It should also be noted that bands 68,000 and 86,000 of *S. japonicum* are not intense in immunoprecipitation by appear

very intense in immunoblotting. It is probable that these bands are "hidden" or not so rich in tyrosine, and therefore not easily accessible to  $^{125}\text{I}$  labelling, while antibodies against them are well generated. There are also other minor bands (eg. MW 128,000; 64,000; 23,000; 19,000) that appear in immunoprecipitation but not in immunoblotting. Furthermore, in comparison to immunoprecipitation, there are no bands detectable at MW below 23,000, while immunoprecipitation patterns always show strong intensity of labelled molecules at MW<15,000 (see Report #2). This region may, therefore, be the lipoproteins or glycolipids that are also labelled with  $^{125}\text{I}$ , and nonspecifically precipitated. In immunoblotting these groups of molecules do not appear since they may not have specific antibodies, or they may simply be lost in the process of blotting due to their small molecular weight. Further work on characterization of immunogenic surface glycolipids will help to resolve this question.

TABLE 1 IMMUNOBLOTTING PATTERNS OF PROTEINS DERIVED FROM FREEZE-THAWED ADULT TEGUMENT OF *S. MANSONI* (MA), *S. JAPONICUM* (CHINESE-CH), *S. JAPONICUM* (PHILIPPINE-PI) AND *S. MEKONGI* (ME) AGAINST MOUSE ANTISERA AGAINST *S. MANSONI* (ISMA), *S. JAPONICUM* (CHINESE-ISCH), *S. JAPONICUM* (PHILIPPINE-ISPH) AND *S. MEKONGI* (ISME); AND CON-A STAINING PATTERN OF THESE PROTEINS

MW	MA**	CH**	ME**	ISMA			ISCH & ISPH			ISME		
	+	+	+	MA	CH&PI	ME	MA	CH&PI	ME	MA	CH&PI	ME
205	205	205		+(*)		+		+(*)				+(*)
200				+	+	+	+	+	+	+	+	+
180		180	185	+(*)	+	+		+(*)				+(*)
160	160	158	155	+	+	+	+	+(*)	+	+	+	+(*)
128		128						+(*)				
115		114		+	+	+	+	+(*)	+	+	+	+
105	105			+(*)	+	+	+	+	+	+	+	+
97			94	+	+	+	+	+(*)	+	+	+	++(*)
86		86	85	+	+	+	+	++(*)	++	+	++	++(*)
80	80			++(*)			+			+		
76				+	+	+	+	+	+	+	+	+
68		68	68	+++	+	+	+	+++(*)	+++	+	+++	+++(*)
64	64	62	64									
58	58							+(*)				
54			54	+	+	+	+	+	+	+	+	+
50				+	+	+	+	+	+	+	+	+
45	42.5	45	43	+	+	+	+	+(*)	+	+	+	+
38	37	38	38.5	++	+	++	+	++	++	+	+	++(*)
34	34	34	35	++	+	+	+	+(*)	++	+	+	+
32				++	+	+	+	+	+	+	+	+
30	29.5	30	31.5	+(*)	+	++	+	+(*)	++	+	++	++(*)
28				+	+		+	+	+	+	+	++
26	26.5	26	26	++	+	+	+	+(*)	+	+	+	+
23	22.5	23						+(*)				
19	19	19										
<15	<15	<15	<15									

"+" signs indicates relative intensity after incubating NC strips in rabbit anti-mouse Ig linked to HRP, and staining with DAB + H<sub>2</sub>O<sub>2</sub>.

\* signs indicates whether the bands also show up when the NC strips are stained with Con A.

MA\*\* CH\*\* ME\*\* are immunoprecipitation patterns of parasites' <sup>125</sup>I labelled surface antigens with corresponding immune sera (See Progress Report #2).

#### 4.2 Identification of lipid components on the surface of *S. japonicum*

Extraction of the tegument using hexane-isopropanol (3:2, V/V) reproducibly recovered majority of the radioactively labelled material in the solvent. The remaining precipitate, presumably proteins, represented about 16% of the total radioactivity (Table 2), indicating that most of the  $^{125}\text{I}$  was incorporated into the lipid components of the *S. japonicum* tegument. After removal of the isopropanol by evaporation, the hexane containing lipid fraction was further extracted by aqueous system to separate polar lipids (27.9%) from non-polar lipids (4.5%).

The presence of large proportion of lipid component in *S. japonicum* tegument was also supported by indirect evidence obtained from SDS-polyacrylamide electrophoresis. It was found that, of total radioactively labelled tegument applied onto the gel, only 17% was precipitated on the gel after the electrophoresis and staining-destaining in acetic acid-methanol solution. The amount of macromolecule remained on the gel was similar to that found in the protein precipitate during lipid extraction (16.1%, Table 2).

Both polar and non-polar lipid fractions were analyzed on thin-layer chromatography of Silica Gel G (F 1500/LS254, 100.µm thick) for 2 hr using butanol-acetic acid - water (3:1:1). The TLC plate was dried and the radioactivity detected by autoradiography. Major components in both fractions were undistinguishable under experimental condition (Figure 2). However, the aqueous fraction (Figure 2, lane 1) appeared to contain some components not observed in the hexane (Figure 2, lane 2). Modification of the chromatographic solvent system is evidently required to obtain better resolution as well as identification of the extracted lipids in comparison with standard lipids, this will provide useful information for future studies.

Antigenic property of the extracted lipids was also preliminarily investigated. Due to material availability, the polar lipids (aqueous fraction) was immunoprecipitated. Starting from  $7.2 \times 10^4$  cpm of the polar lipids, about 74% ( $5.3 \times 10^4$  cpm) was recovered following preabsorption by normal mouse serum and protein A-Sepharose. Following precipitation by *S. japonicum* mouse immune serum and successive washes, 205 cpm from  $5.3 \times 10^4$  cpm (0.4%) was found in the final precipitate. Whether this amount significantly represented the specific immunoprecipitable lipid(s) of *S. japonicum* tegument is presently under investigation.

TABLE 2 EXTRACTION OF LIPID COMPONENTS FROM *S. JAPONICUM* SURFACE TEGUMENT

Fractions	$^{125}\text{I}$ -cpm	% recovery
1. Freezed-thawed tegument	$2.24 \times 10^7$	100
2. Hexane-isopropanol extraction :		
2.1 Precipitate	$0.36 \times 10^7$	16.1
2.2 Supernatant	$1.0 \times 10^7$	44.6
2.2.1 Aqueous	$6.25 \times 10^6$	27.9
2.2.2 Hexane	$1.0 \times 10^6$	4.5

4.3 Detection of released antigens from *S. japonicum* (Chinese) incubated *in vitro* in MEM medium by immunoblotting.

The released antigens collected from MEM culture fluid were detected at various time intervals from 1 to 20 hour by separating the antigens in SDS-PAGE and blotted onto NC strips, which are in turn reacted with mouse antisera against *S. japonicum* (Chinese). The positive bands with determined molecular weight (MW) are shown below in Table 3. The major bands are at MW of 68,000 and 66,000 and 200,000 daltons. In addition there are at least 15 other weak bands ranging in MW from 205,000 to 26,000 dalton. These positive bands are similar in pattern to that obtained from the immunoblotting of freeze-thaw tegument as performed in experiment 3.1 & 4.1.

TABLE 3 RESEALED ANTIGENS OF *S. JAPONICUM* AS DETECTED BY IMMUNOBLOTTING

Protein bands	MW	1 h	1-3 h	3-9 h	9-20 h	CH+ISCH
1	>205K	+	+	+	+	+
2	>205K	-	+	+	+	+
3	200K	+	++	++	++	+
4	180K	-	-	-	++	-
5	160K	-	-	-	+	-
6	115K	-	-	-	-	+
7	105K	+	+	+	+	+
8	97K	+	+	+	+	+
9	86K	+	+	+	+	+
10	76K	+	+	+	+	+
11	68K	++	++	++	++	+++
12	56K	+	+	+	++	+++
13	54K	+	+	+	+	+
14	50K	+	+	+	+	+
15	45K	+	+	+	+	+
16	38K	+	+	+	+	+
17	30K	+	+	+	+	+
18	26K	-	-	+	+	-

+ = low reaction, ++ = moderate intensity, +++ = high intensity

CH+ISCH - antigens from freeze-thawed tegument that appear in immunoblot.

1-20 h - released antigens that appear in immunoblot.

#### 4.4 Production of monoclonal antibodies

Monoclonal antibodies production in the culture supernatants were monitored by ELISA assay, and the results are shown in Tables 4 & 5.

TABLE 4 ELISA ASSAY FOR MONOCLONAL ANTIBODIES IN THE CULTURE SUPERNATANTS OF THE HYBRID CELLS IMMUNIZED WITH *S. JAPONICUM* (CHINESE STRAIN) ANTIGENS.

CODE OF CLONED CELLS	ELISA results (O.D.)			
	1	2	3	4
F51-1	0.084	0.311	0.112	-
F55-1	0.257	0.187	0.106	0.021
F55-2	0.062	-	-	-
F57-1	0.162	0.092	0.052	0.005
F57-2	0.010	-	-	-
F59-1	0.724	0.072	0.016	-
F513-1	0.118	0.023	0.024	-
F553-1	0.0536	0.514	1.333	1.567
F553-2	1.038	0.133	0.07	-
F553-3	0.765	0.033	-	-

TABLE 5 ELISA ASSAY FOR MONOCLONAL ANTIBODY IN THE CULTURE SUPERNATANTS OF THE HYBRID CELLS IMMUNIZED WITH *S. JAPONICUM* (PHILIPPINES STRAIN) ANTIGENS.

CODE OF CLONED CELLS	ELISA results (O.D.)				
	1	2	3	4	5
1G7-2	0.009	1.501			
1G7-3	0.145				
1G7-5	0.021	0.002			
1G7-6	0.194	0.074			
1G7-7	0.192	0.052			
1F11-1	0.064	2.487			
2E2-1	0.014	0.785			
2E2-2	0.109				
2E2-4	0.143				
2G4-2	0.034	0.309	0.168	2.086	
2G4-3	0.014				
2G4-4	2.344	1.731	0.732	2.034	
3F3-1	0.073	1.095			
3F3-2	0.038	0.012			
3B9-1	0.056	1.335			
3B9-2	0.076				
3B9-3	0.162				
3B9-4	0.152				
3D9-1	0.004	1.492			
3D9-2	0.023				
3D10-1	0.218	2.053			
3D10-3	0.251				
3D10-4	0.198				
3E2-1	0.249	1.119			
3E2-2	0.339				
3E2-3	0.391				
3E2-4	0.352				
4F9-1	0.108	0.057	0.015	0.013	
4F9-2	0.203	0.296	0.078		
4F9-3	0.086	0.143	0.026	0.014	
4F9-4	0.192	0.19	0.038		
4F9-5	0.335				

CODE OF CLONED CELLS	ELISA results (O.D.)				
	1	2	3	4	5
4G3-1	1.407	0.580			
4G3-2	2.203	0.768			
4G3-3	2.225	1.173	0.396	0.257	
4G3-4	1.033	1.013	0.054	1.207	1.033
5G2-1	0.220	1.167			
5G2-2	0.213				
5G2-3	0.240				
5G2-4	0.283				
5G2-5	0.283				
5G2-6	0.242				
5G2-7	0.227				
5D4-1	0.115	1.262			
5D4-2	0.102				
5D4-3	0.114				
5D4-4	0.081				
6C7-1	2.552	2.395	2.615	1.427	
6C7-2	1.073	0.079	0.005	0.008	
6C7-4	2.775	2.590	2.425	2.620	
6C7-5	2.751	2.676	2.429	2.757	
6C7-6	2.711	2.644	2.484	2.509	2.751
6C7-7	2.721	2.721			
6C7-8	2.567	2.588	0.834	0.364	0.176
6C7-9	2.749	2.687			
6C7-10	2.725	2.571	1.588	2.640	
6C7-11	1.125	0.070	0.011		
6C7-12	2.634	2.267	1.492	2.761	2.616
6D8-1	0.007	1.836			
6D8-2	0.008				
6D8-3	0.004				
6D8-4	0.026				
6D10-1	0.056	0.893			
6D10-2	0.085				
6E5-1	0.197	0.702			
6E5-2	0.106				
6E5-3	0.133				

NEGATIVE CULTURE FLUID O.D. = 0.09, 0.09, 0.022, 0.013, 0.008, 0.016

POSITIVE CONTROL O.D. = 0.228, 0.228, 0.176, 0.150, 0.159, 0.180

Determination of antibody class

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 subisotypes by ELISA assay using Hybridoma Subisotyping Kit for Mouse Monoclonal Antibodies (CALBIOCHEM) (Table 6).

TABLE 6 ANTIBODY CLASS AND SUBISOTYPES OF THE MONOCLONAL ANTIBODIES AGAINST *S. JAPONICUM* (Chinese and Philippines strains)

CODES OF HYBRIDOMA	ANTIBODY CLASS AND SUBISOTYPES
F553-1	IgM
2G4-2	IgG2b
2G4-4	IgM
6C7-1	IgG2b
6C7-4	IgG2b
6C7-5	IgG2b
6C7-6	IgG2b
6C7-10	IgG2b
6C7-12	IgG2b

The above hybridomas are in the process of expansion *in vivo* to produce large quantities of monoclonal antibodies for further experiments. Moreover, fusions are being performed continuously in order to obtain more hybridomas and more monoclonal antibodies of different classes, subisotypes and specificity.

#### 4.5 Testing activities of monoclonal antibodies

##### 4.5.1 Detection of MAB reactivities to tegument proteins of *S. japonicum* (Chinese) by immunoblotting (Figure 4)

Ten clones of monoclonal antibodies as verified by ELISA assays to be positive were tested for their reactivities with tegument proteins. Freeze-thawed *S. japonicum* (Chinese) tegument were used as immobilized antigens that were separated by SDS-PAGE and blotted onto 10 NC strips. The screening was performed by incubating NC strips in specific MAB obtained either from culture fluid (CF) or ascite fluid (AF). After reacting with peroxidase-conjugated anti-mouse antibody and using DAB + H<sub>2</sub>O<sub>2</sub> as substrate, it was found that only MAB clone No. 6C7-12, as selected from ELISA assays, both from culture fluid and ascite fluid was observed to have immuno-activity to the tegument antigen. The MW of these antigens appeared to be 105,000 dalton for culture fluid and 200 K, 160,000 and 105,000 daltons for ascitic fluid. More MAB clones to be obtained in future will be tested for reactivities in the same manner.

##### 4.5.2 Detection of binding of MAB clone 6C7-12 to the adult parasites by indirect immunofluorescence assay (Figures 5, 6, 7)

The indirect immunofluorescence results using frozen sections of adult *S. japonicum* (Chinese strain) indicate that the monoclonal antibody (MAB) from clone 6C7-12 bind to the epitopes in the muscular layer of the parasite. A much lesser fluorescence was also observed in the internal tissues with the exception of the gut, which show autofluorescence. These MAB from culture fluid and ascitic fluid gave similar fluorescent pattern, however the former showed more intense fluorescence than the latter. The staining of MAB was located specifically in the interstitial area between the muscle bundles and appeared as tiny bright lines, but no fluorescence was observed in the muscle cells. On the other hand, the tegument exhibited only faint fluorescence (Fig. 5, B & C and Fig. 6 C & D). In contrast

the infected mouse serum exhibit a bright green fluorescence over both the tegument and muscular layer and this provide the positive control experiment (Figure 7 A & B). While the negative control section not using primary antibodies was unlabelled and the gut had a yellow autofluorescence appearance (Figure 7 C & D).

It is concluded that the MAB from clone 6 C7-12 may stain proteins in the slender processes of tegument cells and the cell soma themselves that lie between and immediately underneath the muscle layer. In these areas the newly synthesized membrane packed in the form of membranous granules (Mb) are known to accumulate. The faint fluorescence in tegument resulted from MAB stain may be due to the widely scattered Mb granules in this layer.

## 5. CONCLUSION & REMARKS

We have demonstrated that the surface proteins at MW 86,000; 68,000; 38,000 and 34,000 may be major antigenic proteins of *S. japonicum* as shown by immunoblotting, and that these proteins are quite specific to oriental schistosomes. In addition we have obtained at least 10 MAB clones, one of which (clone # 6C7-12) showed reactivity with the tegument proteins at about 105,000 dalton. The patterns of released proteins and the tegument proteins as detected by immunoblotting have been shown to be similar which suggests that the major portion of released (or circulating) proteins are derived from the surface and the tegument of parasites. The profile of these antigens and MAB obtained may be useful for devising the method for future immunodiagnosis.

## 6. WORKPLAN FOR THE NEXT PERIOD

6.1 Immunoprecipitation and immunoblotting patterns of surface antigens in cercariae and schistosomula of *S. japonicum* (Chinese) will be studied and compared to those of adult parasites and other species.

- 6.2 Glycolipids antigens on the surface of parasites will be characterized further.
- 6.3 More MAB clones will be obtained, using immunization with freeze-d-thawed adult tegument, and/or homogenized schistosomula. It is hoped that this method will generate more MAB clones with more specific reactivities to surface proteins.
- 6.4 Screening and assaying of more MAB clones using ELISA, immunoblotting and indirect fluorescence methods will be performed.
- 6.5 Positive clone (such as clone 6C7-12) will be tested for immune killing of schistosomula, using antibody-complement killing assay.

**FIGURE 1** Immunoblottings of antigenic proteins from the tegument of *S. mansoni* (MA), *S. japonicum* (Chinese & Philippine) (CH&PH), *S. mekongi* (ME). The teguments were extracted from the parasites by freeze-thawing, and solubilized in the sample buffer. Electrophoresis was performed in 12.5% SDS-PAGE, and protein bands transferred onto nitrocellulose (NC) paper. The NC strips were overlaid with mouse immune sera ISMA, ISCH, ISPH and ISME, and immunogenic bands were detected by rabbit anti-mouse IgG labelled with horseradish peroxidase (HRP). The color was developed by incubating with DAB + H<sub>2</sub>O<sub>2</sub>.

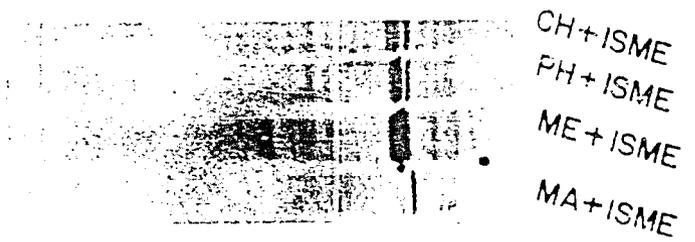
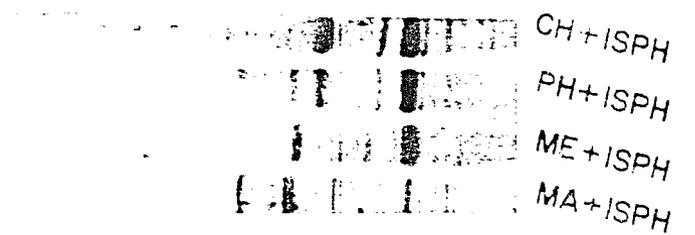
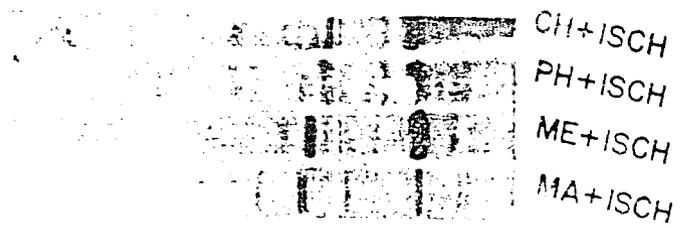
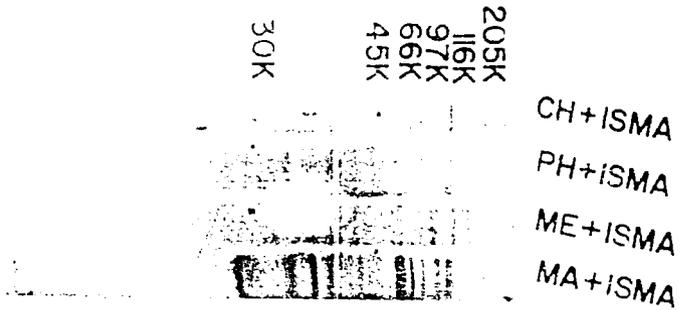


FIGURE 1

FIGURE 2 Autoradiogram of TLC-analysis of polar and non-polar lipids extracted from the tegument of *S. japonicum*.

Lane 1 - polar lipid (aqueous extract)

Lane 2 - nonpolar lipids (hexane extract).

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FIGURE 2

**FIGURE 3** Immunoblotting of released proteins from adult *S. japonicum* (Chinese) incubated in MEM medium at various time intervals, and the detection was performed by using mouse antisera against *S. japonicum* as the primary antibody and rabbit antimouse IgG-HRP as the detector.

CH - freeze-thawed tegument proteins stained with Coomassie Blue  
CH+SCH - freeze-thawed tegument proteins appearing in immunoblot  
1 - 20 h. - released proteins appearing in immunoblot.

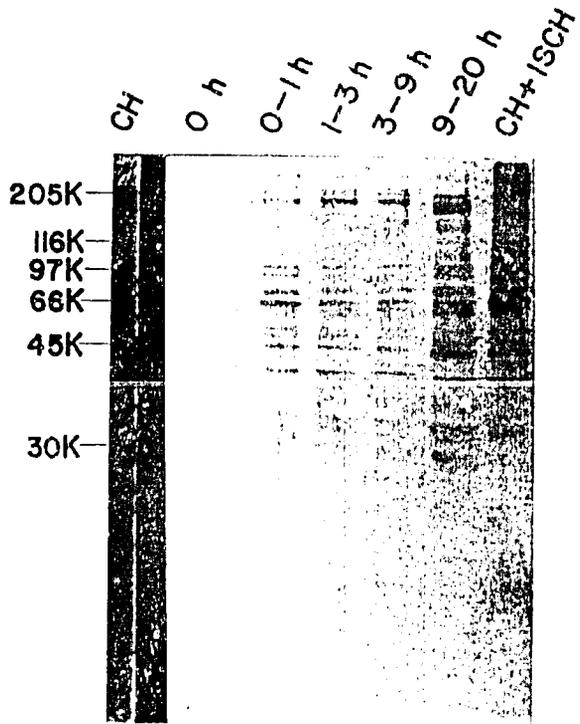


FIGURE 3

FIGURE 4 Testing of monoclonal antibodies (MAB) from ten clones of hybridoma, using proteins from freeze-thawed tegument of *S. japonicum* (CI1) as the antigens, and culture or ascitic fluids from clones of MAB as primary antibodies, and the detector is rabbit-antimouse IgG-HRP.  
(CF - culture fluid; ISCI1 - immune sera against *S. japonicum*; CF 2 B 4 to CF 6 C-7-12 MAB derived from culture fluids, AF 2 D4 & AF 6 C 7-12-MAB derived from ascitic fluids).

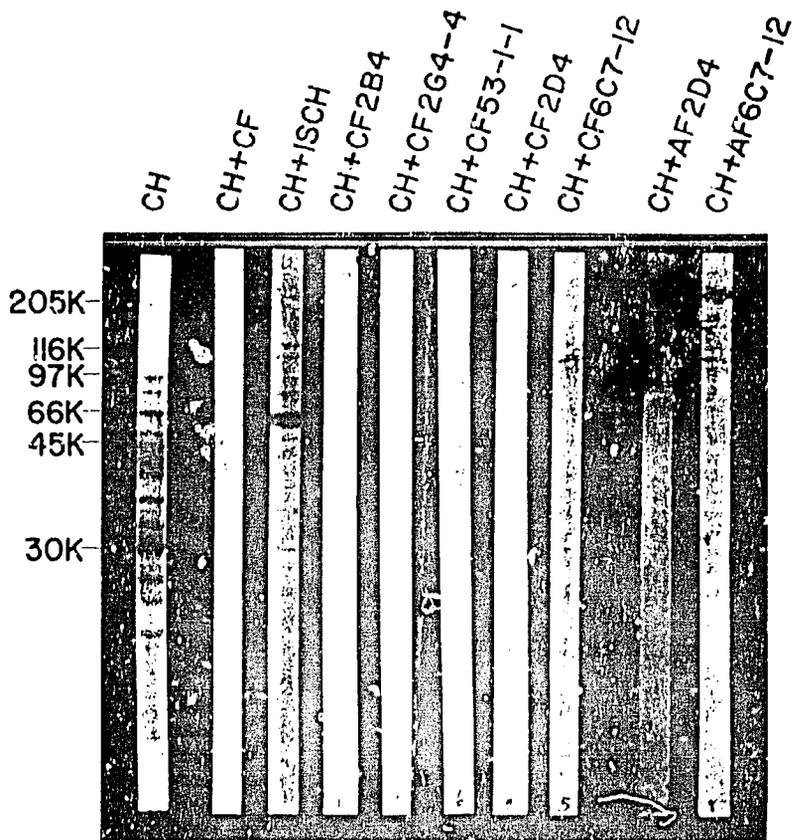


FIGURE 4

FIGURE 5 Photomicrographs of immunofluorescence pattern of frozen-sections of *S. japonicum* (Chinese), stained with monoclonal antibody (MAB) derived from Culture fluid clone No.6C7-12.

A : A heavy staining appear at the level of muscular layer (MU), while a much lower positive staining is also seen on whole section. X 90. B & C : Higher magnification showing positive fluorescence that appears as tiny strands in between the muscle fibers.

Only faint staining appears over the tegument (Te) while the gut (Gu) shows only autofluorescence. X 180, X 360.

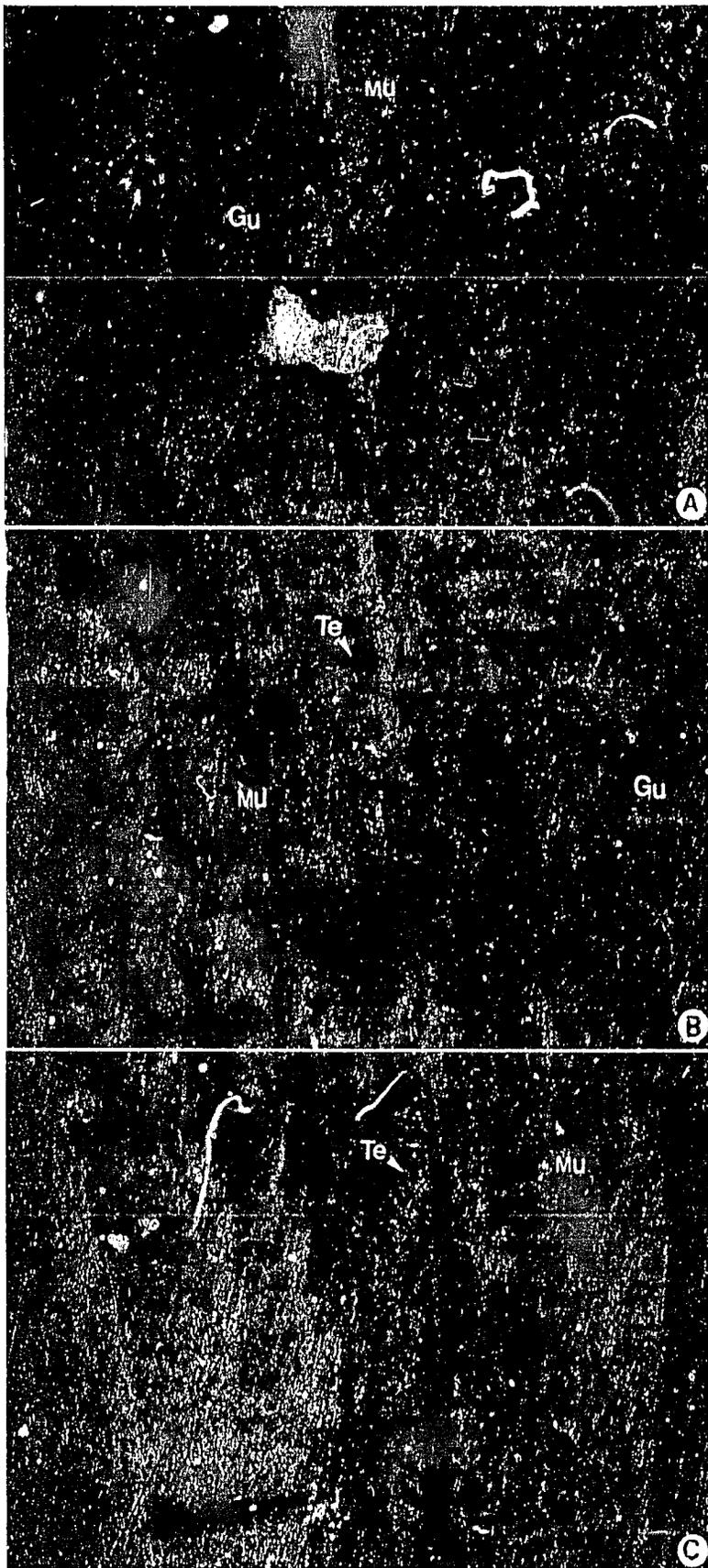


FIGURE 6 Photomicrographs of immunofluorescence pattern of frozen sections of *S. japonicum* (Chinese) stained with monoclonal antibody derived from ascitic fluid of clone No. 6C7-12. A & B : Fluorescence appears only at the muscular layer (Mu) and having similar characteristics as in Figure 5 but with lesser intensity. Only faint fluorescence is observed over the tegument (Te) and the gut (Gu). X 90. C & D : The staining pattern in the muscular layer resembles that in Figure 5. X 180, X 360.

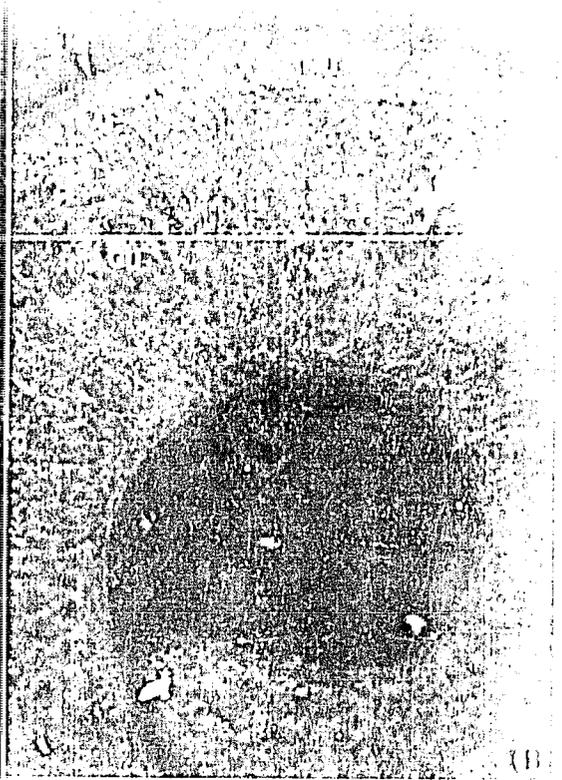
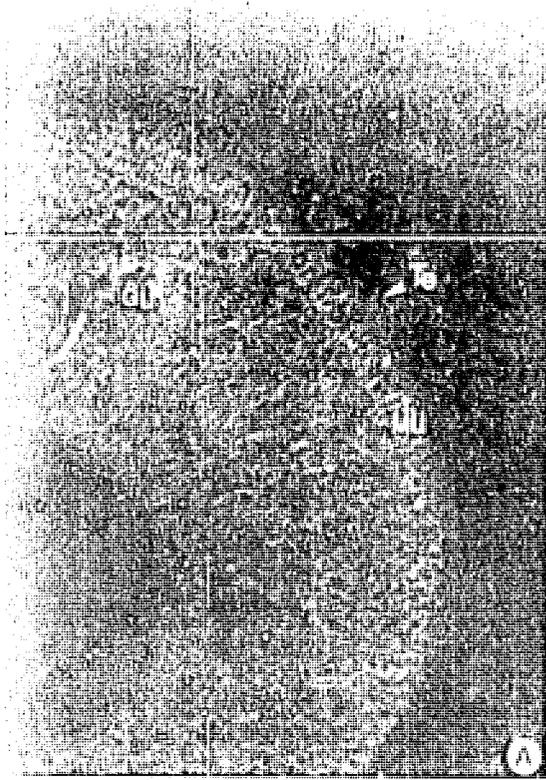


FIGURE 7 Photomicrographs of control sections of *S. japonicum* (Chinese).  
A & B : Positive control using the *S. japonicum* infected mouse serum give strong fluorescence over the entire section and the tegument. X 45, X 90. C & D : Negative control omitting primary antibody, only the gut (Gu) of parasite has a yellow autofluorescence. Other internal tissues and the tegument are unlabelled. X 45, X 180.

