Immune Responses in Mice after Immunization with Antigens from Different Stages of the Parasite *Schistosoma mansoni*

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Mice responses to immunization with *Schistosoma mansoni* antigens were investigated. Priming with cercarial antigen preparation (CAP) induced significant ($P < 0.05$) IgM, IgG, IgG2a, IgG2b, and IgA increases, while booster caused a significant IgG1 increase. A soluble worm antigen preparation (SWAP) caused significant IgG elevation. Priming with soluble egg antigen (SEA) caused significant IgM and IgG2a increases, while booster induced significant IgM, IgG and IgA increases. CAP-immunized mice sera (IMS) recognized CAP peptides ranging from 23–78 kDa. SWAP-IMS recognized SWAP peptides ranging from 40–75 kDa. SEA-IMS recognized SEA peptides ranging from 33–101 kDa. The cross-reactive peptides among the 3 antigens were identified. CAP caused significant increases in mesenteric lymph nodes (MLNs) $CD_{4,8}^+$, B lymphocytes, CD_8^+ thymocytes, CD_4^+ T and B splenocytes. SWAP priming caused significant increases in MLNs $CD_{4,8}^+$ thymocytes and B splenocytes. SWAP booster caused significant increases in MLNs CD_8^+ T and B lymphocytes, $CD_{4,8}^+$ thymocytes and CD_4^+ T and B splenocytes. SEA caused significant increase in CD_4^+ T cells.

Key words: Schistosoma mansoni, Ig Classes and Subclasses, Immunofluoresence, Western Blotting

Introduction

Extensive studies of both humoral and cellular immune responses to cope with the complexity of the *Schistosoma* life cycle, where the parasite undergoes dramatic biochemical and morphological changes, were with limited success.

Cercarial immunogens were expressed for limited periods in very limited quantities that do not support their value as vaccine candidates (Smithers and Terry, 1969). This was later confirmed by genomics results that demonstrated the presence of fewer transcripts in cercariae relative to other stages (Jolly *et al*., 2007). Using irradiated cercariae (IRC) in mice immunization studies induced specific antibodies of IgM, IgA, IgE, total IgG as well as IgG subclasses (Dunne *et al*., 1994) and resulted in resistance to challenge infection in experimental schistosomiasis (Richter *et al*., 1993) as measured by 27–65% reduction in the total worm burdens after challenge infection (El Ridi *et al*., 1997).

Vaccination of mice with crude worm extracts or purified components recorded a reduction in worm burden ranging from 20 to 50% (Murrell *et al*., 1975). Immunizing mice with 24-, 35- and 97-kDa fractions of *S. mansoni* adult worms in the presence of adjuvant induced a significant degree of protection (Gustavson *et al*., 1998). Parasite adult worms continuously released excretory-secretory (ES) antigens (Stevens *et al*., 1983). The large amount of ES antigens is consistent with the abundance of gene transcripts encoded within the adult stage like those encoding protein-degrading enzymes (cathepsin B, cathepsin L), proteins implicated in evasion of the host immune response, and egg production (Caffrey *et al*., 2004).

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S. mansoni eggs stimulated a transient T_H0 response characterized by increased IFN-γ (Vella and Pearce, 1992) and IL-6 production (La Flamme and Pearce, 1999). Moreover, eggs induced strong T_H2 response during natural infection (Grzych *et al*., 1991) and response reached its maximal extent approximately within a week (Vella and Pearce, 1992). Using soluble egg antigens (SEA) in immunization studies resulted in stimulation of significant antigen-specific CD_8^+ T cell response in spleen within 20 days (Pancreà *et al.*, 1999). CD_8^+ T cells played a regulatory role in modulating granulomatous immunopathology associated with eggs trapped in host tissue (Chensue *et al*., 1993). Also, B lymphocytes were involved in regulation of egg pathology (Cheever *et al*., 1985).

It was thought that an effective vaccine should be able to generate an antigen-specific CD_4^+ T cell response, and the number of such cells increased with time after single schistosome infection or upon multiple exposures to infection (Sanderson, 1996; Kourilova *et al*., 2004).

Six candidate parasite antigens were selected by the WHO for advanced evaluation as vaccines (Bergquist and Colley, 1998). Results of independent trials with the six *S. mansoni* vaccine candidates (Hewitson *et al*., 2005), unexpectedly, demonstrated that the maximum induced protection ranged from 30 to 40% obtained by immunization with GST (Balloul *et al*., 1985) or Sm14 (Moser *et al*., 1991).

Approaching optimal protective immune responses against *S. mansoni* requires simultaneous induction and fine tuning of both humoral and cell-mediated effector mechanisms that need to be first better understood at the levels of crude antigens from different parasite stages. Thus, the present work aimed to dissect the changes in both humoral and cellular immune responses after immunizing mice with crude antigens derived from different *S. mansoni* developmental stages. We quantified immunoglobulins (Igs) classes and subclasses levels in sera from immunized mice against the homologous antigens used in immunization. Moreover, we used sera from immunized mice with each of these antigens to characterize the immunogenic peptides and the degree of cross-reactivity among such antigens by Western blotting. In addition, we investigated cellular phenotypic changes in different lymphoid organs in response to immunization.

Materials and Methods

Experimental animals and immunization design

A total of 42 female pathogen-free Swiss albino mice (18–25 g) were used. Animals were fed on standard chew, supplied with water, and maintained at ambient temperature (25 °C). Three test groups received two intraperitoneal injections of cercarial antigen preparation (CAP), soluble worm antigen preparation (SWAP), and soluble egg antigen (SEA) (Rutitzky *et al.,* 2001), respectively. For the three antigens, the used concentration in each injection was 50 μg/100 μl PBS and the second immunization was one week later. The control group received two injections each with 100 μl of PBS. Post immunization, animals were sacrificed, lymphoid organs were dissected and freshly used for further immunophenotyping analyses. Blood samples were collected by penetrating the retro-orbital plexus/sinus with a heparin-treated glass capillary tube from individual mice and centrifuged at 14,000 × *g* at 4 ºC for 20 min. Sera were separated and frozen at –80 ºC till being used.

Enzyme-linked immunosorbent assay (ELISA) for the detection of immunoglobulin classes and subclasses

Levels of IgG in sera from immunized and control animals were detected by ELISA (Maghraby and Bahgat, 2004) on individual U-shaped wells of 96-well polyvinyl-coated plates (Alto, Italy) with 50 μl/well of CAP (62.5 μg/ml), SWAP (250 μg/ml) and SEA (125 μg/ml) diluted in coating buffer (20 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6). After coating, plates were incubated overnight at room temperature, washed three times using 0.01 M PBS, pH 7.4, containing 0.05% Tween-20 (PBST). Antigen-free sites were blocked against non-specific binding using 100 µl/well of PBST containing 1% bovine serum albumin (PBST-BSA) and incubated at 37 ºC for 1 h. After three washes with PBST, diluted sera in PBST-BSA (1:100 for CAP and SEA and 1:200 for SWAP) were applied at 50 μl/well, and the plates were incubated at 37 ºC for 2 h. For total IgG detection 50 μl/well at 1:500 dilution (in PBST-BSA) peroxidase-conjugated anti-mouse IgG was added, and the plates were incubated at 37 ºC for 1 h. For IgA, IgM and IgG1, -2a, -2b, and -3, rabbit 50 μl/well monoclonal anti-

immunoglobulin classes and subclasses antibodies (ZYMED Laboratories Inc, San Francisco, CA, USA) were added, and the plates were incubated at 37 ºC for 1 h. After 3 washes with PBST, peroxidase-labeled anti-rabbit IgG was applied (50 μl/well) to the plates that were incubated at 37 ºC for 1 h followed by 3 washes. For visualizing the antigen-antibody binding, plates were dried, and the peroxidase-specific colourimetric substrate *O*-phenylenediaminediamine dihydrochloride (Sigma, St. Louis, MO, USA) was used in the presence of H_2O_2. To avoid a possible increase in the background of the enzyme-substrate reaction, 2 M HCl was applied (25 μl/well). The changes in optical densities were measured at λ_{max} = 490 nm using a microwell plate reader (TECAN-SUNRISE, Austria).

Western blotting

Immunoblotting was carried out according to Towbin *et al.* (1979) to detect the immunereactivity in sera from mice immunized with different schistosomal antigens against their homologous and heterologous antigens. Following SDS-PAGE of CAP, SWAP, and SEA according to Laemmli (1970) through 4% stacking and 10% resolving gels (55 × 85 × 1 mm) in an electrophoresis chamber (Mini-protean II; Bio-Rad Laboratories, Munich, Germany), resolved proteins were electrophoretically transferred from the gel to a nitrocellulose sheet (BA85, pore size 0.45 μm; Schleicher and Schüll, Dassel, Germany) at 60 V for 3 h in transfer buffer. Membranes were cut into individual strips that were washed three times each for 5 min with PBS/0.3% T followed by blocking against non-specific binding for 1 h in PBS/0.3% T/1% BSA at room temperature.

Strips were washed three times as above mentioned and incubated for 2 h with sera from immunized mice sera (IMS) with different schistosomal antigens as well as control mice sera (CMS) (1/100 in PBS/0.3% T). After three successive washes for 5 min each, strips were rinsed for 2 h in peroxidase-conjugated anti-mouse IgG (1/500 in PBS/0.3%T) at room temperature. Visualization of the immune reaction on the nitrocellulose strips was done by incubation with a peroxidase-specific substrate (22 mg diaminobenzidine and 2.2 μl 30% H_2O_2 in 22 ml PBS).

Immunophenotyping of different lymphocyte populations

Mesenteric lymph nodes (MLNs), thymus and spleen were excised and gently teased in Petri dishes containing PBS using glass slides. Cells were washed three times with PBS by centrifugation at 1500 × *g* at 4 ºC for 10 min. To remove red blood cells, splenocytes were treated with lysis buffer (Hunt, 1987). The total number of lymphocytes was counted on a haemocytometer under a light microscope (400 × magnification).

Percentage viability was calculated using the following formula:

viability (%) = (number of viable cells/total number of lymphocytes) · 100,

total number of lymphocytes = N · 2 · 10^4/ml,

where N is the number of lymphocytes counted in 16 large squares of the haemacytometer, and 2 is the dilution factor since the cell suspension was diluted 1:1 in 4% trypan blue.

CD_4^+, CD_8^+ T and B lymphocytes were labeled with fluorescin isothiocyanate (FITC)-conjugated monoclonal anti-mouse CD_4^+, CD_8^+ and IgM μ chain, respectively (Sigma). To calculate the percentage of each cell population, green fluorescence cells were counted in a minimum of 100–200 viable cells using a fluorescence microscope (Zeiss Axioskop, Jena, Germany) (Bahgat *et al.,* 2005).

Statistical analysis

All obtained data were analyzed by the student's t-test using the GraphPad InStat software.

Results

Immunoglobulin classes and subclasses levels in sera from immunized mice

Using CAP as an antigen preparation, CAP-IMS one week post 1st immunization showed significantly ($P < 0.05$) higher levels of IgM, IgG, IgG2a, IgG2b, and IgA than CMS. The levels of IgG1 and IgG3, although they did not significantly vary, were 2- to 9.6-fold higher in IMS than CMS against CAP (Table I). One week post 2nd immunization, the IgG1 levels were significantly ($P < 0.05$) higher in IMS than CMS against CAP. The levels of IgM, IgG, IgG2a, IgG2b, IgG3, and IgA were, respectively, 8.5-, 3-, 7.9-, 4.8-, 3.2-, and 6.7-fold higher in IMS than CMS against CAP; yet, such increase was not significant (Table I).

Table I. ELISA levels of immunoglobulin classes and subclasses in mice sera against CAP one week post 1st immunization and 2nd immunization.

CAP 1st immunization

Antibody	IgM		IgG		IgA	
Mice group	C	T	C	T	C	T
Mean	0.14	0.46	0.3	0.88	0.02	0.14
SD	0.031	0.22	0.3	0.8	0.009	0.06
P value	0.042		0.25		0.0062	

Antibody	IgG1		IgG2a		IgG2b		IgG3	
Mice group	C	T	C	T	C	T	C	T
Mean	0.14	0.28	0.08	0.53	0.20	0.88	0.3	0.34
SD	0.13	0.17	0.04	0.3	0.14	0.25	0.04	0.29
P value	0.2172		0.023		0.0022		0.084	

CAP 2nd immunization

Antibody	IgM		IgG		IgA	
Mice group	C	T	C	T	C	T
Mean	0.01	0.07	0.30	0.6	0.1	0.88
SD	0.02	0.5	0.8	0.3	0.005	0.11
P value	0.0251		0.091		0.2218	

Antibody	IgG1		IgG2a		IgG2b		IgG3	
Mice group	C	T	C	T	C	T	C	T
Mean	0.05	0.3	0.06	0.53	0.15	0.7	0.01	0.4
SD	0.01	0.17	0.04	0.5	0.15	0.37	0.01	0.3
P value	0.0336		0.1859		0.0628		0.1221	

C, control mice group; T, immunized mice group. Significant *P* value < 0.05.

Using SWAP as an antigen preparation, SWAP-IMS one week post 1st immunization had significantly ($P < 0.05$) higher levels of IgG than CMS. Levels of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA, although did not significantly differ, were, respectively, 1.37-, 1.87-, 1.36-, 1.86-, 1.58-, and 1.44-fold higher in IMS than CMS against SWAP (Table II). One week post 2nd immunization, the IgA levels nearly did not change, while the IgG level remained significantly ($P < 0.05$) higher in IMS than CMS against SWAP. The levels of IgM IgG1, IgG2a, IgG2b, and IgG3 were, respectively, 1.29-, 1.75-, 1.4-, 1.58-, and 1.35-fold higher in IMS than CMS against SWAP; yet, such increase was not significant (Table II).

Using SEA as an antigen, SEA-IMS one week post 1st immunization had significantly ($P < 0.05$) higher levels of IgM and IgG2b than CMS. The levels of IgG, IgG1, IgG2a, IgG3, and IgA, although did not significantly vary, were 5.3-, 3-, 5-, 5-, and 2.8-fold higher in IMS than CMS against SEA (Table III). One week post 2nd immunization, the levels of IgA, IgG, and IgM were significantly ($P < 0.05$) higher in IMS than CMS. Also, the levels of IgG1, IgG2a, IgG2b, and IgG3, although did not significantly differ, were, respectively, 6.1-, 3.5-, 2.8-, and 6.5-fold higher in IMS than CMS against SEA (Table III).

Characterization of the immunogenic peptides in the three S. mansoni antigen preparations

The IgG reactivity of CAP-IMS was further tested against SDS-PAGE-fractionated CAP on Western blots (Fig. 1a). Results demonstrated that individual sera from mice that received single immunization (Fig. 1A, lanes 1–4) recognized peptides at 65, 78, 85, 92, and 97 kDa. Only two individuals recognized peptides at 31 kDa (lanes 2 and 3) and 40 kDa (lanes 1 and 2). Booster CAP-IMS (lanes 5–7) recognized peptides at 23, 48, 63, 71, 78, 85, 92, and 97 kDa. Only two individuals strongly reacted to a 40-kDa peptide (lanes 6 and 7). It is noteworthy, that CAP-peptides at 65, 80, 85, 92, and 97 kDa were poorly specific as they

Table II. ELISA levels of immunoglobulin classes and subclasses in mice sera against SWAP one week post 1st immunization and 2nd immunization.

SWAP 1st immunization

Antibody	IgM		IgG		IgA	
Mice group	C	T	C	T	C	T
Mean	0.49	0.67	0.16	00.4	0.19	0.28
SD	0.14	0.02	0.05	0.16	0.04	0.16
P value	0.36		0.048		0.4	

Antibody	IgG1		IgG2a		IgG2b		IgG3	
Mice group	C	T	C	T	C	T	C	T
Mean	0.24	0.46	0.34	0.47	0.75	0.89	0.17	0.28
SD	0.04	0.4	0.01	0.21	0.1	0.24	0.02	0.14
P value	0.49		0.361		0.38		0.258	

SWAP 2nd immunization

Antibody	IgM		IgG		IgA	
Mice group	C	T	C	T	C	T
Mean	0.49	0.71	0.16	0.38	0.19	0.23
SD	0.16	0.21	0.05	0.08	0.04	0.11
P value	0.17		0.005		0.61	

Antibody	IgG1		IgG2a		IgG2b		IgG3	
Mice group	C	T	C	T	C	T	C	T
Mean	0.24	0.33	0.3	0.6	0.75	1.05	0.17	0.23
SD	0.04	0.12	0.016	0.3	0.1	0.22	0.02	0.05
P value	0.27		0.19		0.06		0.14	

C, control mice group; T, immunized mice group. Significant *P* value < 0.05.

Table III. ELISA levels of immunoglobulin classes and subclasses in mice sera against SEA one week post 1st immunization and 2nd immunization.

SEA 1st immunization

Antibody	IgM		IgG		IgA	
Mice group	C	T	C	T	C	T
Mean	0.07	0.34	0.34	0.6	0.05	0.14
SD	0.02	0.12	0.3	0.3	0.03	0.12
P value	0.01		0.06		0.04	

Antibody	IgG1		IgG2a		IgG2b		IgG3	
Mice group	C	T	C	T	C	T	C	T
Mean	0.06	0.3	0.1	0.5	0.3	0.8	0.04	0.23
SD	0.02	0.2	0.04	0.3	0.1	0.2	0.05	0.1
P value	0.11		0.07		0.001		0.09	

SEA 2nd immunization

Antibody	IgM		IgG		IgA	
Mice group	C	T	C	T	C	T
Mean	0.07	0.59	0.3	0.6	0.05	0.2
SD	0.002	0.3	0.03	0.26	0.03	0.12
P value	0.04		0.02		0.04	

Antibody	IgG1		IgG2a		IgG2b		IgG3	
Mice group	C	T	C	T	C	T	C	T
Mean	0.06	0.3	0.1	0.3	0.27	0.8	0.04	0.28
SD	0.02	0.24	0.01	0.26	0.12	0.36	0.01	0.2
P value	0.08		0.16		0.05		0.1	

C, control mice group; T, immunized mice group. Significant *P* value < 0.05.

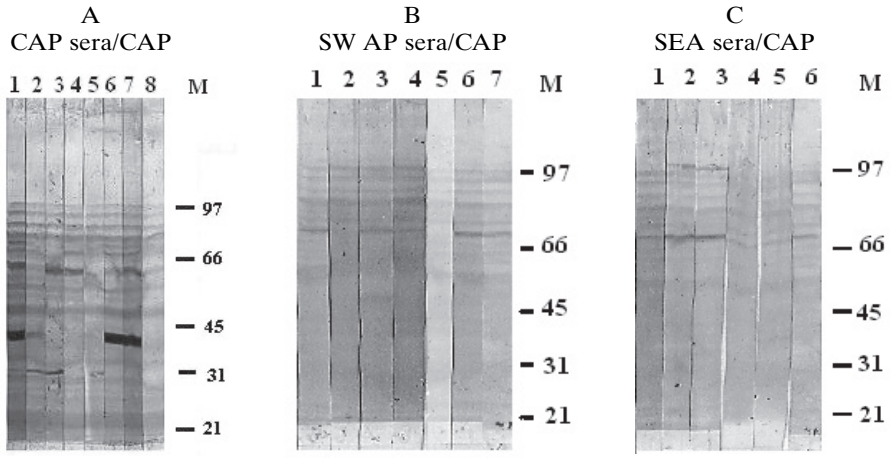

Fig. 1. Characterization of immunogenic peptides in *S. mansoni* crude cercarial antigen preparation (CAP) using sera from mice immunized with different *S. mansoni* antigens. IgG reactivity of CAP-IMS was tested against SDS-PAGE-fractionated CAP on Western blots (A). Mice that received single immunization (A, lanes 1–4) recognized peptides at 65, 78, 85, 92, and 97 kDa. Only two individuals recognized peptides at 31 kDa (lanes 2 and 3) and 40 kDa (lanes 1 and 2). Booster CAP-IMS (lanes 5–7) recognized peptides at 23, 48, 63, 71, 78, 85, 92, and 97 kDa. Only two individuals strongly reacted to a 40-kDa peptide (lanes 6 and 7). It is noteworthy that CAP peptides at 65, 80, 85, 92, and 97 kDa were poorly specific as they cross-reacted with CMS (A, lane 8), SWAP (B, lane 7), and SEA-IMS (C, lane 6).

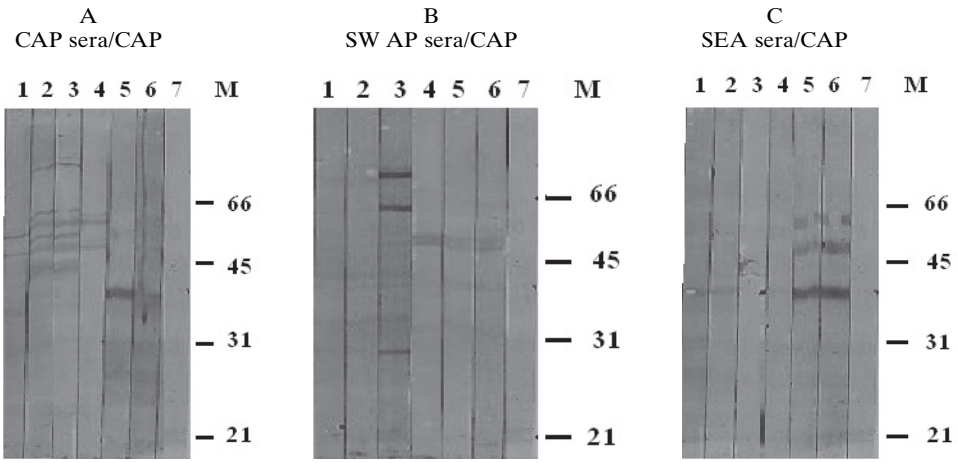

Fig. 2. Characterization of immunogenic peptides in *S. mansoni* crude soluble worm antigen preparation (SWAP) using sera from mice immunized with different *S. mansoni* antigens. Among the sera from mice that received single immunization with SWAP (A), two (lanes 2 and 3) recognized SWAP peptides at 45, 50, 53, 59, and 75 kDa, while the rest recognized peptide bands at 48 and 53 kDa (lane 1). Of the SWAP booster IMS, two recognized a SWAP peptide band at 40 kDa (lanes 5 and 6), whereas the rest reacted with peptide bands at 50 and 57 kDa (lane 4). The CMS (lane 7) did not react to any of the above-mentioned SWAP peptides. IgG-reactive CAP (B) or SEA-IMS (C) in ELISA were tested for their cross-reactivity against SWAP. In (B), lanes 1–3 were treated with individual priming CAP-IMS, while lanes 4–6 with individual booster CAP-IMS, and lane 7 with CMS. In (C), lanes 1–3 were treated with individual priming SEA-IMS, while lanes 4 and 5 with booster SEA-IMS, and lane 6 with CMS. Among the CAP-IMS, only one serum (B, lane 3) cross-reacted with SWAP peptides at 30, 59, and 70 kDa. All booster IMS recognized a peptide band at 50 kDa (B). Both the 50- and 59-kDa bands were previously recognized by SWAP-IMS. Single IMS with SEA did not react with any of the SWAP-fractionated peptides, whereas two individual sera from booster immunization (C, lanes 4–6) recognized a band at 40 kDa, which was also recorded by SWAP-IMS. CMS did not show any cross-reactivity with any of the SWAP peptides (C, lane 7).

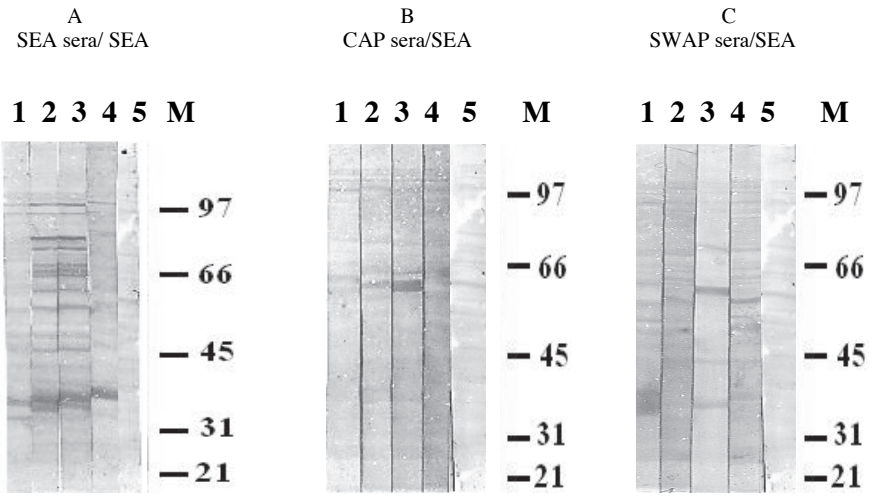

Fig. 3. Characterization of immunogenic peptides in *S. mansoni* crude soluble egg antigen (SEA) using sera from mice immunized with different *S. mansoni* antigens. Priming (A, lanes 1 and 2) or booster SEA-IMS (lanes 3 and 4) recognized SEA peptides at 33, 55, 96, and 101 kDa, while only two IMS (lanes 2 and 3) strongly reacted to additional peptides at 44, 61, 69, 71, 78, and 84 kDa. The CMS (A, lane 5) did not react to any of the above-mentioned bands. IgG-reactive CAP (B) or SWAP-IMS (C) in ELISA were tested for their cross-reactivity against SEA. In (B) and (C) lanes 1 and 2 were treated with priming IMS, while lanes 3 and 4 with booster IMS, and lane 5 with CMS. CAP-IMS weakly cross-reacted to 101, 107, and 101 kDa SEA peptides, while only two individual CAP-IMS (B, lanes 2 and 3) cross-reacted with two extra bands at 63 and 66 kDa. It is noteworthy that the 101-kDa SEA peptide was previously recognized by SEA-IMS. The CMS (B, lane 5) did not react with the above-mentioned bands. Priming SWAP-IMS weakly cross-reacted with 51, 55, 73, and 103-kDa SEA peptides (C, lanes 1 and 2), while booster IMS recognized two sharp bands at ~ 55 and 71 kDa (C, lanes 3 and 4). The 71-kDa band was previously recognized in SEA-IMS and the CMS did not react to any of the above-mentioned SEA peptides (C, lane 5).

cross-reacted with CMS (Fig. 1A, lane 8), SWAP (Fig. 1B, lane 7) and SEA-IMS (Fig. 1C, lane 6).

Among the SWAP-IMS in Fig. 2A, lanes 2 and 3 recognized SWAP peptides at 45, 50, 53, 59, and 75 kDa, while the rest recognized peptide bands at 48 and 53 kDa (lane 1). Of the SWAP booster IMS, two recognized a SWAP peptide band at 40 kDa (lanes 5 and 6), whereas the rest reacted with peptide bands at 50 and 57 kDa (lane 4). The CMS (lane 7) did not react to any of the above-mentioned SWAP peptides. IgG-reactive CAP (Fig. 2B) or SEA-IMS (Fig. 2C) in ELISA were tested for their cross-reactivity against SWAP. In Fig. 2B, lanes 1–3 were treated with individual priming CAP-IMS, while lanes 4–6 with individual booster CAP-IMS, and lane 7 with CMS. In Fig. 2C, lanes 1–3 were treated with individual priming SEA-IMS, while lanes 4 and 5 with booster SEA-IMS, and lane 6 with CMS. Among the CAP-IMS, only one serum (Fig. 2B, lane 3) cross-reacted with SWAP peptides at 30, 59, and 70 kDa. All booster IMS recognized a peptide band at 50 kDa (Fig. 2B). Both the 50- and 59-kDa band were previously recognized by SWAP-IMS. Single IMS with SEA did not react with any of the SWAP-fractionated peptides, whereas two individual sera from booster immunization (Fig. 2C, lanes 4–6) recognized a band at 40 kDa, which was also recorded by SWAP-IMS. CMS did not show any cross-reactivity with any of the SWAP peptides (Fig. 2C, lane 7).

Priming (Fig. 3A, lanes 1 and 2) or booster SEA-IMS (lanes 3 and 4) recognized SEA peptides at 33, 55, 96, 101 kDa, while only two IMS (lanes 2 and 3) strongly reacted to additional peptides at 44, 61, 69, 71, 78, and 84 kDa. The CMS (Fig. 3A, lane 5) did not react to any of the above-mentioned bands. IgG-reactive CAP (Fig. 3B) or SWAP-IMS (Fig. 3C) in ELISA were tested for their cross-reactivity against SEA. In Figs. 3B and 3C, lanes 1 and 2 were treated with priming IMS, while lanes 3 and 4 with booster IMS and lane 5 with CMS. CAP-IMS weakly cross-reacted to 101, 107, and 111 kDa SEA peptides, while only

Table IV. MP of CD_4^+ T, CD_8^+ T and B lymphocytes in different lymphoid organs post 1st and 2nd immunization with CAP.

Post 1st immunization

MLN cells	CD_4^+		CD_8^+		B	
Mice group	C	T	C	T	C	T
Mean	17.3	33.4	15.8	32.9	18.4	33.6
SD	2.6	3.76	2.5	4.3	3.6	2.66
P value	0.0038		0.0041		0.0044	

Thymus cells	CD_4^+ T		CD_8^+ T	
Mice group	C	T	C	T
Mean	22.25	31.32	23.8	29.94
SD	6.1	1.9	2.6	0.53
P value	0.0701		0.0169	

Spleen cells	CD_4^+		CD_8^+		B	
Mice group	C	T	C	T	C	T
Mean	14.5	29.1	16.5	25.5	16.1	34.0
SD	2.7	3.1	6.2	1.01	1.6	3.46
P value	0.003		0.0702		0.0013	

Post 2nd immunization

MLN cells	CD_4^+		CD_8^+		B	
Mice group	C	T	C	T	C	T
Mean	17.3	40.1	15.8	42.7	18.44	30.72
SD	2.69	8.2	2.5	16.1	3.68	1.46
P value	0.0102		0456.0		0.0058	

Thymus cells	CD_4^+		CD_8^+	
Mice group	C	T	C	T
Mean	22.2	32.8	23.8	25.2
SD	6.1	5.5	2.6	2.4
P value	0.0889		0.5254	

Spleen cells	CD_4^+		CD_8^+		B	
Mice group	C	T	C	T	C	T
Mean	14.5	29.1	16.5	28.0	16.1	34.7
SD	2.7	3.1	6.2	6.5	1.6	6.28
P value	0.003		0.09		007.0	

C, control mice group; T, immunized mice group. Significant P value < 0.05.

two individual CAP-IMS (Fig. 3B, lanes 2 and 3) cross-reacted with two extra bands at 63 and 66 kDa. It is noteworthy, that the 101-kDa SEA peptide was previously recognized by SEA-IMS. The CMS (Fig. 3B, lane 5) did not react to any of the above-mentioned bands. Priming SWAP-IMS weakly cross-reacted to 51, 55, 73, and 103 kDa SEA peptides (Fig. 3C, lanes 1 and 2), while booster IMS recognized two sharp bands at ~ 55 and 71 kDa (Fig. 3C, lanes 3 and 4). The 71-kDa band was previously recognized in SEA-IMS, and the CMS did not react to any of the above-mentioned SEA peptides (Fig. 3C, lane 5).

Cellular immune responses after immunization

One week post priming with CAP, the mean percentages (MP) of CD_4^+, CD_8^+ T and B lymphocytes from MLNs, CD_8^+ T thymocytes and CD_4^+ as well as B splenocytes were significantly higher ($P < 0.05$) in IMS than CMS. Although the MP of CD_4^+ T thymocytes and CD_8^+ T splenocytes were almost 1.5-fold higher in tissues from CAP-IMS than CMS, this difference was not significant (Table IV). One week after boosting with CAP, The MP of MLNs CD_4^+, CD_8^+ T and B lymphocytes, CD_4^+ T and B splenocytes remained

Table V. MP of CD_4^+ T, CD_8^+ T and B lymphocytes in different lymphoid organs post 1st and 2nd immunization with SWAP.

Post 1st immunization

MLN cells	CD_4^+ T cells		CD_8^+ T cells		B cells	
Mice group	C	T	C	T	C	T
Mean	17.3	28.56	15.81	27.26	18.44	26.85
SD	2.6	16.9	2.5	6.7	11.3	3.6
P value	0.3201		0.0493		0.2900	

Thymus cells	CD_4^+ T cells		CD_8^+ T cells	
Mice group	C	T	C	T
Mean	22.25	43.4	23.9	31.01
SD	6.1	4.7	2.6	8.8
P value	0.0078		0.2491	

Spleen cells	CD_4^+ T cells		CD_8^+ T cells		B cells	
Mice group	C	T	C	T	C	T
Mean	14.51	36.92	16.55	21.4	16.2	37.31
SD	2.7	9.5	6.2	8.2	1.6	4.4
P value	0.0174		0.4663		0.0015	

Post 2nd immunization

MLN cells	CD_4^+ T cells		CD_8^+ T cells		B cells	
Mice group	C	T	C	T	C	T
Mean	17.3	34.1	15.8	28.5	18.44	30.73
SD	2.6	3.8	2.5	3.7	3.6	3.6
P value	0.3201		0.0493		0.2900	

Thymus cells	CD_4^+ T cells		CD_8^+ T cells	
Mice group	C	T	C	T
Mean	22.25	35.9	23.83	34.16
SD	6.1	2.5	2.6	5.8
P value	0.0234		0.0484	

Spleen cells	CD_4^+ T cells		CD_8^+ T cells		B cells	
Mice group	C	T	C	T	C	T
Mean	14.51	35.15	16.55	28.47	16.2	30.14
SD	2.7	1.6	6.2	7.3	1.6	4.3
P value	0.0004		0.0969		0.0064	

C, control mice group; T, immunized mice group. Significant *P* value < 0.05.

significantly higher ($P < 0.05$) in IMS than CMS. Although the MP of CD_4^+ T thymocytes and CD_8^+ T splenocytes were 1.47- and 1.69-fold higher in CAP-IMS than CMS this difference was not significant, while the MP of CD_8^+ T thymocytes was nearly unchanged in IMS (Table IV). One week post priming with SWAP, the MP of MLNs CD_8^+ T lymphocytes, CD_4^+ T thymocytes and B splenocytes were significantly ($P < 0.05$) higher in IMS than CMS. Although the MP of CD_4^+ T and B lymphocytes from MLNs, CD_8^+ T splenocytes and thymocytes in SWAP-IMS were 1.65-, 1.45-, 1.3-, and 1.29-fold higher than in CMS, this difference was not significant (Table V). One week after boosting with SWAP, the MP of CD_8^+ T and B lymphocytes from MLN, CD_4^+, CD_8^+ T thymocytes, CD_4^+ T, and B splenocytes from IMS were significantly higher ($P < 0.05$) than CMS. Although the MP of MLNs CD_4^+ T lymphocytes and CD_8^+ T splenocytes were 1.97- and 1.72-fold higher in SWAP-IMS than CMS this difference was not significant (Table V). One week post priming with SEA, the MP of CD_4^+ T cells from all tested lymphoid organs and B splenocytes were significantly higher ($P < 0.05$) in IMS than CMS. Although the MP of MLNs CD_8^+ T, B lymphocytes and CD_8^+ T

splenocytes were 1.4-, 1.6-, and 1.6-fold higher in SEA-IMS than CMS, these differences were not significant (Table VI). One week after boosting with SEA, the MP of MLNs CD_4^+ T thymocytes as well as CD_8^+ T and B splenocytes were significantly ($P < 0.05$) higher in IMS than CMS. Although the MP of CD_4^+ T thymocyte was 1.4-fold higher in SEA-IMS than CMS this increase was not significant (Table VI). It is noteworthy, that CD_8^+ T thymocytes remained nearly unchanged after both priming and booster immunizations with SEA.

Discussion

Upon immunization with CAP, the IgM level was elevated. This agrees with the elevation of such Ig class upon vaccinating mice with IRC (Mazza *et al*., 1990). We also recorded the elevation in the IgG level upon immunization with CAP which is consistent with the induced isotype response by carbohydrate antigens from cercarial stage (Nyame *et al*., 2002). Immunization with CAP caused an elevation of both IgG2a and IgG2b, and the same elevation was recorded in both subclasses by others upon immunization with a purified 30-kDa cercarial antigen (Atallah *et al*., 2004).

Immunization with SWAP caused poor response in IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA. It might be attributed to acquiring host antigens by the worm stage as immune evasion (Pearce and Sher, 1987). The total IgG level elevated upon immunization with SWAP, and one might attribute that to the presence of some proteins like hemoglobinases (Sm31/32) which are known to be immunogenic in both humans (Ruppel *et al*., 1990) and animals (Chappell and Dresden, 1988).

The resulting increase in the IgM and IgG levels after immunization with SEA can be attributed to the abundant polysaccharide antigens (Mazza *et al*., 1990) and glycan epitopes in eggs which stimulate the same response in infected mice (Nyame *et al*., 1999). The same increases were for IgG1 and IgG2a following immunization with SEA. This agrees with the reactivity of the same subclasses in sera from *S. mansoni*-infected mice toward SEAs (Mountford *et al*., 1994). The detected increase of the IgG3 level in SEA-IMS is consistent with the results obtained by Mazza *et al*. (1990) upon immunization with antigens from the egg stage. The increases in the IgA level following immunization with SEA are consistent with the detected IgA response against egg antigens in natural infection (Poulain-Godefroy *et al*., 1996).

In Western blots, CAP-IMS recognized a 31-kDa CAP peptide and a 30-kDa SWAP peptide. When a 30-kDa *S. mansoni* antigen was previously tried in vaccinating mice, it elicited high IgG levels (predominantly IgG2a and IgG2b) and resulted in significant protection against infection (Atallah *et al*., 2004). Also, immunizing mice with *S. mansoni* adult worm 31- to 32-kDa antigens resulted in significant protection against infection (Chlichlia *et al.,* 2001). In addition, in CAP-IMS 40-kDa CAP peptide was detected. This is approximately compatible with the recorded ~ 38-kDa schistosomula surface protein that was recognized in sera from immunized rats with *S. mansoni* IRC (Verwaerde *et al*., 1985). In addition, in CAP-IMS, a 71-kDa CAP peptide was detected. An antigen with approximately similar molecular weight (70 kDa) (Zhang *et al*., 1999) was previously identified by Western blotting using sera from multiple vaccinated mice with IRC and characterized as a heat shock protein.

In SWAP-IMS, a 57-kDa SWAP peptide was detected. A SWAP peptide with the same molecular weight was detected in *S. mansoni* using sera from mice immunized by either virulent or IRC (Hanna *et al*., 1994). A 62-kDa antigen, calreticulin, was recognized in *S. mansoni* SWAP using infected patients sera (El Naglaa *et al*., 2004) which was defined as a good T and B cell antigen and represents a potential vaccine candidate. We also detected other SWAP peptides of 70 and 75 kDa in the SWAP- and CAP-IMS. Immunogenic adult *S. mansoni* glycoproteins of approximately similar molecular weights (70 kDa) were previously recognized by Western blotting using sera from mice vaccinated with IRC (Strand *et al*., 1987).

SEA-IMS recognized SEA peptides at 44, 61, 69, and 96 kDa; these results are consistent with another study which detected SEA immunogenic peptides at 45, 64, and 92 kDa using naturally infected rat sera (Alarcón de Noya *et al*., 1997). In SEA-IMS, we also recognized a 71-kDa SEA peptide. An immunogenic peptide of ~ 75 kDa molecular weight was previously recognized in *S. mansoni* egg ES antigens using polyclonal antisera raised in rabbits against processed urine (Shaker *et al*., 1998) or feces (Attallah *et al*., 1997) of S. *mansoni*-infected patients.

Table VI. MP of CD_4^+ T, CD_8^+ T and B lymphocytes in different lymphoid organs post 1st and 2nd immunization with SEA.

Post 1st immunization

MLN cells	CD_4^+ T cells		CD_8^+ T cells		B cells	
Mice group	C	T	C	T	C	T
Mean	17.3	25.8	15.8	25.8	18.4	26.6
SD	2.6	3.9	2.5	7.4	3.6	4.3
P value	0.0360		0.0922		0.0775	

Thymus cells	CD_4^+ T cells		CD_8^+ T cells	
Mice group	C	T	C	T
Mean	22.25	40.18	23.9	25
SD	6.1	0.8	2.6	9.8
P value	0.007		0.86	

Spleen cells	CD_4^+ T cells		CD_8^+ T cells		B cells	
Mice group	C	T	C	T	C	T
Mean	14.5	36.9	16.5	21.3	16.1	37.3
SD	2.7	9.5	6.2	8.2	1.6	4.4
P value	0.0174		0.4663		0.0015	

Post 2nd immunization

MLN cells	CD_4^+ T cells		CD_8^+ T cells		B cells	
Mice group	C	T	C	T	C	T
Mean	17.3	28.8	15.8	25.1	18.4	29.4
SD	2.6	1.8	2.5	2.1	3.6	0.9
P value	0.0036		0.0081		0.0074	

Thymus cells	CD_4^+ T cells		CD_8^+ T cells	
Mice group	C	T	C	T
Mean	22.25	31.2	23.83	27.87
SD	6.1	2.1	2.6	2.8
P value	0.067		0.14	

Spleen cells	CD_4^+ T cells		CD_8^+ T cells		B cells	
Mice group	C	T	C	T	C	T
Mean	14.5	35.1	16.5	28.7	16.1	30.1
SD	2.7	1.61	6.26	7.38	1.6	4.35
P value	0.0004		0.0969		0.0064	

C, control mice group; T, immunized mice group. Significant *P* value < 0.05.

The increase in B, CD_4^+ and CD_8^+ T lymphocytes of MLNs in response to immunizations with CAP confirm the previously reported *in vivo* proliferation of T cells in the skin draining LNs of mice immunized with IRC (Pearce *et al.*, 1988). The increase of the MP of CD_8^+ T lymphocytes from MLNs, B, CD_4^+ T splenocytes, B, CD_4^+ CD_8^+ T thymocyte upon immunization with SWAP matches with the reported induced cellular immune response upon immunization with rSm14 (Ribeiro *et al.*, 2002) and paramyosin from adult worms (Pearce *et al.*, 1988). The increase of B cells from both MLNs splenocytes upon immunization with SEA might support the previously reported role of B cells against egg antigens to down-modulate granuloma formation in infected mice (Kwatia *et al.*, 2000).

The generalized increase of the MP of CD_4^+ T cells upon immunization with SEA agrees with previous reports (Pearce and Macdonald, 2002) where SEA-derived antigens could activate CD_4^+ T cells that were in turn suggested to play a role in regulating the granuloma formation. The nearly absent change in the MP of CD_8^+ thymocytes after immunization with SEA agrees with a previous report (Mathew and Boros, 1986) where schistosomiasis infection was demonstrated to be less able to mount specific CD_8^+ T response, and

with another report where such cell population was shown not to be required for down-modulating the granuloma formation in mice (Ferru *et al*., 1998).

Our pairwise comparisons of the stimulated immune responses by the three different antigens indicated a positive correlation and cross-reactivity that was more obvious for responses obtained from both CAP and SEA antigens. This agrees with the reported cross-reactivity between different parasite antigen preparations (Eberl *et al*., 2001a) where the detected immune responses in natural infection were attributed to repeated exposure to common antigens expressed in different parasite stages but not due to re-exposure to infection.

Our results clearly demonstrated common B and T cell responses towards both CAP and SEA, and one may attribute this to the presence of common glycan epitopes in both parasite stages (Eberl *et al*., 2001b). This might also be supported by the recognized cross-reactivity among both antigens by Western blotting in our hands.

In conclusion, although antigens from the infective stage, cercariae, were reported to be transiently present (Smithers and Terry, 1969), the CAP was the most immunogenic as it stimulated B, CD_4^+, CD_8^+ T cells as well as Ig subclasses were protective as suggested in previous vaccination trials. In contrast, SWAP was the poorest stimulator for antibody response; it mildly stimulated the cellular response confirming the repeated disappointing protection results upon using worm stage antigens in immunization trials.

The SEA-specific CD_4^+ T response confirms the evidence that egg antigens have a regulatory effect on modulating the granuloma formation. Since both cellular and humoral responses are required for protection against schistosomiasis, we believe that there is a need for more studies to define and characterize the appropriate antigens' combinations capable of stimulating both responses regardless of the source stage.

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