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Immune Events Associated with Protection in C57BL/6 Mice Immunized with Anti-Idiotypic Antibodies Mimicking Protective Antigens Shared Between Gamma-Irradiated Cercariae Vaccine and Human Resistance Model of *Schistosoma haematobium*

Sherif H. Abdeen

Department of Zoology, Faculty of Science, Mansoura University, Mansoura, Egypt

Immunoregulation is central for successful manipulation of schistosomiasis. Unlike schistosome vaccine development strategies that relied on direct selection of antigens from crude responses leading to selection of mildly protective antigens, the present study tested the utility of selection of potentially protective antigens encompassed rounds of immunoregulation via idiotypic network. Anti-idiotypic antibodies (Ab2) were purified from sera of New Zealand white rabbits multiply immunized with gamma-irradiated cercariae of *S. haematobium*, using adult worm specific idiotypes (Ab1) purified from sera of subjects resistant to reinfection. Ab2 was used for immunization of C57BL/6 mice and consequences of immunization were monitored before and after challenge infection with *S. haematobium*. Results showed an increase of splenic T cell expression of intercellular adhesion molecule-1 (ICAM-1) and very late antigen-4 (VLA-4) upon immunization (average % stimulated cells 54.9 vs. 20.4, $P < 0.05$ for ICAM-1 and 31.1 vs. 6.6, $P < 0.01$ for VLA-4) and challenge, especially at day 6 (83.5 vs. 45.6, $P < 0.01$) for ICAM-1 and day 10 (50.4 vs. 20.8, $P < 0.05$) for VLA-4. Thereafter, both adhesion molecules declined at day 28 through 90. Similarly, lymphoproliferation was manifested upon immunization ($OD_{570-630}$ 0.27 vs. 0.09, $P < 0.01$) and challenge at day 6 (0.5 vs. 0.17, $P < 0.01$) through day 10 (0.49 vs.0.18, $P < 0.05$), then declined at day 28 through 90. Moreover, sera of Ab2-immunized mice exhibited an anti-anti-Ids (Ab3) reactivity against antigens of approximate molecular weight 40, 80 and 160 kDa of adult worms, which were also recognized by Ab1. However, in contrast to Ab1, Ab3 showed no surface binding to 3 hr schistosomula. Strikingly, mice immunized with Ab2 showed strong resistance to challenge infection (~ 82% reduction in worm burden, $P < 0.001$). Taking all, this alternative vaccine development strategy appears to filter out non-protective antigens. Indeed Ab3 recognizes much fewer numbers of antigens, which passed through two rounds of immune regulation. These antigens appear to represent a significant proportion of the protective response in the gamma-irradiated cercariae vaccine and human resistance model as well, providing the basis for an alternative vaccine for schistosomiasis.

Schistosomiasis is a chronic parasitic disease caused by blood flukes of the genus *Schistosoma*. More than 207 million people worldwide are infected, with an estimated 700 million people at risk in 74 endemic countries (Bergquist, 2008). Chemotherapeutic campaigns is the only containment measure available, however, the development of a vaccine would be an effective long-term option for future control of the disease due to the emerging drug resistance and the inability of chemotherapy to protect against reinfection (Bergquist *et al*., 2002; Kasinathan *et al*., 2010). Although several antigens have been identified as schistosome vaccine candidates, none of such antigens reached the level close to sterile immunity seen after vaccination with irradiated cercariae (Kouriba *et al*., 2010). However, the use of anti-idiotypic antibodies (Ab2) mimicking protective antigens may help reaching higher levels of resistance to the parasite and define a novel mix of protective antigens selected on the basis of their manipulation via idiotypic network regulatory circuits (Eichmann, 2008).

According to idiotypic network theory, as suggested by Jerne (1974) and later supported

by experimental results (Shoenfeld, 2004), idiotypes (Ab1) are unique amino acid sequences located in the variable region of the antibody. They direct antigen binding but also act as antigenic determinants for a second set of antibodies termed Ab2, to which a third set of antibodies (Ab3) are induced and so on. Interactions of these complementary antibodies are intimately involved in regulation of the immune response (Kaufmann, 2004).

Active specific immunotherapy with Ab2 represents a promising approach for management of a number of autoimmune diseases, cancers, inflammations, allergies, viruses and other pathologies including; Guillain-Barré syndrome (Usuki *et al*., 2010); autoimmune central nervous system (CNS) demyelination (von Budingen *et al*., 2010), nasopharyngeal carcinoma (Wang *et al*., 2010), breast cancer (Guthmann *et al*., 2006; Alvarez-Rueda *et al*., 2009), lymphomas (Alvaro *et al*., 2010), ovarian cancer (Chang *et al*., 2008), osteosarcoma (Pritchard-Jones *et al*., 2005), HIV (Mader and Kunert, 2010) and prion disease (Colja Venturini *et al*., 2009) and schistosomiasis (Velge-Roussel *et al*., 1991; Nyindo *et al*., 1995; Abdeen *et al*., 2003; Feng *et al*., 2001; 2004).

Ab2 vaccination offers some advantages over antigen-based or recombinant subunit vaccines since it can induce both humoral and cellular immune responses (Saha and Chatterjee, 2010) even when the critical epitopes required are carbohydrates or lipids (Reagan *et al*., 1983; McNamara *et al*., 1984). In addition, it is capable of overcoming the immunogenetic barriers that strongly regulate human resistance to schistosomiasis, which may result in lowered immune responses causing severe clinical disease (Dreesman and Kenedy, 1985; Marriott *et al*., 1987).

On the other hand, the potentiation of the immunological response require accumulation of inflammatory cells and induction of several interdependent processes, such as cell-cell and cell-extracellular matrix (ECM) adhesion, cell signaling and cytokine secretion (Makgoba *et al*., 1989; Springer, 1991). Among the molecules mediating adhesive cellular events are intercellular adhesion molecule-1 (ICAM-1, CD54) and very late antigen-4 (VLA-4, CD49d/CD29). ICAM-1 affinity for *Plasmodium falciparum*-infected erythrocytes and its role as the binding site for entry of human rhinovirus (HRV) suggested a possible implication in infectious diseases (Bella *et al*., 1998; Chakravorty *et al*., 2005). Likewise, VLA-4 has been suggested to have a role in experimental leishmaniasis (Stanley *et al*., 2008).

In schistosomiasis, ICAM-1 and/or VLA-4 play an important accessory role in inflammatory cytokine production (Wilson *et al*., 1999). They are implicated in periportal fibrosis in advanced human schistosomiasis mansoni (Ellis *et al*., 2009) and correlate with schistosome granuloma formation in the murine model (Jacobs and Van Marck, 1998; Jacobs *et al*., 1998; el-Ahl *et al*., 2000; Hassanein *et al*., 2001). Furthermore, parasite-induced suppression of ICAM-1 and VLA-4 may help the parasite for immune evasion (Ramaswamy *et al*., 1997; Trottein *et al*., 1999) and contrarily up-regulation of ICAM-1 and VLA-4 is suggested to induce a protective immunologic response (Abdeen *et al*., 1999; Abdeen & Kamal, 2003).

Yet, promising models of resistance against schistosomiasis included the irradiated cercarial vaccine and natural human resistance. Radiation attenuated-cercariae induce the highest levels of protection that reach up to 90% reduction of *S. mansoni* (Reynolds and Harn, 1992), and 99% reduction of *S. haematobium* challenge infections in mice (Dean *et al*., 1996). However, human acquired immunity has been evidenced after prolonged exposure to dying schistosome worms, but requires several years

of experience to infection (Black *et al*., 2010). Based on these models, most of the candidate antigens were identified from crude humoral responses leading to selection of tens of antigens that engendered, in most cases, disappointing resistance results.

The present study investigates the utility of Ab2 mimicking protective moiety of adult worm antigens shared between gamma irradiated-cercariae vaccine and resistant humans, to protect against experimental schistosomiasis haematobium in mice. Such merge may help dissecting different immune responses as putative effector mechanisms and selecting for potentially protective antigens that passed rounds of immunoregulation via idiotypic networks. Spleen T cell expression of ICAM-1 and VLA-4, lymphoproliferation as well as humoral responses will be investigated.

Materials and Methods

Reagents

All reagents were supplied from Sigma, St. Louis, MO, USA unless otherwise noted.

Animals and Parasitic Materials

All host and parasite materials were supplied from Biologicals Production Unit (BPU), Theodore Bilharz Research Institute (TBRI, Cairo, Egypt) including six weeks old female C57BL/6 (C57) mice, two months old male New Zealand white rabbits, cercariae and adult worms of *S. haematobium*. All animal experiments were performed according to the guidelines of the National Research Council (NRC, 1997) for laboratory animal use.

Antigen Preparation

Soluble antigens of adult worms (SWAP) were prepared by homogenization in ice-cooled 20 mM Tris-HCl buffer containing 2 mM phenylmethyl-sulfonyl fluoride (PMSF), pH 7.2 using Teflon glass homogenizer for 30 min (Boctor and Shaheen, 1986). Antigen preparation was clarified by centrifugation at 50000 × g for 60 min at 4°C using a Sorvall RC 5 series super speed centrifuge. The supernatant was collected, total protein content was determined by Lowry's method (Lowry *et al*., 1951) and stored at -70°C until used.

Human and Rabbit Sera, and Preparation of Anti-Idiotypic Antibodies (Ab2)

After obtaining informed consent from all participants, chronic human sera (CHS) were collected from 43 patients, aged 18 to 37 years, presumed resistant to schistosomiasis haematobium reinfection (i.e. with no detectable eggs in stool in spite of continuous exposure to infection), according to duplicate Kato-Katz thick faecal smears (Kato and Tazaki, 1967). They were diagnosed free of any concomitant viral hepatitis B, C infections, giardiasis or amoebiasis. They all reported a history of anti-schistosomal treatment with praziquantel. Normal human serum (NHS) samples were collected from 20 age-matched individuals with no history of schistosomiasis or other helminthic infections. Vaccinated rabbit sera (VRS) included that pooled from two New Zealand white rabbits multiply immunized with 10,000 *S. haematobium* gamma-irradiated cercariae (4 times at monthly interval). Cercariae were attenuated at 20 krad and used for immunization according to the method of Reynolds and Harn (1992). Normal rabbit sera (NRS) included that pooled from two age and sex matched New Zealand white rabbits that received no immunizations.

Ab2 was immunoaffinity purified from VRS, using adult worm-specific idiotypes (Ab1) purified from CHS against SWAP. Ab2 was extensively depleted (4 times) over normal human immunoglobulins (NHIgs) affinity column to remove all isotypic and allotypic antibodies that might present with Ab2 preparation. NHIgs were prepared from NHS by 80% saturated ammonium sulphate precipitation according to the method of Ahlstedt *et al*. (1973). All affinity chromatography procedures were carried out using packed Cyanogen bromide-activated Sepharose 4B affinity column (Pharmacia Fine Chemicals, Upsala, Sweden) according to manufacturer's manual. Full characterization of Ab1 and Ab2 is considered for publication elsewhere.

Mice Immunizations and Challenge Infection

In two independently repeated protection experiments, two groups (25 mice each) of female six weeks old C57 mice were immunized with Ab2 or saline (naive control). Mice were percutaneously injected with 100 µg/animal of Ab2 divided in three equal doses. The initial immunization dose was emulsified (1:2 v/v) in complete Freund's adjuvant (CFA). The second booster immunization (wk 2) was emulsified (1:2 v/v) in incomplete Freund's adjuvant (IFA), whereas the third booster immunization (wk 4) was in saline. Two weeks after the last immunization, animals of both groups were percutaneously challenged with 500

normal *S. haematobium* cercariae. Three animals from each group were sacrificed one day prior to challenge and at days 6, 10, 28 and 90 after challenge. Sera and splenocytes were collected at the different time points. At day 90 post-challenge, adult worms were recovered from the hepatic portal system and liver according to the method of Smithers and Terry (1965). The number of worms recovered from the immunized group was compared with controls, and protection expressed as a percentage according to the formula: % protection = (C-I)/C × 100

Where, C is the mean number of worms in the control group and I is the mean number of worms in the immunized group.

Spleen Cell Preparation

Splenocytes were collected individually from three mice of each group and cultured as previously described by Chikunguwo *et al*. (1991) in complete RPMI 1640 (Gibco BRL, MO) medium supplemented with 2 mM L-glutamine, 4.2 mM $NaHCO_3$, 100 Units/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, 0.2 mM non-essential amino acids, 20 mM HEPES and 10% heat-inactivated fetal calf serum. T cell enrichment was performed with packed nylon wool (Cellular Products, Buffalo, NY). Erythrocytes were lysed with Tris ammonium chloride buffer, pH 7.2, for 15 minutes on ice. Cells that excluded trypan blue were resuspended in 1×10^6 cells per ml.

Flow Cytometric Analysis

Spleen cells were cultured in medium with or without 10 µg/ml of lipopolysaccharide of *Shegella* (LPS), Ab2 or SWAP in 37°C, 5% CO_2. After 6 hr culture, the cells were harvested and washed twice in fluorescence-activated cell sorting (FACS) buffer (PBS containing 0.1% BSA and 0.01% NaN_3) and incubated at 4ºC for 15 min in FACS blocking buffer (FACS buffer containing 1 µg/ml rat IgG $_{2b,}\kappa$, clone 2.4G2) for inhibition of nonspecific antibody binding. Cells were then incubated on ice for 30 min in dark with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD54 (ICAM-1, clone 3E2), FITC-conjugated hamster IgG anti-TNP (ICAM-1 isotype control, clone G235-2356), FITC-conjugated anti-mouse CD49d (Integrin α_4 chain = VLA-4, clone R1-2) and FITC-conjugated Rat IgG $_{2b,}\kappa$ (VLA-4 isotype control, clone A95-1). Monoclonal antibodies were optimally diluted in FACS blocking buffer following BD PharMingen's staining protocol. Labeled cells were washed twice in FACS buffer, fixed for 1 hr in 1% paraformaldehyde, then washed twice again with FACS buffer and acquired on flow cytometer. Cell acquisition and analysis of data were carried out using PC-Lysis [TM] software version 1.0 [9/92] (Becton Dickinson). Unstained cells and cells stained with irrelevant isotype-matched antibodies were included as controls to assess the amount of nonspecific staining. Spleen cells were defined and gated based on forward and right angle light scatter characteristics "lymphocyte gate" (Fig. 1). The specificity of the test was controlled by the lack of expression in the absence of stimulus. Accordingly, the background expression of unstimulated cells was subtracted from their respective stimulated cells. All assays were performed in triplicates and experiments were independently repeated twice. Average percent stimulation was calculated based on the mean ± SD.

Lymphoproliferation Assay

Splenocytes prepared for adhesion molecule measurements were also used for lymphocyte proliferation assays. Lymphoproliferation assay was performed according to the method of Mosmann (1983) using CellTiter 96TM non-radioactive cell proliferation assay kit (Promega). After optimization of spleen cell number and stimulus concentration, 100 µl of 1×10^6 cells per ml was incubated in the presence or absence of 5 µg/ml of LPS, Ab2 or SWAP diluted in culture medium. Cells were incubated for three days at 37°C, 5% CO2 in water-jacketed incubator (Jouan IG 150). After culture, 15 µl of the dye solution were added to each well and the plates were incubated for 4 hours at 37°C, 5% CO2. 100 µl of the solubilization/stop solution was added to each well and plates were allowed to stand overnight at room temperature to completely solubilize the formazan crystals. Absorbance values were recorded at 570 nm using ELISA reader (GDV, Roma, Italy). A reference wavelength of 630 nm was used to reduce the background contributed by cell debris and other nonspecific absorbance. The specificity of the proliferative response was controlled by the lack of response in the absence of stimulus. Accordingly, the background proliferation of unstimulated cells was subtracted from their respective stimulated cell proliferation. All assays were performed in triplicates and experiments were independently repeated twice.

Western Blotting Analysis

100 µg of SWAP were separated on 12% SDS-PAGE under denaturing condition and transferred to nitrocellulose (NC) sheets according to the method of Towbin *et al*. (1979). Primary antibodies included; 10 µg of Ab1 and 1:100 diluted immunized mouse sera (IMS), normal mouse sera (NMS), collected before challenge, and their corresponding sera after challenge

($IMS_{challenged}$ and $NMS_{challenged}$). Secondary antibodies included alkaline phosphatase labeled polyvalent goat anti-human (IgG, IgM, IgA and IgE) and anti-mouse (IgG and IgM) conjugates, diluted to 1:1000. Molecular weights (MWs) were determined in comparison to broad range protein MW standards (BioRad). Gel Analyzer 1.0 software was used to measure the MWs.

Indirect Immunofluorescence Assay (IIF)

IIF assay was performed on formalin-fixed mechanically transformed 3 hr schistosomula of *S. haematobium* according to the method of Gregoire *et al*. (1987). Mechanical transformation was carried out according to the method of Lazdins *et al*. (1982). Primary antibodies included 10 µg of Ab1 and 1:50 diluted IMS and NMS. Secondary antibody used was 1:128 diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (IgG and IgM) polyvalent conjugate. Fluorescence was examined using Leitz fluorescence microscope. Each experiment was repeated three times independently.

Statistical Analysis

All statistical analyses were performed using SPSS 13 statistical software. A significant level of 0.05 at 95% confidence interval was chosen. Independent student's *t*-test was used to determine the statistical significance of resistance to challenge infection. However, one-way ANOVA test was used to determine the statistical significance of changes in the expression of adhesion molecules and lymphoproliferation. In addition, multiple regression analysis was used to determine the relationship between lymphoproliferation and expression of adhesion molecules in one hand and protection on the other hand.

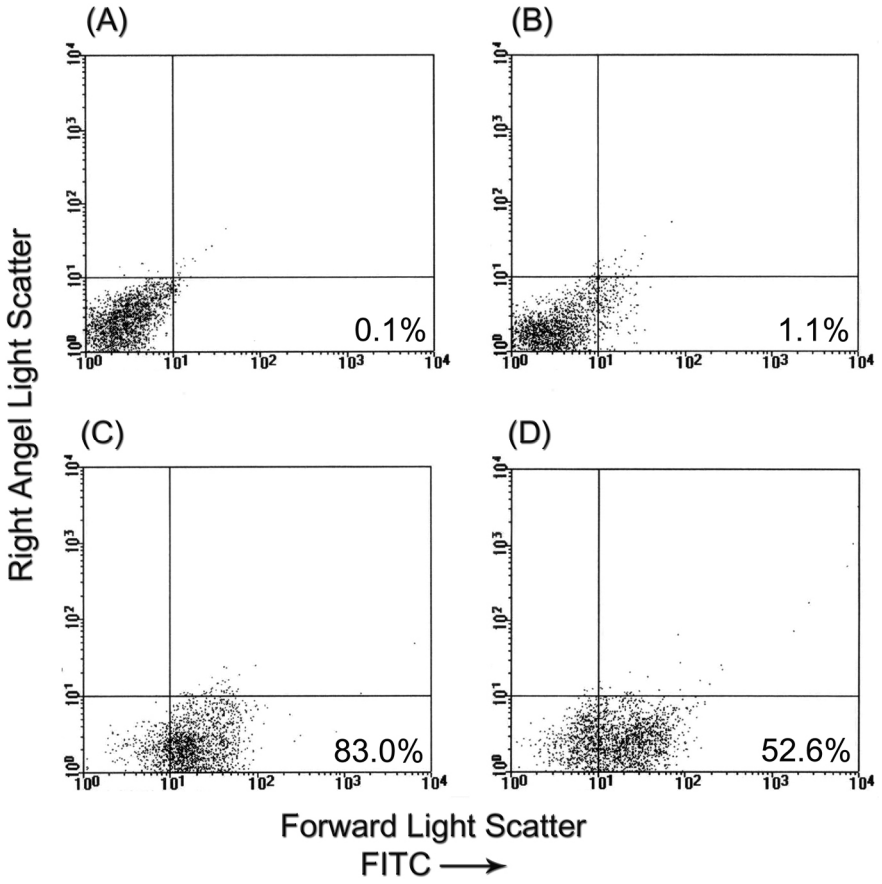

Figure 1. One-parameter dot plot to identify adhesion molecules expressing cells; plots are representatives of (A) unstimulated cells, (B) isotype control-stained cells, (C) Ab2- stimulated cells stained for ICAM-1 and (D) Ab2-stimulated cells stained for VLA-4, at the time of maximum expression.

Results

Splenocyte Expression of ICAM-1 and VLA-4

Splenocytes were obtained from tested mice groups one day prior to challenge and after challenge at days 6, 10, 28 and 90. Results in figure (2) showed that splenocytes of Ab2-immunized mice could be stimulated *in vitro* to express both ICAM-1 and VLA-4, not only by Ab2, to which the cells were primed *in vivo*, but also by SWAP and LPS. The fluorescence intensity obtained following *in vitro* stimulation with LPS was the highest among other stimuli with no significant difference between the different time points.

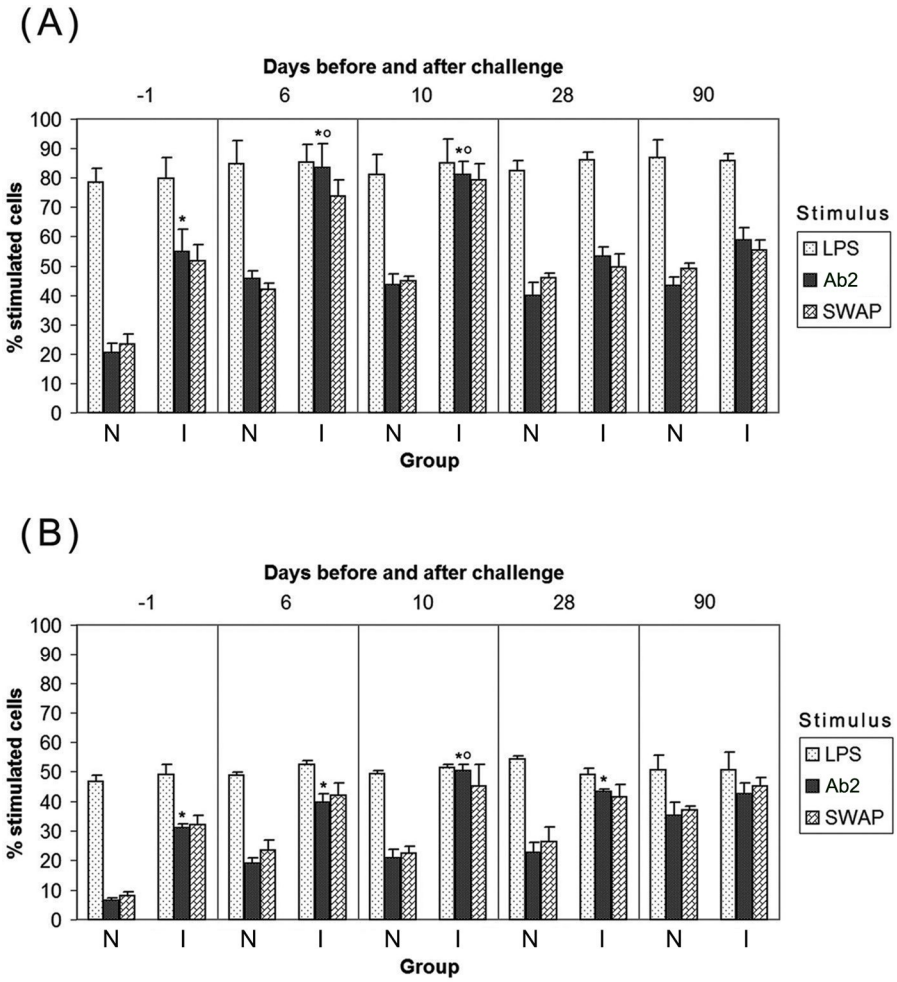

Figure 2. Splenocyte expression (average % stimulated cell ± SD) of (A) ICAM-1 and (B) VLA-4. Histograms are representing the mean of triplicate assays in two experiments repeated independently with standard deviations shown in bars.
NOTE: N = naive mice, I = Ab2-immunized mice.
* Significant or $P < 0.05$, compared to naive controls.
^{o} Significant or $P < 0.05$, compared to its corresponding level before challenge.

Concerning Ab2-driven expression, splenocytes of Ab2-immunized mice showed high levels of expression of both adhesion molecules before challenge (average % stimulated cells 54.9 vs. 20.4, $P < 0.05$ for ICAM-1 and 31.1 vs. 6.6, $P < 0.01$ for VLA-4), compared to naive controls. However, the expression of both adhesion molecules continued to increase after challenge and maximize at day 6 for ICAM-1 (83.5 vs. 45.6, $P < 0.01$) and day 10 for VLA-4 (50.4 vs. 20.8, $P < 0.05$). Thereafter, both adhesion molecules declined at day 28 through day 90 in levels comparable with controls, however, to lower levels with ICAM-1. On the other hand, SWAP-induced expression of both adhesion molecules followed somehow the kinetics of Ab2-induced expression.

In general, ICAM-1 expression was higher than that of VLA-4. Moreover, the expression of adhesion molecules in Ab2-immunized mice was higher than that of controls, especially during the time points: before challenge, days 6 and 10 for ICAM-1 and before challenge, days 6, 10 and 28 for VLA-4.

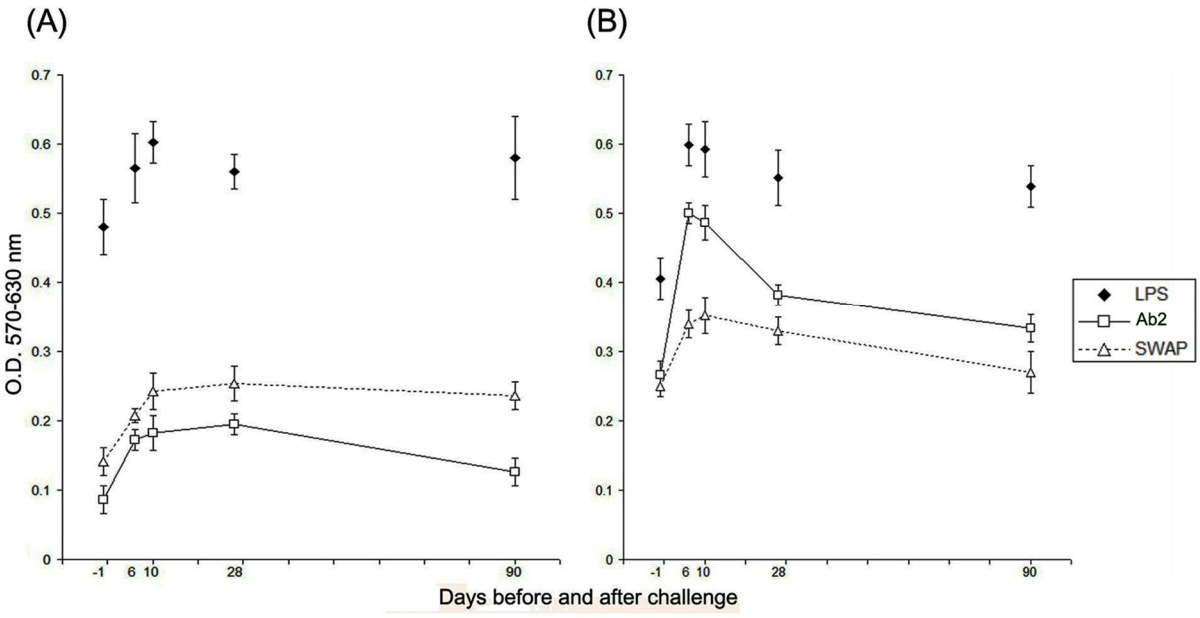

Figure 3. Lymphoproliferative responses of splenocytes from (A) naive and (B) Ab2-immunized mice, before and after challenge infection with *S. haematobium* normal cercariae. Stimuli included lipopolysaccharide of *Shegella* (LPS) as a mitogen, Ab2 and SWAP for specific stimulation. Points are the mean of triplicate assays in two experiments repeated independently with standard deviations shown in bars.

Lymphoproliferative Consequences of Ab2 Immunization

Before challenge, splenocytes of Ab2-immunized mice showed diminished LPS-driven proliferation, compared to controls. After challenge, LPS-driven responses increased on day 6 and sustained in the subsequent days. Generally, mitogen-induced responses were the most pronounced among other responses.

On the other hand, Ab2- and SWAP-induced lymphoproliferation showed

somehow similar kinetics with increased responses upon immunization and challenge, compared to controls. However, Ab2-driven response showed enhanced levels, especially on day 6 ($OD_{570-630}$ 0.5 vs. 0.17, $P < 0.01$) and day 10 (0.49 vs.0.18, $P < 0.05$) post-challenge, and then declined later.

Relationship between Expression of Adhesion Molecules, Lymphoproliferation and Protection

In two independently repeated protection experiments, Ab2-immunized mice showed a significant ($P < 0.001$) reduction of worm counts of ~ 82%, compared to controls. Multiple regression analysis revealed direct relationship between levels of adhesion molecules expressed on splenocytes of and protection gained in individual mice at day 90 (regression coefficient or $\beta = 0.87$, $P < 0.01$) for ICAM-1, ($\beta = 0.81$, $P < 0.05$) for VLA-4 and ($\beta = 0.79$, $P < 0.05$) for the lymphoproliferation (Table 1).

Table 1. Correlation of splenic T lymphoproliferative responses and expression (average % Ab2-stimulated cells) of ICAM-1 and VLA-4 with protection.

% Protection (*P*-value)		Regression coefficient (β) (*P*-value)
82.3 ($P < 0.001$)	ICAM-1	0.87 ($P < 0.01$)
	VLA-4	0.81 ($P < 0.05$)
	Proliferation	0.79 ($P < 0.05$)

Data were considered significant at $P < 0.05$.

Reactivity of Ab3 in Sera of Ab2-immunized Mice

Ab3 in immunized mouse sera (IMS) recognized SWAP antigenic bands, which were also recognized by Ab1 (Fig. 4). Ab3 reactivity included three bands of approximate MW of 40, 80 and 160 kDa and other faint bands of approximate MW of 110, 99, 95 and 60 kDa. However, Ab1recognized also a broad range of antigens in the range of 22.6 to > 200 kDa.

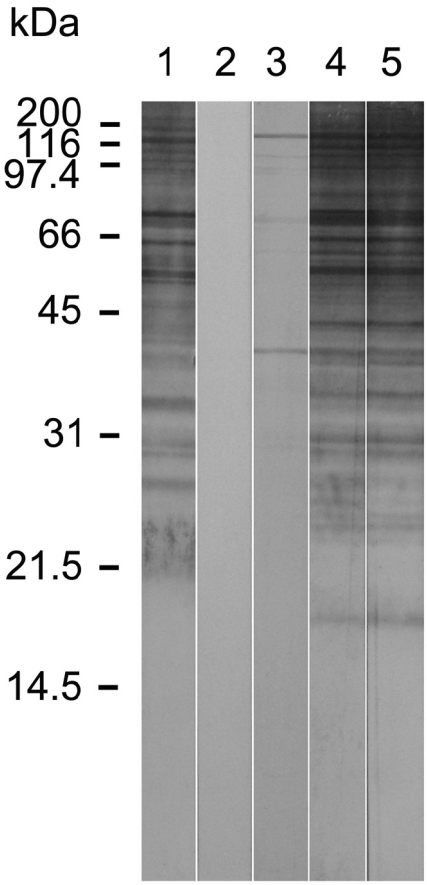

Figure 4. Western blotting analysis of (1) Ab1, (2) NMS, (3) IMS, (4) $NMS_{challenged}$ and (5) $IMS_{challenged}$ against SWAP. Secondary antibodies included (1) anti-human and (2, 3, 4, 5) anti-mouse polyvalent conjugates.

Comparison of the reactivity of sera from naive and Ab2-immunized mice after challenge ($NMS_{challenged}$ and $IMS_{challenged}$, respectively) revealed that both recognized a similar broad range of antigens in the range of 18 to > 200 kDa, except for the disappearance of the bands of approximate MW of 27 and 25.5 kDa in $IMS_{challenged}$.

Furthermore, IIF assay was used to determine the binding of Ab3 to the surface of *S. haematobium* 3 hr schistosomula. Ab3 showed no surface binding to 3 hr schistosomula, compared to a moderate and uniform surface binding for Ab1. Image

analysis (Pixcavator IA 5.0) of IIF revealed an average fluorescence intensity of 43 for Ab3, compared to 166 and 41 for Ab1 and NMS, respectively (Fig. 5).

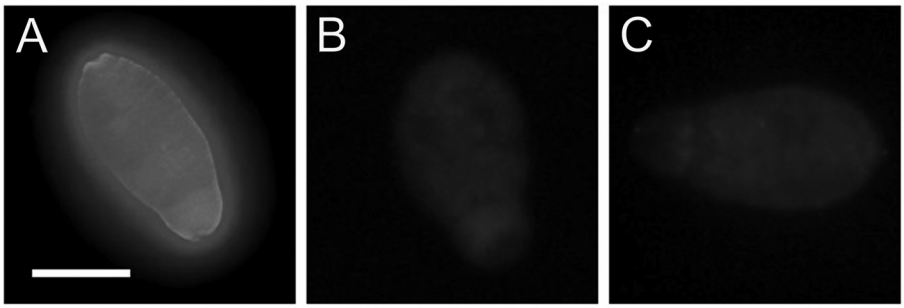

Figure 5. Surface-binding of Ab3 to formalin-fixed 3 hr schistosomula of *S. haematobium*. The assay included (A) Ab1, (B) Ab3 and (C) NMS. The bar is representing 50 µm.

Discussion

The currently available schistosome vaccine antigens were identified and isolated empirically using attenuated larvae, protective monoclonal antibodies, or by analysis of antibody and cytokine responses of naturally resistant humans (Driguez *et al*., 2010). Unfortunately, most of the identified vaccines may lack the required efficacy (McManus and Loukas, 2008). A possible insufficient antigen formulation or presentation, and a negative impact conducted by the immunoevasive strategies of the parasite have been suggested (Pearce and MacDonald, 2002; Bergquist *et al*., 2005). Nevertheless, the research area is still open for the use of other protective antigens, newly developed immunization techniques that may help optimization of the immune response and reach higher levels of resistance.

The present study used adult worm-specific idiotypes (Ab1) of presumed resistant individuals who were treated and estimated to be negatively egg passers in spite of their continuous exposure to contaminated water, to select for anti-idiotypes (Ab2) from gamma-irradiated cercarial vaccine. Ab1 was selected due to it recognition to antigens possibly following worm death upon chemotherapeutic treatment and presumed to be implicated in resistance against reinfection. Ab2 was purified from sera of New Zealand white rabbits multiply immunized with gamma-irradiated cercariae of *S. haematobium* and used for immunization of C57BL/6 mice. Consequences of immunization including splenic T cell expression of intercellular adhesion molecule-1 (ICAM-1) and very late antigen-4 (VLA-4), lymphoproliferative responses, as well as anti-anti-Ids (Ab3) reactivity were assessed, before and after challenge infection with normal *S. haematobium* cercariae. Investigated time points included one day prior to challenge (six weeks post initial-immunization) and days 6, 10, 28 and 90 post-challenge, corresponding to the end of immunization, beginning and end of lung stage, liver and egg depositing stages of the parasite.

To ensure better selection for the maximum number of antigenic Ab2 from sera of immunized rabbits, polyclonal Ab1 recognizing a broad range of adult worm antigens from 22.6 to > 200 kDa were used (Fig. 4). It seems that Ab1-recognized 22.6 kDa antigen is an analogous to one long been

known to be associated with resistance to reinfection (Fitzsimmons *et al*., 2004). It is expressed primarily in the adult worm and to which IgE levels increased after treatment of infected individuals (Fitzsimmons *et al*., 2007). After cloning of the recombinant *S. mansoni* tegument allergy like (SmTAL-1, formerly Sm22.6), it has been hypothesized that this antigen is one of the antigens sequestered during active infection and released upon worm death (Webster *et al*., 1996).

In the present study, splenocytes of Ab2-immunized mice showed, before challenge, a significant increase of Ab2-induced expression of ICAM-1 (average % stimulated cells 54.9 vs. 20.4, $P < 0.05$) and VLA-4 (31.1 vs. 6.6, $P < 0.01$), compared to naive controls. This may indicate the involvement of both cell-cell and cell-extracellular matrix (ECM) interactions with the elicitation of immune responses upon immunization. However, after challenge, Ab2-immunized mice showed another significant up-regulation of both ICAM-1 and VLA-4 that peaked by day 6 for ICAM-1 (83.5 vs. 45.6, $P < 0.01$) and day 10 for VAL-4 (50.4 vs. 20.8, $P < 0.05$), compared to naive challenged controls. This may indicate a possible role for ICAM-1 and VLA-4 in the early and late phases of immune response during parasite residence in the lungs. Thereafter, both adhesion molecules declined to some extent at day 28 through day 90.

On the other hand, Ab2-induced lymphoproliferative responses somehow coincide with adhesion molecule profiles. They showed a significant increase upon immunization ($OD_{570-630}$ 0.27 vs. 0.09, $P < 0.01$) and challenge, however, to higher levels after challenge, especially during day 6 (0.5 vs. 0.17, $P < 0.01$) and day 10 (0.49 vs.0.18, $P < 0.05$). Thereafter, they declined in days 28 through 90 (Fig. 3). Furthermore, multiple regression analysis revealed significant direct relationship between expression of adhesion molecules and lymphoproliferation in one hand and protection on the other hand. Such relationship agrees with previous studies reported an important accessory role for ICAM-1 and/or VLA-4 in lymphocyte activation (Lejoly-Boisseau *et al*., 1999; Wilson *et al*., 1999; Stavitsky and Xianli, 2002), proliferation (Langley and Boros, 1995) and consequently, induction of protective immunity (Abdeen *et al*., 1999; Abdeen & Kamal, 2003).

Up-regulation of cellular responses of immunized mice during early infection may indicate a possible role of the investigated adhesion molecules in activation of Th1 cell subset that orchestrate the response during these times (Pearce and MacDonald, 2002). Conversely, the slight down-regulation of the investigated cellular responses during days 28 and 90 could be owing to the parasite's immunoevasive mechanisms that flourish upon maturation, mating and egg laying (Ramaswamy *et al*., 1997; Trottein *et al*., 1999; Tong *et al*., 2004). This was evidenced more in controls since they had higher burden of mature parasites.

Moreover, sera of Ab2-immunized mice exhibited specific Ab3 response, which was also recognized by Ab1 including antigens of approximate molecular weight 40, 80 and 160 kDa (Fig. 4). These antigens are probably not expressed on the surface of 3 hr schistosomula as judged by null indirect immunofluorescence activity (Fig. 5). Interestingly, mice immunized with Ab2 showed strong resistance to challenge infection (~ 82% reduction in worm burden, $P < 0.001$).

In Ab2-immunied mice, the elevation of T cell splenic responses during lung stage parasite suggested that not only T cells recruited in the lungs are called in action but also T cells of the spleen far from the site of attrition. Together with the negative binding

of Ab3 to 3 hr schistosomular surface, this may indicate that exacerbated antigens during lung stage may belong to the excretory-secretory (E-S) products of the parasite and elevation of splenic T cell response at this time may indicate parasite destruction in the lungs of Ab2-immunized mice since naive challenged controls showed no such elevation. Indeed spleen and liver are principally implicated in handling E-S antigens (Burke *et al*., 2010) that have been suggested as good candidates for vaccine development (El Ridi *et al*., 2010).

Unlike most of the previous schistosomiasis vaccine approaches that used Ab1 to select for antigens, the current study used Ab3 for definition of potentially protective antigens. Since Ab2 can recognize antigenic determinants in an antibody's combining site that was in contact with the original antigen, the binding site of the Ab2 could carry an internal image of the antigen (Hantusch *et al*., 2006). This antigen selection strategy is suggested to be advantageous since immune response has encompassed two rounds of immunoregulation and probably selection and maturation via Ab1/Ab2 regulatory circuits. Indeed, immunoregulation is central for successful management of schistosomiasis (Kojima, 1997; Montesano *et al*., 2002; Yazdanbakhsh and Sacks, 2010); a concept evidenced in most (> 90%) infected individuals in endemic areas who seem to successfully regulate their responses over time to prevent severe morbidity during infection (Pearce and MacDonald, 2002).

In conclusion, the findings together indicated that Ab2 could mimic, at the humoral and cellular levels, the protective properties of antigens shared between irradiated-cercariae vaccine and human resistance model, priming T cells for expression of adhesion molecules and lymphoproliferation, and inducing humoral responses, all needed for optimization of the immune response that was evidenced by high-level protection. This may provide an alternative approach to select for candidate antigens for the development of a future vaccine against schistosomiasis.

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