Protective Immunity Induced in Mice by F8.1 and F8.2 Antigens Purified from *Schistosoma mansoni* Eggs

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Schistosoma mansoni *soluble egg antigens (SEA) were fractionated by isoelectric focusing, resulting in 20 components, characterized by pH, absorbance and protein concentration. The higher absorbance fractions were submitted to electrophoresis, and fraction 8 (F8) presented a specific pattern of bands on its isoelectric point. Protein 3 was observed only on F8, and so, it was utilized to rabbit immunization, in order to evaluate its capacity of inducing protective immunity. IgG antibodies from rabbit anti-F8 serum were coupled to Sepharose, and used to obtain the specific antigen by affinity chromatography. This antigen, submitted to electrophoresis, presented two proteic bands (F8.1 and F8.2), which were transferred to nitrocellulose membrane (PVDF) and sequenciated. The homology of F8.2 to known proteins was determined using the Basic Local Alignment Search Tool program (BLASTp). Significant homologies were obtained for the rabbit cytosolic* Ca^{2+} *uptake inhibitor, and for the bird* a_1*-proteinase inhibitor. Immunization of mice with F8.1 and F8.2, in the presence of* Corynebacterium parvum *and* $Al(OH)_3$ *as adjuvant, induced a significant protection degree against challenge infection, as observed by the decrease on worm burden recovered from portal system.*

Key words: *Schistosoma mansoni* - egg antigen purification - immunity - vaccine

Schistosoma mansoni infection is characterized by the presence of adult worms in the portal and mesenteric veins as result of a complex migratory cycle, initiated by cutaneous penetration of cercariae, transformed into schistosomula in the skin of an appropriate host (Boros 1989). In mice, the pathology of schistosomiasis is associated with the daily production of viable eggs by the intravascular worms. Many eggs do not reach external environment and cause an inflammatory response when trapped in liver sinusoids, leading to predominantly cell-mediated granuloma formation and ultimately to hepatic fibrosis (Phillips & Lammie et al. 1986). Numerous efforts over the last decade have focused the elaboration of a reliable vaccine able to protect humans against natural infection by *S. mansoni*. It is essential to have available defined, purified parasite antigens, allowing a better understanding of its immunogenic and biological functions (Kambara & Wilson 1990, Hirsch et al. 1997). Some antigens have been purified from parasite eggs, using either conventional purification techniques (Lukacs & Boros 1992, Hirsch & Goes 1996) or affinity chromatography with human monoclonal antibodies (Hirsch et al. 1997). It has been previously demonstrated that fractions of *S. mansoni* soluble egg antigens were able to sensitize and elicit granulomatous hypersensitivity in mice (Harn et al. 1989, Lukacs & Boros 1991). In this study, we report the characterization of *S. mansoni* egg antigens fractionated by liquid isoelectric focusing and their capacity to induce protection against a challenge infection.

MATERIALS AND METHODS

Mice and parasites - Adult female BALB/c mice, purchased from Centro de Bioterismo, ICB, UFMG, Belo Horizonte, Brazil, and maintained under standard laboratory care, were used throughout this study. Cercariae of *S. mansoni* were obtained from *Biomphalaria glabrata* snails, previously infected with miracidia of the L.E. strain, from Belo Horizonte, Brazil. Anesthetized mice (10/group) were exposed to cercariae on the abdomen, using the ring method of Smithers and Terry (1965). Vaccinated and control mice challenged with cercariae were perfused eight weeks after exposure (Smithers & Terry 1965). The protective immunity of each group was evaluated by comparing the recovery of worms from immunized and control mice.

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SEA fractionation - Soluble eggs anigen (SEA) was fractionated by isoelectric focusing (IEF) in a liquid cell (Rotofor Cell, BioRad, Richmond, CA) according to manufacture's instructions. Briefly, 5 mg SEA were diluted tenfold with 3 M urea including a final ampholyte (pH 3-10, Pharmacia, Piscataway, NJ) concentration of 4%. SEA was applied and focused for approximately 500 volts for 3 hr. Subsequently, 20 fractions, designated F1 to F20, were harvested and the pH of each fraction was measured (Fig. 1). To remove ampholytes, fractions were dialysed against phosphate buffer saline (PBS). Protein concentration of each fraction was measured according to Bradford microassay (Bradford 1976).

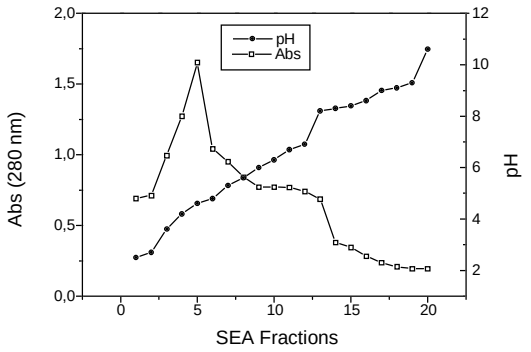

Fig. 1: elution profile of SEA by liquid isoelectric focusing in rotofor cell. 5 mg of SEA were diluted tenfold with 3 M urea including a final ampholyte (pH 3-10) concentration of 4% at 500 volts for 3 hr. This procedure resulted in 20 fractions, characterized by pH, absorbance and protein concentration.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot - Isoeletric fractions separated by SDS-PAGE (Laemmli 1970) were either Coomassie blue stained or electrophoretically transferred to nitrocellulose membrane (BioRad), as described by Towbin et al. (1979).

Production of rabbit anti-serum to Protein 3 - The protein 3 (M.W. of 68 kDa) of F8 fraction (pI 5.8) was excised from SDS-PAGE and used as immunogen. For immunization, Coomassie blue-stained protein was cut of two to three gels, washed with PBS, and homogenized with 0.5 ml of PBS. The antiserum was obtained from rabbits immunized by i.m. injections of sample emulsified with complete Freund adjuvant at a 1:1 ratio, and boosted twice at 2-week intervals with identical antigen amounts but in incomplete Freund adjuvant. Specific rabbit antibodies were coupled to cyanogen bromide-activated Sepharose-4B, and F8.1 and F8.2 proteins were purified as described by Goding (1986).

Amino-terminal sequencing - The SDS-PAGE Protein 3 was electrophoretically transferred to polyvinylidine difluoride membranes (PVDF, 0.2 mm, BioRad) with the transblot apparatus, at a constant current of 50 mA, overnight. Transfers were stained for 1 min with 1% Ponceau S in 10% acetic acid, briefly destained in distilled water, and air-dried. Protein was cut from membranes, and amino-terminal sequencing were performed by Centro Brasileiro de Sequenciamento de Proteínas, Universidade de Brasília, Brasília, DF, Brazil. To determine sequence homology, amino acid sequences were compared to protein sequences in non-redundant data bases, using the Basic Local Alignment Search Tool program (BLASTp) (Altschul et al. 1990), at National Center for Biotechnology Information (NCMI).

Immunization of mice with F8.1 and F8.2 - Groups of five BALB/c mice were immunized by s.c. injections of 10 mg of each F8.1 and F8.2 preparation, in presence of 100 mg of *Corynebacterium parvum* and 1 mg of aluminium hydroxide [$Al(OH)_3$] as adjuvant. Animals were boosted twice, at 2-week intervals, with identical amounts of antigen, but the last injection was done by i.p. without adjuvant.

RESULTS

Fractionation of SEA by IEF and purification of F8.1 and F8.2 by affinity chromatography - In the present study, utilizing two different procedures, we purified F8.1 and F8.2 from *S. mansoni* egg antigens. Results presented in Fig. 1 depict the fractionation of SEA utilizing liquid isoelectric focusing in a Rotofor Cell system. This procedure resulted in 20 fractions, characterized by pH, absorbance and protein concentration. The higher absorbance fractions were submitted to electrophoresis, and fraction 8, denominated F8, pI 5.8, presented a specific pattern of bands on its isoeletric point. Protein 3 was observed only on F8, and so, was utilized to rabbit immunization.

Immunoaffinity chromatography was applied to purify Protein 3 from F8, prepared in the absence of protease inhibitors, using specific rabbit antibodies coupled to Sepharose-4B. The purified antigen, submitted to SDS-PAGE and Western blot analysis, revealed to have two proteic bands, denominated F8.1 and F8.2, with respectively M.W. of 70 kDa and 68 kDa (Fig. 2). The amino acid sequence data obtained for 20 amino-terminal residues of F8.2 protein are presented in Table. This partial amino acid sequencing indicated strong sequence homology to rabbit cytosolic Ca^{2+} uptake inhibitor antagonist and to a 1 - proteinase inhibitor (100% homology).

Immunization of mice with F8.1 and F8.2, and their effect on a subsequent infection with S. mansoni cercariae - The protective effect of immunization with F8.1 and F8.2, in the presence of *C. parvum* and $Al(OH)_3$ as adjuvant, was additionally investigated in mice. As shown in Fig. 3, the number of worms, recovered eight weeks after challenge infection of immunized animals, was significantly reduced when compared to that observed on adjuvant-immunized or not-immunized control mice. Immunization with F8.1 and F8.2 established a protection of 29% and 35%, respectively.

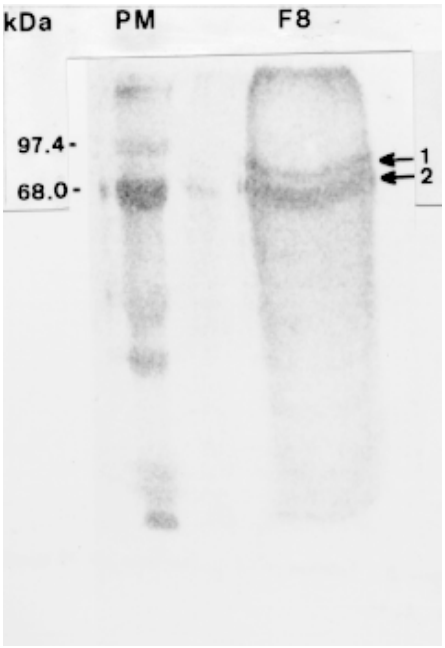

Fig. 2: SDS-PAGE of *Schistosoma mansoni* egg antigen affinity purified Protein 3. Samples (F8) and molecular weight marker (PM) were applied to 10% SDS-PAGE under reducing conditions, followed by Coomassie blue stain. Two separated proteins were evidenciated: F8.1 and F8.2.

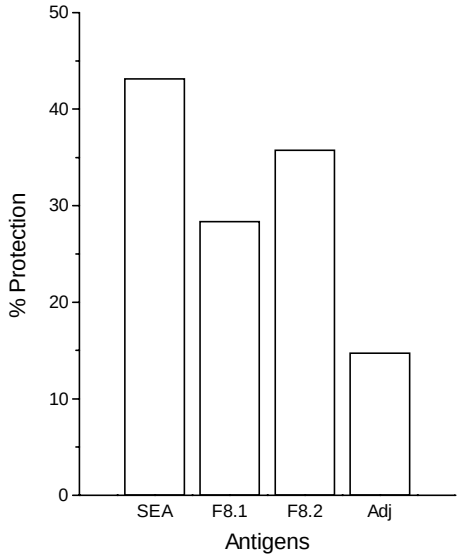

Fig. 3: protective effect of F8.1 and F8.2. BALB/c mice were immunized with 10 mg of each antigen, in presence of *Corynebacterium parvum* and $Al(OH)_3$, as described on Materials and Methods. Worm burden was determined by perfusion of portal system, at eight weeks after challenge infection, in five mice/group. % protection = 100 - experimental/control x 100.

TABLE

Protein sequence homology between F8.2 of *Schistosoma mansoni* and other species

Homology[a]	Animals	Similarity (%)	Identity (%)	Size (aa)	Score	Probability
Antagonist protein, cytosolic Ca^{2+} uptake inhibitor antagonist/serum albumin homolog	rabbits	100	100	13	69	0.00014
a_1-proteinase inhibitor	birds	100	83	12	59	0.0043
70 kDa seizure activity-linked albumin-like glycoprotein	rats	92	84	13	60	0.0047
TB66=66kda seroreactive protein	*Mycobacterium tuberculosis*	92	83	14	56	0.017
Serum albumin (fragment)	human	92	76	13	59	0.0054
49 kDa protein (cornea peptide)	rats	92	76	13	59	0.0062

a: N-Terminal sequence of F8.2: E A H K S E I A H R F N D V Q X E H F I G L V L
This protein was compared to protein sequences deposited in non-redundant databases, using the Basic Local Alignment Search Tool program, BLASTp.

DISCUSSION

In this study we identified immunogenic components of *S. mansoni* egg antigen preparation essential to the formulation of a schistosomiasis vaccine. In order to conduct this issue we used two approaches to obtain purified antigens: isoelectric focusing and affinity chromatography.

The technique of liquid isoelectric focusing has many features which make it a valuable tool in our search for antigens involved in the expression of protective immunity to schistosomes (Mountford & Wilson 1993). It permits fractionation of relatively small quantities of soluble protein mixtures, with a recovery rate greater than 90%. The system has the capacity to fractionate complex antigen preparations such as SEA, which encompass a wide range of protein sizes into 20 samples, each one containing a slightly different range of proteins. The eluted proteins are in aqueous form, in small volume, and at a relatively high concentration. In this system, we were able to identify Protein 3 only on fraction 8, and so, it was utilized to rabbit immunization.

Another procedure utilized for purification of Protein 3 involved affinity chromatography using rabbit anti-Protein 3 IgG coupled to Sepharose. The analysis of SDS-PAGE purified antigen showed that Protein 3 was constituted by two antigens, F8.1 and F8.2, with respectively M.W. of 70 kDa and 68 kDa. Amino acid sequence data obtained for the 20 amino-terminal residues of F8.2 protein showed a 100% homology with a rabbit antagonist protein, cytosolic Ca^{2+} uptake inhibitor antagonist (Xu & Narayanan 1994), and with a_1-proteinase inhibitor (Kuhn et al. 1994).

The role of F8.1 and F8.2 as protective antigens was supported by mice immunization. Three successive immunizations, in the presence of *C. parvum* and $Al(OH)_3$, gave a significant level of resistance to subsequent cercarial challenge. Several groups have demonstrated the potential vaccinating effect of different *S. mansoni* antigens and obtained various levels of protection. Smith and Clegg (1985) vaccinated mice with two schistosome surface antigens of 155 and 53 kDa, and obtained a level of protection around 30%. Other groups have identified potential antigens for vaccination using indirect methods, particularly by production of monoclonal antibodies associated with protective activity in passive transfer experiments (Smith et al. 1982, Harn et al. 1984, Hirsch et al. 1997). Sher et al. (1986) identified a 97 kDa immunogen relevant to the induction of protection in mice. Recently, other vaccine candidates were described: a 22.6 kDa membrane-associated antigen (Jeffs et al. 1991), and 44.7/56.8 kDa egg antigens recognized by human monoclonal antibodies (Hirsch et al. 1997).

Data presented here clearly demonstrate that F8.1 and F8.2 might be of help in the formulation of a vaccine, which could reduce worm burden.

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