Induction of Resistance to *Schistosoma mansoni* Infection in Mice by Purified Parasite Paramyosin

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Abstract

Freeze-thaw (FT)-disrupted schistosomula or their membrane extract induced significant resistance in mice to *Schistosoma mansoni* infection (34 and 25%, respectively) without the use of adjuvant. Antigens identified in schistosome extracts by sera from immunized animals were then evaluated for protective potential. Immunization with schistosomal antigens of 97 and 68–70 kD resulted in significant protection that was equivalent to that obtained by FT schistosomula. Since the 97-kD antigen was suggested to be parasite paramyosin, we used a biochemical technique to purify this muscle protein. Purified schistosome paramyosin ran as a single band on 10% SDS-PAGE and was recognized both by sera from mice immunized with FT schistosomula and a polyclonal antiserum raised against the 97-kD parasite protein. Preincubation of schistosome paramyosin with sera from mice immunized with FT schistosomula resulted in the removal of reactivity with the 97-kD protein in crude worm extracts. Paramyosin was identified by Western blotting to be in the tegument of schistosomula. The purified schistosome paramyosin resulted in significant protection in three separate experiments (24, 46, and 53%) without the use of adjuvant. Addition of BCG to paramyosin resulted in enhanced protection.

Introduction

Induction of resistance against human schistosome infection represents a major goal for control programs. The multicellular and complex nature of these organisms results in divergent immunologic responses in the mammalian host that can be either harmful or beneficial (1). For example, purified glycoprotein antigens of the ova induce granuloma formation around parasite eggs deposited in host tissues (2). In contrast, immunomodulatory responses such as induction of T suppressor cells that may limit the extent of disease are the result of host reactivity to different components of the parasite (3). Similarly, resistance to schistosomiasis has been shown to be based on several mechanisms including innate and acquired with both specific and nonspecific components (4). Although the effector constituents of the host responses contributing to protection have been examined thoroughly (5), characterization of the protective parasite antigens is only beginning.

Earlier attempts to define protective schistosome antigens from crude parasite preparations produced conflicting results (6, 7). In the present study we have demonstrated that freeze-thaw (FT)[1]-disrupted schistosomula or their membrane extract induced significant resistance in mice without the use of adjuvants. Two candidate parasite antigens of 97 and 68–70 kD were shown to give equivalent degrees of protection. The purified 97-kD antigen appeared to be schistosome paramyosin; upon injection into mice it induced significant protection without adjuvant.

Methods

Parasites. A Puerto Rican strain of *Schistosoma mansoni,* maintained in our laboratory, was used in these studies. Schistosomula were obtained by cercarial penetration of isolated mouse skin (8). The organisms were washed extensively in PBS and counted. They were then centrifuged at 400 *g* at 4°C for 2 min and subjected to four cycles of FT before use in protection studies. Mechanically transformed schistosomula were prepared as previously described (9). The organisms were then incubated at 37°C in an atmosphere of 5% CO_2 for 3 h in serum-free media. To prepare membrane extract of schistosomula, 50,000 mechanically transformed organisms were added to 0.25 ml of 1% Triton X-100 (Polysciences Inc., Warrington, PA) with 1 mM PMSF (Sigma Chemical Co., St. Louis, MO) and 5 mM iodoacetamide (Sigma Chemical Co.). The organisms were kept at 4°C and vortexed every 5 min for 30 min and then centrifuged at 3,000 *g.* Clear supernatant was removed and immediately used for vaccination and immunochemical studies.

Soluble worm antigen preparation (SWAP) was obtained as previously described (10). SWAP was prepared in the presence of protease inhibitors by incubating adult worms at 4°C for 30 min in 1 mM diisopropyl fluorophosphate (Sigma Chemical Co.). The organisms were then homogenized with 5 mM PMSF, 5 mM *N*-α-D-tosyl-L-lysine chloromethylketone (Sigma Chemical Co.), and 5 mM iodoacetamide.

Induction and assay of resistance. CF1 female mice (Charles River Breeding Laboratories, Wilmington, MA) weighing 18–20 g were used in groups of 6–10 animals. For protection experiments using FT schistosomula, each animal was injected subcutaneously with ~ 2,000 organisms twice at 2-wk intervals and challenged with cercariae after another 2 wk. Control mice received similar injections of PBS. BCG was used at a dose of 5×10^6 CFU/dose (University of Illinois, Chicago, IL). Induction of protection by subcutaneous injection of schistosomula extract or individual antigen preparations followed the same time schedule. Resistance in vaccinated and control animals was assayed by exposure of mice to freshly shed cercariae. Groups of animals were anesthetized and their abdomens shaved, and then they were individually exposed to 100 or 500 cercariae. Lung recovery of schistosomula was performed at day 5 and adult worm perfusion was performed at 6–8 wk (11). Protection was calculated as

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1. *Abbreviations used in this paper:* FT, freeze-thaw; SWAP, soluble worm antigen preparation.

$$1 - \frac{\text{mean number of worms in immunized mice}}{\text{mean number of worms in control mice}} \times 100.$$

Differences between the means were analyzed by the 2-tailed *t* test.

Characterization of protective antigens. Sera from immunized mice were used as probes in Western blotting studies to determine the antigenic components of crude schistosome preparations. Aliquots of 50–100 μg of SWAP or schistosomula extract were mixed with equal volumes of sample buffer containing 2-mercaptoethanol. Polypeptides were separated on 10% SDS-PAGE and transferred electrophoretically to nitrocellulose paper (12, 13). Strips of nitrocellulose with the transferred proteins were reacted with 1:10–1:25 dilution of sera from normal or vaccinated mice. Bound antibodies were detected with horseradish peroxidase goat anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA) as conjugate and 4-chloronaphthol as substrate.

To determine if antigens recognized by immune sera were protective, parasite proteins were separated on SDS-PAGE gels and slices of gel were cut to correspond to the molecular weights of desired antigens. The gel slices were homogenized in PBS and injected subcutaneously. Control animals received gel slices without electrophoresed proteins. Immunization schedule was similar to that outlined above.

Purification and testing of schistosome paramyosin. Two proteins of 97 and 68–70 kD were found to be protective. The 97-kD protein was selected for further evaluation, as the work of Lanar et al. indicated it may be schistosome paramyosin (14). A polyclonal rabbit serum was raised by repeated subcutaneous injections at 2-wk intervals of 10% SDS-PAGE slices containing the 97-kD protein. Furthermore, we extracted parasite paramyosin from adult schistosomes according to the procedure of Harris and Epstein (15). Briefly, adult worms were homogenized in PBS in the presence of protease inhibitors (1 mM ethylenediaminetetracetic acid, 1 mM PMSF, and 5 mM DTT) (Sigma Chemical Co.). Actomyosin was solubilized in 0.6 M KCl and paramyosin was then separated from actomyosin by ultracentrifugation at 100,000 *g* for 3 h at 4°C. Solubilization in 0.6 M KCl and then reprecipitation in 0.1 M KCl removed several minor contaminants (16). The extent of purification of the isolated paramyosin was confirmed on a 10% SDS-PAGE and by Western blot with the anti–97-kD rabbit serum and sera from mice immunized with FT schistosomula. Furthermore, to assess the immunologic identity of the purified schistosome paramyosin, 100 μl of sera from mice immunized with FT schistosomula was incubated overnight at 4°C with 100 μg of paramyosin and then analyzed by Western blot. The protective effect of the parasite protein was then evaluated by injection of 5 μg of paramyosin in groups of mice at 2-wk intervals. Mice were challenged and adult worm burden assessed at 6 wk as previously described. Serum from mice injected with paramyosin or PBS was assessed for specific antibody by Western blot analysis.

Results

Induction of resistance by crude schistosome preparations. Groups of mice (6–10 each) were immunized twice at 2-wk intervals with ~ 2,000 FT schistosomula per injection. Control animals received injections of PBS according to the same schedule. In six separate experiments significant protection was observed upon recovery of lung schistosomula on day 5 after challenge (Table I). In individual experiments the mean protection in immunized mice varied from 21–47%. The optimal parasite dose was then evaluated. Groups of mice were immunized twice with ~ 1,000, 2,000, or 5,000 schistosomula. Significantly higher protection (30%) was demonstrated in animals immunized with 2,000 organisms ($P < 0.01$). Subsequently, we used detergent extract of schistosomula to determine if it was equally protective. An extract of 5,000 schistosomula given twice at 2-wk intervals to CF1 mice resulted in 27% protection ($P < 0.02$) as compared with immunization

Table I. Induction of Resistance by FT Schistosomula

Experiment	No. of mice in each group	Mean no. of schistosomula recovered (±SD)		% Protection (P value)
		Control	FT schistosomula	
1	5	146±18	114±6	22% ($P < 0.001$)
2	5	190±19	100±10	47% ($P < 0.001$)
3	5	112±4	88±7	27% ($P < 0.001$)
4	5	105±12	77±5	29% ($P < 0.001$)
5	5	189±12	121±4	36% ($P < 0.001$)
6	9	145±13	86±5	41% ($P < 0.001$)
Mean of six experiments		148±36	98±17	34% ($P < 0.020$)

Each animal was injected subcutaneously with ~ FT 2,000 organisms at days 1 and 14 and then exposed to 500 cercariae percutaneously on day 28. Recovery of lung schistosomula was performed on day 5 after challenge.

with 1% Triton X-100 alone as control (Table II). These results were confirmed by performing adult worm perfusion at 6 wk; a mean of 22% protection ($P < 0.05$) was again observed.

Sera from vaccinated and control mice were analyzed by Western blot against schistosomula extract and SWAP prepared with protease inhibitors. Sera from mice immunized with FT schistosomula consistently recognized in SWAP a single band at 97 kD (Fig. 1 *A*), or a doublet at 97 and 92 kD (Fig. 1 *E*). A similar pattern was seen when the sera were reacted with schistosomula extract. Sera from mice immunized with schistosomula detergent extract recognized bands at 200, 97, and 68–70 kD in SWAP (data not shown).

Due to the simple humoral response and the availability of SWAP, the protective effect of the 97- and 68–70-kD proteins recognized in SWAP by immune sera were examined further. After electrophoresis of SWAP, gel slices corresponding to 92–97 and 65–70 kD mol wt were excised and used to immunize groups of CF1 mice. The 97- and the 68–70-kD antigens resulted in a respective mean reduction of schistosomula recovery of 40% ($P < 0.001$) and 48% ($P < 0.001$) (Table II).

Characterization of protective potential of schistosome paramyosin. Extraction and purification of paramyosin from adult *S. mansoni* resulted in isolation of a single band at 97 kD on Coomassie Blue staining on 10% SDS-PAGE (Fig. 1 *B*). Western blot analysis showed that this preparation was recognized both by sera from mice immunized with FT schistosomula and by a polyclonal rabbit sera raised against the 97-kD antigen (Fig. 1 *C*). Furthermore, schistosome paramyosin absorbed the immunological recognition of the 97-kD antigen by sera from mice immunized with FT schistosomula (Fig. 1 *E*). Injection of 5 μg of this purified material twice in groups of CF1 mice in three separate experiments resulted in a mean protection of 24, 46, and 53% ($P < 0.02$) as assessed by adult worm perfusion at 7 wk (Table III).

To determine whether paramyosin-induced protection can be enhanced with BCG, studies summarized in Table III were performed. Paramyosin alone resulted in 24% protection and the addition of BCG enhanced this protection to 52% ($P < 0.01$). BCG alone resulted in 24% protection ($P < 0.05$). Furthermore, sera from immunized mice recognized two

Table II. Induction of Resistance by Schistosomula Membrane Extract and Purified Schistosome Proteins

Experiment	Parasite preparation	No. of mice in each group	Mean no. of schistosomula recovered (±SD)		% Protection (*P* value)
			Control	Immunized	
1	1% Triton X-100 extract of 5,000 schistosomula	5	159±9	115±11	27% ($P < 0.02$)
2	Gel slice with 97-kD protein	4	166±13	117±12	30% ($P < 0.001$)
3	Gel slice with 97-kD protein	6	174±8	91±11	48% ($P < 0.001$)
4	Gel slice with 97-kD protein	9	145±13	86±5	41% ($P < 0.001$)
5	Gel slice with 68–70-kD protein	6	174±8	91±8	48% ($P < 0.001$)

Each animal was injected subcutaneously with various doses of antigen on days 1 and 14 and then exposed to 500 cercariae percutaneously on day 28. Recovery of lung schistosomula was performed on day 5 after challenge. Control animals in experiment 1 received PBS and control animals in experiments 2–5 received polyacrylamide gel slice alone. Animals in experiment 2 received only one dose of antigen and were then challenged on day 14.

bands at 92–97 kD in SWAP and reacted strongly with parasite paramyosin (Fig. 1 *D*).

Localization of paramyosin. To assess if paramyosin is present in the membrane component of schistosomula, we examined the reactivity of sera from mice immunized with a mild nonionic detergent membrane extract of schistosomula against purified paramyosin. Sera from immunized mice reacted with purified paramyosin by Western blot analysis, whereas control mouse sera raised against 1% Triton X-100 alone did not react to paramyosin (data not shown).

Discussion

The multiplicity of antigens and of host responses to helminthic parasites poses several problems in identifying those of potential protective relevance. Although resistance to *S. mansoni* in mice can be induced by irradiated cercariae (17), the protective parasite components are unknown. On the other hand, the development of monoclonal antischistosome antibodies that adoptively confer resistance has led to characterization of several potential protective antigens. Smith and Clegg have reported the isolation of two protective antigens of 155 and 53 kD that in conjunction with adjuvant have induced significant protection of 21% (18). King et al. have isolated an antigen of 68 kD from SWAP that induced protection of 30–66% without adjuvant (19). Furthermore, Balloul et al. have described immunization with a recombinant fusion protein containing a portion of a 28-kD schistosome antigen that provides 52 and 67% protection in hamsters and mice, respectively (20).

In the current study we made use of another model of inducing resistance to *S. mansoni* in mice without adjuvant that we originally described (21). FT preparations of schistosomula and a Triton X-100 extract of the parasite without

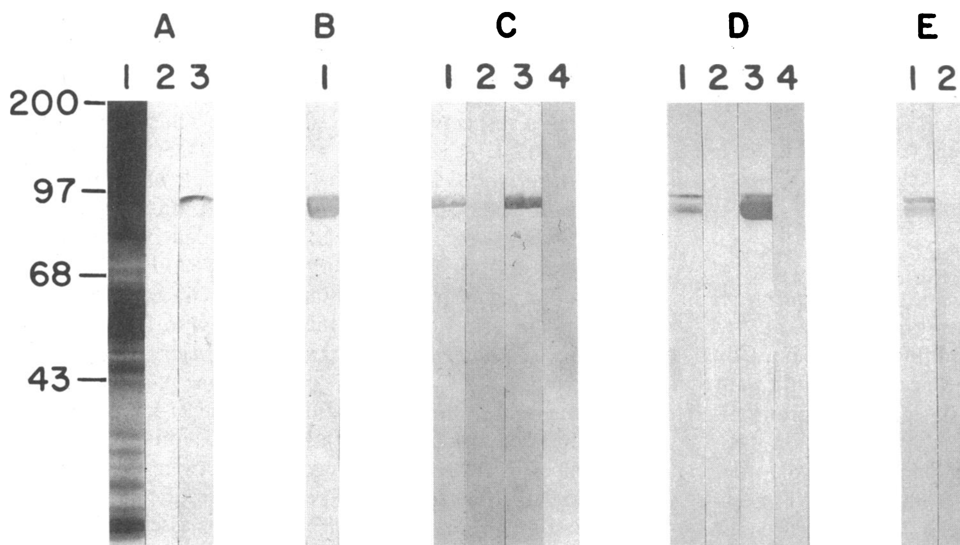

Figure 1. Immunologic reactivity of sera from mice immunized with various schistosome proteins. (*A*) Sera were reacted with SWAP separated on 10% SDS-PAGE in the presence of 2-mercaptoethanol. Lane *1*, sera from mice chronically infected with *S. mansoni.* Lane *2*, sera from mice immunized with PBS. Lane *3*, sera from mice immunized with FT schistosomula recognized a single band at 97 kD. (*B*) Coomassie blue staining of a 10% SDS-PAGE of purified schistosome paramyosin shows a broad band at 97 kD. (*C*) Reactivity of various sera against schistosome paramyosin. Lane *1*, sera from mice immunized with FT schistosomula reacted with paramyosin. Lane *2*, sera from mice immunized with PBS. Lane *3*, a polyclonal rabbit serum raised against the 97-kD schistosome protein reacts with paramyosin. Lane *4*, preimmune rabbit sera. (*D*) Lane *1*, sera from mice immunized with paramyosin recognized a doublet at 92 and 97 kD when reacted with SWAP. Lane *3*, sera from mice immunized with paramyosin reacted with schistosome paramyosin. Lanes *2* and *4*, sera from mice immunized with PBS showed no reactivity with SWAP and paramyosin, respectively. (*E*) Preincubation of sera from mice immunized with FT schistosomula with schistosome paramyosin absorbed the immunologic reactivity to the 97- and 92-kD protein. Lane *1*, sera from mice immunized with FT schistosomula reacted against SWAP. Lane *2*, reactivity of the above sera with SWAP after it has been preincubated with schistosome paramyosin.

Table III. Induction of Resistance by Paramyosin Purified from Adult S. mansoni

Experiment	Preparation	No. of mice in each group	Mean no. of adult worms recovered (±SD)	% Protection (P value)
1	PBS	8	19±7	
	Paramyosin	7	9±6	53% ($P < 0.01$)
2	PBS	8	24±10	
	Paramyosin	8	13±10	46% ($P < 0.05$)
3	PBS	8	38±8	
	Paramyosin	8	29±8	24% ($P < 0.05$)
	BCG	7	29±5	
	*‡Paramyosin + BCG	7	18±7	52% ($P < 0.01$)

Each animal was injected subcutaneously with 5 μg of paramyosin in 100 μl of PBS on days 1 and 14 and then exposed to 100 cercariae on day 28. Mice were perfused after 6 wk to assess adult worm burden. Control mice (eight in each group) received 100 μl of PBS alone.
* Immunization with paramyosin + BCG when compared with BCG alone was significantly protective (38%, $P < 0.02$).
‡ Immunization with paramyosin + BCG when compared with immunization with paramyosin alone was significantly protective (38%, $P < 0.05$).

adjuvant produced a significant degree of protection (21–47%). James et al. have shown that immunization of mice with FT schistosomula in conjunction with BCG results in significant protection (22). Although immunization with FT schistosomula and Triton X-100 extract of schistosomula provide a multitude of parasite antigens, a strong monospecific humoral response was observed in vaccinated mice. For example, sera from mice immunized with Triton X-100 extract of schistosomula recognized antigens of ~ 200, 97, and 68–70 kD mol wt, whereas sera from mice immunized with FT schistosomula recognized only a 97-kD antigen or a doublet at 97 and 92 kD in SWAP. Immunization of mice with gel slices containing the 97- or the 68–70-kD antigens indicated that both were significantly protective. The unique features of our immunization protocol are that no adjuvant is used and that only minute quantities of antigen are needed (~ 5 μg/injection per mouse).

Sher et al., using sera from mice immunized with FT schistosomula and BCG, detected antigens at 180, 95, and 78 kD in SWAP run on a nonreducing gel (23). When SWAP was prepared in the presence of protease inhibitors and run on a reducing gel, the same sera recognized only a single band at 97 kD (23). Recently it was reported that a DNA that encoded about half of the 97-kD protein has been cloned and sequenced (14). The deduced amino acid sequence shares homology with invertebrate paramyosin. Furthermore, a MAb to the 97-kD protein recognized purified schistosome paramyosin (14, 24). To characterize further the biochemical nature of the protective schistosome antigens, we isolated parasite paramyosin. Using the biochemical procedure of Harris and Epstein (15), the purified preparation was demonstrated as a single band on SDS-PAGE and recognized on Western blot both by sera from mice immunized with FT schistosomula and by a rabbit serum raised against the 97-kD native protein isolated from schistosomes. Sera from paramyosin immunized mice recognized two bands at 97 and 92 kD when reacted by Western blot against SWAP. Furthermore, purified schistosome paramyosin absorbed completely the reactivity of sera from mice immunized with FT schistosomula with the 97- and 92-kD parasite antigens. The 92-kD protein is probably a breakdown product of paramyosin, as antibody to the 92-kD protein is absorbed by schistosome paramyosin. The protective potential of purified schistosome paramyosin was subsequently evaluated. Two injections of 5 μg/mouse of the purified schistosome protein at 2-wk intervals resulted in 24, 46, and 53% protection as assessed by adult worm recovery. Addition of BCG to paramyosin resulted in a mean protection of 52%. When compared with immunization with BCG alone, protection was significantly enhanced ($P < 0.02$).

Paramyosin has previously been well characterized in invertebrates as an integral muscle protein that allows for extremely prolonged and powerful muscle contraction (25). We demonstrated that antibody to a mild nonionic detergent membrane extract of schistosomula reacted to purified paramyosin on Western blot analysis. The presence of the protein in the tegument of schistosomula may explain its immunogenicity in the FT schistosomula model. Pearce et al. found binding of a MAb against paramyosin just beneath and possibly within the surface of adult schistosomes, though it was suggested that paramyosin is not surface exposed (24). Ultrastructural studies using electron microscopy and a polyclonal antibody against paramyosin demonstrated paramyosin firmly embedded within the tegument (26).

Our data indicate that immunologically defined components of schistosome extracts are capable of inducing significant protection without the use of adjuvant. Two schistosome antigens of 97 and 68–70 kD resulted in equivalent protection to that obtained by FT schistosomula. We purified a 97-kD antigen from worm extracts that corresponded to parasite paramyosin. It was recognized by sera from mice immunized with FT schistosomula and by a polyclonal antiserum raised against the 97-kD native parasite protein. Preincubation of schistosome paramyosin with sera from mice immunized with FT schistosomula resulted in the removal of reactivity with the 97-kD protein. Finally, the purified schistosome paramyosin resulted in significant protection (without the use of adjuvant) comparable to that obtained by any previously described protective schistosome antigens.

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Note added in proof. Since the submission of this manuscript, Pearce et al. (27) have reported that paramyosin administered with BCG at a total dose of 4–40 μg/mouse conferred significant protection against *S. mansoni* challenge infection.

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