Induction of protective immunity and modulation of granulomatous hypersensitivity in mice using PIII, an anionic fraction of *Schistosoma mansoni* adult worm

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SUMMARY

This study was performed in order to define *Schistosoma mansoni* antigens that are able to function as modulator agents in the granulomatous hypersensitivity to parasite eggs in BALB/c and C57BL/6 mice. A fraction of *S. mansoni*, designated PIII, derived from adult worm antigen preparation (SWAP) was obtained using anion-exchange chromatography on an FPLC system. Immunization of mice with PIII in the presence of *Corynebacterium parvum* and $Al(OH)_3$ as adjuvant induced an immune response in these animals as determined by ELISA and spleen cell proliferation assays against *S. mansoni* antigens SEA, SWAP and PIII. In addition, PIII caused a significant degree of protection against a challenge infection in immunized mice as observed by the decrease on worm burden recovered from the portal system. We also showed that PIII profoundly inhibited the vigorous anamnestic granulomatous response to eggs in the liver and lungs. This suppression correlated with a significant decrease in granuloma size. From these results we conclude that the PIII preparation contains antigens that can mediate protective anti-parasite immunity and downregulate granulomatous hypersensitivity to *S. mansoni* eggs.

Key words: *Schistosoma mansoni*, modulation, granuloma, worm, immunity.

INTRODUCTION

Parasite egg-granulomas are the primary pathogenic lesions in experimental and human schistosomiasis mansoni (Warren, 1982; Boros, 1989). This cell-mediated granulomatous response is specific for soluble egg antigen and appears to be mediated predominantly by CD4+ Th2 cells (Grzych *et al*. 1991; Chensue *et al*. 1992). As infection progresses from the acute to the chronic phase, the cell-mediated anti-soluble egg antigen response attenuates in a process called modulation. Experimental murine schistosomiasis has been widely used to investigate parasite biology, host–parasite interactions, including immunology and morbidity, and strategies for treating or preventing this common tropical disease of man (Phillips & Colley, 1978). Therefore, the identification of *Schistosoma mansoni* antigens and an assessment of their role in host–parasite interaction, notably in the prevention or decrease in size of granuloma formation by immunization procedure is essential for the development of an anti-schistosome vaccine (Bergquist, 1990). Future progress in this field might depend on the understanding of the complex immunoregulatory events that modulate the evolution of granulomatous hypersensitivity to *S. mansoni* eggs and the basis of protective immunity in man (Butterworth, 1992). Various experimental approaches have been carried out by investigators to identify *S. mansoni* antigens that elicit T cell proliferation and granuloma formation. These include parasite antigens purified by conventional purification techniques (Carter & Colley, 1979; Payares *et al*. 1985; Harn *et al*. 1989; Lukacs & Boros, 1992), affinity chromatography with monoclonal antibodies (Harn *et al*. 1985; Smith & Clegg, 1985; Hsu *et al*. 1986; Dissous, Grzych & Capron, 1982; Goes *et al*. 1989) or by recombinant DNA technology (Boulanger *et al*. 1991; Jeffs *et al*. 1991). Several aspects of the model used have not been adequately defined because of conflicting results reported by different laboratories, notably the type of antigens which yield the highest levels of resistance, a single or cocktail antigen that gives significantly increased resistance and whether such a vaccine would aggravate or ameliorate the development of granulomatous egg pathology (Warren, Domingo & Cowan, 1967). Our laboratory has produced PIII, an anionic fraction from soluble adult worm antigen preparation (SWAP). This fraction was able to induce high levels of proliferation and small granuloma formation *in vitro* by human peripheral blood mononuclear cells from chronic schistosomiasis patients (Hirsch & Goes, 1996). We have extended these studies in this paper to

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investigate whether PIII would protect mice and modulate granuloma formation resulting from challenge schistosome infections.

MATERIALS AND METHODS

Mice and parasites

Adult female BALB/c and C57BL/6 mice purchased from Centro de Bioterismo, ICB, UFMG, Brazil, were used throughout this study. The mice were maintained under standard laboratory care. The cercariae of *S. mansoni* were obtained from *Biomphalaria glabrata* snails previously infected with miracidia of the L.E. strain, from Belo Horizonte, Brazil. Cercariae were shed from infected snails under bright artificial illumination. Anaesthetized mice (8/group) were exposed to normal cercariae on the abdomen by using the ring method of Smithers & Terry (1965). Vaccinated and control mice challenged with normal cercariae were perfused 8 weeks after exposure (Smithers & Terry, 1965). The protective activity of each group assayed was evaluated by comparing the difference between the recoveries of worms from immunized and control mice.

Antigens

Concanavalin A (Con-A, Sigma, St Louis, MO, USA) was used at a concentration of 12·5 μg/ml in culture medium. Antigenic preparations were obtained from schistosome eggs (SEA) and adult worms (SWAP). These antigens were prepared as soluble supernatant fluids from buffered saline homogenates of the respective life-cycles stages (Goes *et al*. 1989). Other antigens used were PIII, obtained from SWAP by anion-exchange chromatography on an FPLC system, and bovine serum albumin (BSA). These materials were used based on their protein content to give the maximum response in blastogenesis assays with most mice (25 μg/ml). All stimulants were sterilized by filtration and stored at −70 °C.

PIII preparation

PIII was prepared as previously described by Hirsch & Goes (1996). Briefly, SWAP (30 mg) was dialysed against 20 mM Tris–HCl, pH 9·6, and filtered using an acrodisc 0·2 μm filter. Separation was performed by FPLC (Pharmacia, Uppsala, Sweden) on Q-Sepharose anion-exchange chromatography (5 mm × 90 mm glass columns, packed with Q-Sepharose; bead size distribution 45–165 μm; Pharmacia). Proteins were eluted with 20 mM Tris–HCl, pH 9·6, in a multistep increasing gradient up to 1 M NaCl, interrupted by hold-gradient intervals at 0 (PI), 100 (PII), 280 (PIII), 450 (PIV), 600 (PV) and 750 mM (PVI). Flow-through fractions were concentrated by lyophilization. The concentrated material was dialysed against 0·15 M phosphate-buffered saline (PBS), pH 7·4, sterilized by filtration and stored at −70 °C. The protein content of SWAP fractions was measured according to the Bradford microassay (Bradford, 1976).

Immunization of mice with PIII

BALB/c and C57BL/6 were immunized by s.c. injections of 10 μg PIII in the presence of 100 μg of *Corynebacterium parvum* and 1 mg of aluminium hydroxide [$Al(OH)_3$] as adjuvant. The animals were boosted twice at 2-week intervals with identical amounts of antigen, but the last injection was done by i.p. injection without adjuvant.

Induction and measurement of pulmonary granulomas elicited by S. mansoni *eggs*

The induction of synchronous egg granulomas was performed as described previously (Lukacs *et al*. 1994). Briefly, *S. mansoni* eggs were extracted from the livers of infected mice. To induce granulomas, normal, infected or PIII-immunized mice were injected i.v. with 2000 intact living *S. mansoni* eggs. After 4 days, animals were killed and their lungs were inflated and fixed with 4% paraformaldehyde in phosphate buffer and then embedded in paraffin. Sections were stained with haematoxylin and eosin or Heidenhein's azan (Hirata *et al*. 1993). Only lesions with a single well-defined egg nidus were measured. The results were expressed as mean of area (μm^2) calculated from 30–50 granulomas.

Measurement of hepatic granuloma formulation

PIII-immunized and infected mice were killed 8 weeks after challenge infection and the livers were removed and fixed with 4% paraformaldehyde in phosphate buffer. Histological sections were stained with haematoxylin and eosin or Heidenhein's azan (Hirata *et al*. 1993). The areas of granulomas surrounding single, mature eggs were measured by the same procedure used for pulmonary granuloma determination.

Spleen cell preparation

Single-cell suspensions, which had been prepared from spleens of normal, infected or PIII-immunized mice, were treated with Tris–ammonium chloride, pH 7·2, to lyse erythrocytes, and then were washed and counted. The spleen cells were resuspended in culture medium (RPMI 1640 with 20% foetal bovine serum, 1·6% L-glutamine, 300 U/ml of penicillin, 0·6 mg/ml of streptomycin and 0·05 mg/ml of gentamycin).

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Table 1. Antibody response and cellular reactivity induced in mice immunized with PIII

Sera and spleen cells from C57BL/6 mice	Antibody reactivity*			Cellular reactivity†		
	SWAP‡	PIII	SEA	SWAP	PIII	SEA
Normal	0·083	0·203	0·210	0·800	0·635	0·927
Chronic infection	1·012	1·698	2·000	4·327	3·734	8·004
PIII-immunized	1·230	0·977	0·699	13·513	7·133	12·836

* Antibody reactivity determined by ELISA assay. Data are reported as means of optical density at 1/640 serum dilution in each experimental group performed twice ($n = 5$ determinations).
† Cellular reactivity was determined by cell proliferation assay. Data are reported as means $\times 10^3$ cpm of [^{3}H]thymidine incorporation in each experimental group performed twice ($n = 8$ determinations).
‡ SWAP, Adult worm antigen preparation; PIII, anionic fraction derived from SWAP; SEA, soluble egg antigen.

Table 2. Protective effect of PIII on vaccinated mice against a challenge infection with *Schistosoma mansoni* cercariae

(Protective effect of PIII was studied in BALB/c and C57BL/6 mice immunized with 10 μg of antigen in the presence of *Corynebacterium parvum*/Al(OH)$_3$ as adjuvant.)

Treatment	Worm burden*	
	C57BL/6	BALB/c
Infection	45 ± 2	40 ± 4
Adjuvant	42 ± 4·4	38 ± 6
PIII	25 ± 6†	25 ± 4†
% Reduction‡	44	37

* Worm burden was determined by perfusion of portal system at 8 weeks after challenge infection in 8 mice/group performed 3 times.
† $P < 0{\cdot}05$.
‡ %Reduction = 100 × control − experimental/control.

Cell proliferation assays

Spleen cell proliferation assays in response to Con-A, PIII and other *S. mansoni* antigens (SWAP and SEA) were performed. Briefly, $7{\cdot}0 \times 10^5$ spleen cells were cultured in 200 μl of culture medium in 96-well flat-bottomed plates. Every experiment was set up in triplicate. Cultures were stimulated with 25 μg/ml of each antigen and maintained at 37 °C in a 5 % CO_2 incubator for 3 days. For the last 18 h of incubation, 0·5 μCi/well of tritiated thymidine (specific activity, 37 Ci/mmol; New England Nuclear, Boston, MA, USA) were pulsed. The cells were harvested for scintillation counting and the data were calculated as the mean of cpm values.

Enzyme-linked immunosorbent assays (ELISA)

To evaluate the specificity of (i) normal, and (ii) *S. mansoni* chronically infected and immunized mice sera against PIII, flat-bottomed microtitre plates (Immulon II, Dynatec Corp., Alexandria, VA, USA) were coated overnight with 100 μl of a 10 μg/ml solution of each antigen (SWAP, PIII and SEA) in 0·5 M carbonate buffer, pH 9·6. Plates were washed 3 times in 0·05 M PBS containing 0·05 % Tween 20 (PBS–T), and blocked with 200 μl of 2 % bovine serum albumin (BSA) in PBS, at room temperature. After incubation for 1 h, plates were filled with 100 μl of serial dilutions of mice sera and re-incubated for 1 h. Plates were washed with PBS–T and incubated for 1 additional hour with 100 μl of a 1/5000 dilution of goat anti-mouse Ig peroxidase-conjugated antibody (Sigma, St Louis, MO, USA). Plates were then washed and peroxidase activity was assayed with 150 μl of *o*-phenylenediamine dihydrochloride (OPD) solution (34 mg of OPD and 20 μl of hydrogen peroxide to 100 ml of citrate/phosphate buffer, pH 5·0). Colour development was stopped with 50 μl of 5 % H_2SO_4. The optical density at 492 nm was measured with an automated ELISA reader (Bio-Rad 2550 Reader EIA).

Statistical analysis

Data were analysed statistically by the Student's *t*-test with the level of significance set at $P < 0{\cdot}05$.

RESULTS

Antibody response induced by PIII

Mice were immunized with PIII using *C. parvum* and Al(OH)$_3$ as adjuvant, and the immune response was analysed by ELISA assay. The data from Table 1 show that PIII immunization induces a high antibody response against PIII and SWAP. The antibody reactivity for PIII and SWAP determined in sera from PIII-immunized mice was in the same level as observed for sera from chronically infected ones. We observed that sera from infected mice

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Table 3. Reduction of granulomatous hypersensitivity of mice immunized with PIII

	Granuloma area*			
	Hepatic		Pulmonary	
Conditions	C57BL/6	BALB/c	C57BL/6	BALB/c
Non-infected	—	—	7·1 ± 4·1	3·7 ± 2·5
Infected	77·6 ± 39·1	108·5 ± 55·1	39·1 ± 14·1	32·1 ± 20·5
PIII-immunized	42·6 ± 24·3†	61·7 ± 30·0†	19·1 ± 8·5†	9·9 ± 8·6†
%Reduction‡	45	43	51	69

* Granuloma area was calculated assuming a spherical shape. The results were reported as the mean $\times 10^3$ $\mu m^2 \pm$ S.D. from 30–50 granulomas for each mouse of 2 separated experiments ($n = 5$ mice/group).
† $P < 0{\cdot}05$.
‡ %Reduction = 100 × control − experimental/control.

present a significant reactivity to SEA. However, this was not observed in sera from PIII-immunized mice. Normal mice sera did not react with either SEA, SWAP or PIII. On the other hand, sera from mice vaccinated with PIII without adjuvant or with adjuvant only did not present any reaction with all tested antigens (data not shown). Therefore, in the following experiment we concentrated on the further analysis of cellular activity induced by PIII.

Stimulatory capacity of PIII for S. mansoni*-specific spleen cells* in vitro

Splenic cells from infected and PIII-immunized mice were tested for proliferative responses to *S. mansoni* antigens SEA, SWAP and PIII. The results in Table 1 demonstrate considerable variation in degree of proliferative responses between the antigens tested. Crude SEA promoted strong responses of splenic cells from chronically infected mice and PIII-immunized mice. We observed that SWAP induced a significant ($P < 0{\cdot}05$) proliferation of cells from infected mice but this was not at the same level as observed to SEA. A significant cell proliferation was also induced by PIII in the same group of mice. PIII was subsequently tested in proliferation assays with cells from PIII-immunized mice. The data indicated that the proliferation induced by PIII was smaller than that obtained with SWAP or SEA on cells from immunized mice (Table 1). Spleen cells derived from non-infected mice did not respond to any *S. mansoni* antigens used.

Immunization of mice with PIII and its protective effect on a subsequent infection with S. mansoni *cercariae*

The protective effect of immunization with PIII in the presence of *C. parvum* and $Al(OH)_3$ as adjuvant was additionally investigated in mice. As shown in Table 2 the number of worms recovered 8 weeks after challenge infection of PIII-immunized mice was significantly reduced when compared to that observed in adjuvant only or with non-immunized control animals. Immunization with PIII established a protection of 44 and 37 % respectively to C57BL/6 and BALB/c.

Immunization of mice with PIII and its effect on pulmonary and hepatic granuloma formation

Immunization of mice with PIII resulted in a significant reduction in pulmonary granuloma size of BALB/c and C57BL/6 subsequently injected with *S. mansoni* eggs (Table 3). The granuloma reaction was tightly compact and focal aggregates of cells were observed around or close to intravascular and intra-alveolar eggs. We did not detect any focal aggregation in normal mice injected with eggs only (Fig. 1). The sizes of pulmonary granulomas of vaccinated mice were $9{\cdot}9 \times 10^3$ and $19{\cdot}1 \times 10^3$ μm^2 to BALB/c and C57BL/6 respectively, and those infected with cercariae alone were $32{\cdot}1 \times 10^3$ and $39{\cdot}1 \times 10^3$ μm^2 (Table 3). The percentages of granuloma reduction in these mice were 69 and 51 %. Comparison of means showed very significant differences between any two experimental treatments. In addition, we investigated the effect of PIII immunization in hepatic granuloma reaction after challenge infection. The foci in the livers of infected mice with cercariae were more numerous and frequently very large (Fig. 2), extending to up to 77×10^3 μm^2 and 108×10^3 μm^2 respectively in C57BL/6 and BALB/c (Table 3). The hepatic granulomas in the PIII-vaccinated group presented small foci and the size was reduced to $42{\cdot}6 \times 10^3$ μm^2 and $61{\cdot}7 \times 10^3$ μm^2 for C57BL/6 and BALB/c (Table 3). The percentages of granuloma reduction in these animals were 45 and 43 %. Each was significantly different from the controls at $P < 0{\cdot}05$.

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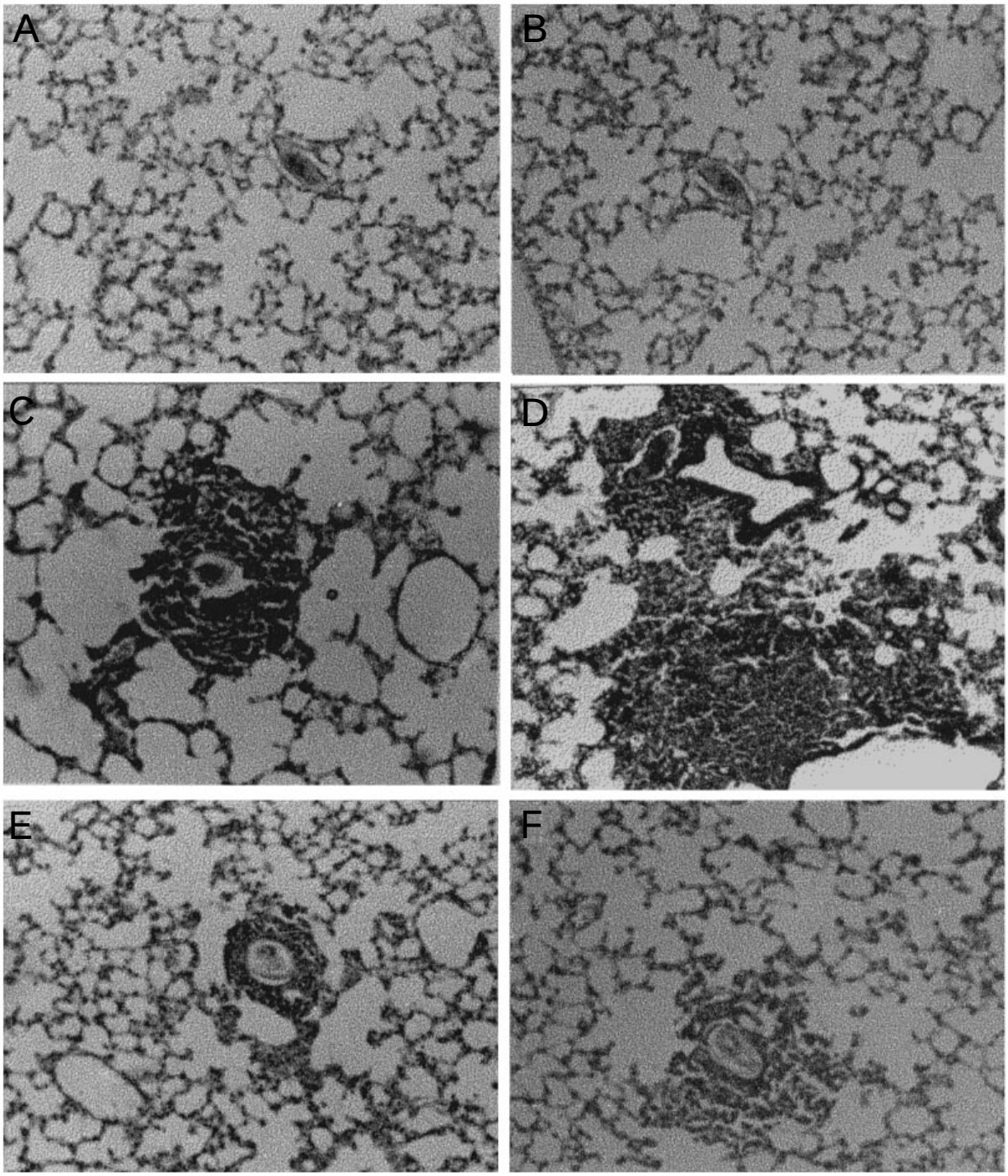

Fig. 1. Pulmonary granuloma photomicrographs (×250) of BALB/c (A, C, E) or C57BL/6 (B, D, F) mice after 4 days of i.v. egg injection in either normal (A, B), chronically infected (C, D) or immunized with PIII (E, F) mice. Histological sections were stained with haemotoxylin–eosin.

DISCUSSION

The present study describes an antigenic protein fraction PIII, obtained from *S. mansoni* adult worms, which fails to induce vigorous granulomatous hypersensitivity to eggs on liver and lungs, but also causes protection against a challenge infection in mice after local (s.c.) immunization. Our results demonstrated that mice vaccinated with PIII can alter the formation of granulomatous hypersensitivity to the parasite eggs. The vaccinated mice developed pulmonary and hepatic granulomas that were statistically smaller than those formed in the unvaccinated controls. In addition, spleen cells from these mice had a strong proliferative response to SEA and SWAP and even to PIII. It appears that PIII components are responsible for inducing heterogeneity in cell-mediated responses. These

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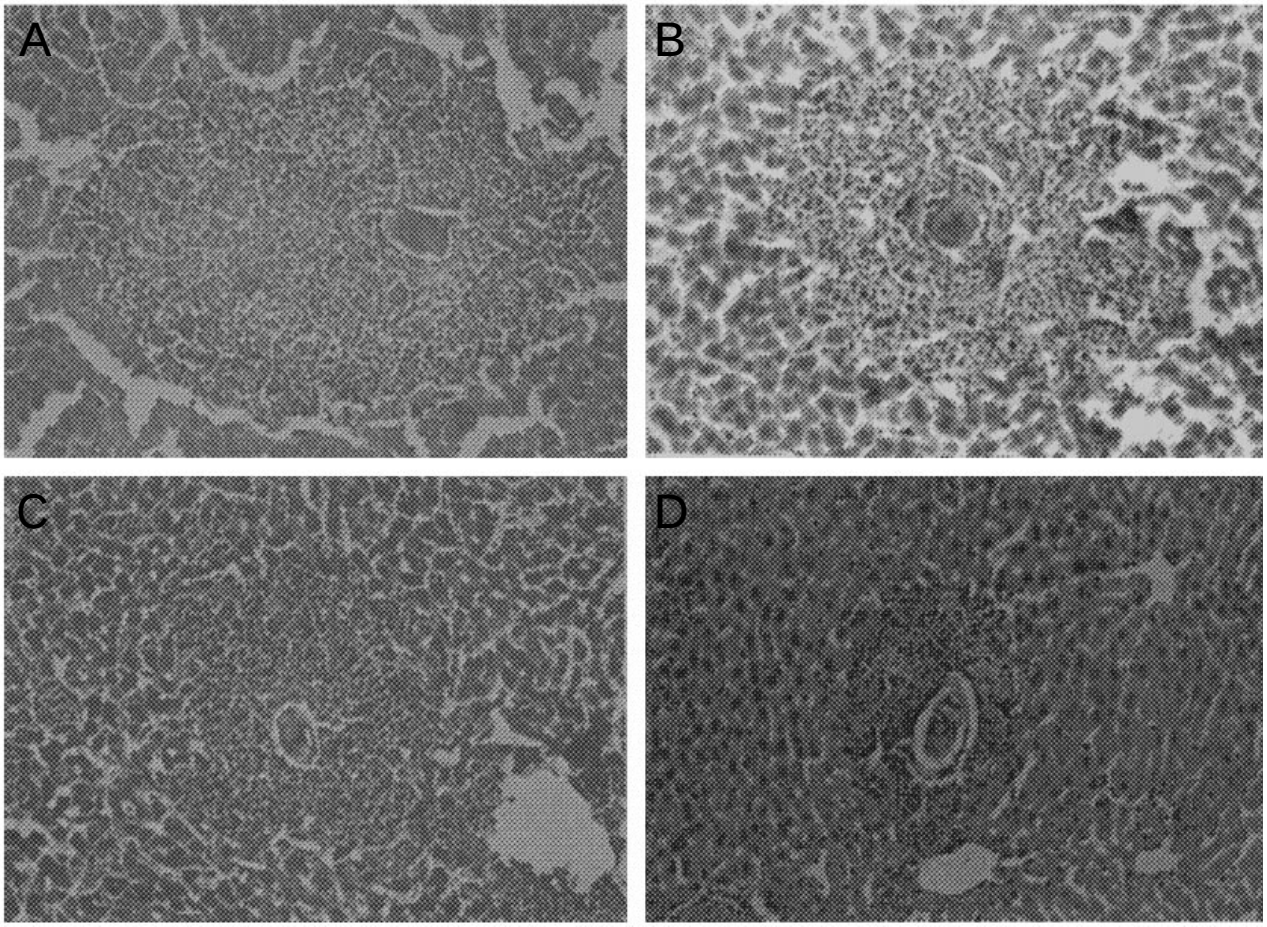

Fig. 2. Hepatic granuloma photomicrographs (× 250) of BALB/c (A, C) or C57BL/6 (B, D) mice 8 weeks after challenge infection in either non-immunized (A, B) or PIII immunized mice (C, D). Histological sections were stained with haematoxylin–eosin.

findings are in accord with previous results reported both by Hirsch & Goes (1996), using an *in vitro* granuloma formation system and cell proliferation of peripheral blood mononuclear cells from human schistosomiasis patients, and by Wynn *et al*. (1994) in mice. The ability of PIII to suppress both pulmonary and hepatic egg-induced granulomatous responses suggested the possibility of prophylactically immunizing mice against granulomatous inflammation by sensitizing them with adult worm antigens in the presence of *C. parvum* and $Al(OH)_3$. This indicated the presence, within adult worm antigens, of several molecules that might play a role in cell activation and modulation of granulomatous hypersensitivity. The existence of an SEA cross-reactive humoral immune response prior to egg deposition is well documented (Dissous & Capron, 1983; Goes *et al*. 1989). In contrast, there is a scarcity of data on T cell cross-reactivity between larval, adult worms and egg antigens. Early publications reported a lack of granulomatous responsiveness in mice injected intravenously with eggs or immunized with irradiated cercariae (Warren, 1982). Recently, Contigli *et al*. (1994) demonstrated that human T cell clones selected from PBMC stimulated with SEA preferentially proliferated in response to SWAP or CAP and that these clones were able to induce *in vitro* granulomas in cooperation with autologous antigen-presenting cells.

Conversely, post-schistosomal hepatic lesions have been shown to be caused by the formation of egg granulomas in the portal area. However, studies done by others suggest the contribution of adult worm antigen in part to the development of the hepatic lesion. The essential role of adult worms in the formation of egg granulomas *in vitro* (Doughty & Phillips, 1982; Hirsch *et al*. 1997) and the strong association between vigorous response to adult worm antigen and the development of hepatic lesions (Deelder *et al*. 1980; Elner *et al*. 1980; Ohta *et al*. 1982) support this concept, although it had been assumed that the strength of the immune response to the adult worm antigen depends on the intensity of infection. However, additional studies by Barsoum *et al*. (1982) demonstrated that the responsiveness to the adult worm antigen is determined mainly by immunoregulatory mechanisms. Indeed, there is considerable debate concerning the immunological effector mechanisms responsible for the rejection of challenge parasites in resistant animals with evidence

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supporting a function for humoral responses (Capron & Capron, 1994) and other findings indicating a role for cell-mediated immunity (James & Boros, 1994). Therefore, an important strategy for improving vaccine efficacy is to define mechanisms of immunity operating in a given vaccination protocol and to attempt to enhance the response for young parasite forms and decrease the granulomatous hypersensitivity to eggs trapped in host tissue.

The data presented here clearly demonstrate that PIII might be of help in the formulation of a vaccine which both reduces worm burden and inhibits the pathology resulting from egg deposition. Examination of the production of circulating anti-PIII antibody revealed an increase in levels in PIII-immunized mice. Since antibody production was maintained in the face of moderate inflammation, this tends to argue in favour of the notion of antibody playing a direct role in the regulation of granuloma formation (Goes *et al*. 1991; Parra *et al*. 1991; Rezende *et al*. 1993).

A recent report of vaccines against schistosomiasis (Butterworth, 1992) showed that certain facts or aspects of the host immune response were important for the practical evaluation of the usefulness of schistosomiasis vaccines. When vaccines have been tried in mice, the end result has always been that a few organisms of a challenge infection develop into adult worms. Because adult worms do not divide, any antigen candidate that will reduce the number of worms and the granuloma size in humans would be beneficial (Colley & Colley, 1989; Butterworth, 1992). It is also possible that a vaccine candidate in humans will be a cocktail of antigens that will be used to protect against schistosomiasis. Therefore, PIII has this advantage because this fraction is constituted by 5 principal components (Hirsch & Goes, 1996). The immunization with a mixture of PIII in *C. parvum*/$Al(OH)_3$ and administered subcutaneously is capable of inducing significant parasite killing mechanisms and downregulation of granulomatous hypersensitivity involving stimulation of both humoral and cell-mediated immunity against further infection without exacerbating cell-mediated tissue pathology.

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