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Protection against experimental *Schistosoma mansoni* schistosomiasis achieved by immunization with schistosomula released products antigens (SRP-A): role of IgE antibodies

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SUMMARY

Schistosomula-released products (SRP-A) have been shown to induce preferentially a significant IgE response against *Schistosoma mansoni* schistosomula when injected into rats, in the absence of adjuvant. The present work provides additional evidence of the *in-vivo* relevance of the anti-SRP-A target antigens. Two strains of rat (Brown Norway and Fischer) were immunized with SRP-A and infected percutaneously. A significant level of protection (up to 83% reduction in worm burden) was observed. Passive transfer experiments carried out with anti-SRP-A or IgE-depleted anti-SRP-A sera suggested the preponderant role of antibodies and particularly of IgE in the protective immunity developed by Fischer rats. Platelets and macrophages recovered from such immunized rats had surface IgE as demonstrated by immunofluorescence analysis with FITC anti-IgE, and have been shown to be directly cytotoxic for schistosomula. The chemiluminescence observed when the macrophages were incubated with anti-IgE suggested the presence of IgE on the surface of these cells.

Keywords *Schistosoma mansoni* ADCC IgE protection

INTRODUCTION

The mechanisms of resistance to *Schistosoma mansoni* in various mammalian species are not yet entirely understood. Nevertheless, the analysis of the components of the immune response in rats and humans reveals: firstly, the dramatic production of high levels of antibodies and particularly of anaphylactic antibodies; secondly, the importance of ADCC mechanisms involving macrophages (Capron *et al.*, 1975), platelets (Joseph *et al.*, 1983) and eosinophils (Capron *et al.*, 1981) suggests that the schistosomula, which are the immature forms of the parasite, are the targets of the protective immune response. At the moment efforts are currently being directed towards the development of a vaccine using irradiation attenuated cercariae (Hsu, Hsu & Osborne, 1969; Minnard *et al.*, 1978; Majid *et al.*, 1980; Bickle *et al.*, 1979; Sher *et al.*, 1983). This living material has been demonstrated to induce a high degree of immunity in laboratory animals. Other attempts to vaccinate have consisted of immunization with dead parasite material. Partial protection of mice was observed with cercarial sonicates absorbed on aluminium hydroxide gel adjuvant (Horowitz,

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Smolarsky & Arnon, 1982). Smith & Clegg (1982) have partially protected mice or monkeys by immunizing them with soluble antigens isolated from young or adult schistosomes.

On the basis of ADCC reactions using immune rat serum and monoclonal antibody probes several potentially protective antigens have been recently characterized. A polypeptide antigen of 38 kD exposed on the surface of schistosomula and recognized by a monoclonal antibody conferring immunity by passive transfer has been described (Grzych *et al.*, 1982). This antigen defined by a rat IgG2a monoclonal antibody seems therefore to represent one of the major targets of IgG-dependent eosinophil cytotoxicity and can be considered as a potentially protective antigen. Recently, a 28 kD antigen has been identified (Balloul *et al.*, 1985) among the *in-vitro* translation products from adult worm RNA and among ^{125}I-labelled surface antigens of *S. mansoni* schistosomula. The 28 kD antigen induced the production of IgG antibodies, highly cytotoxic in presence of eosinophils, and thus appeared as a target of the potentially protective immune response as shown for the 38 kD molecule.

It is now clear that not only IgG but also IgE antibodies participate in protective immunity (Capron, Dessaint & Capron, 1978; Capron & Dessaint, 1985). Indirect evidence of their role *in vivo* was provided by the very close correlation between the existence of specific IgE in sera during experimental schistosomiasis in the rat and the development of immunity to reinfection (Capron, Dessaint & Capron, 1978), and more directly by passive transfer experiments (Capron *et al.*, 1984).

In a previous report, we have demonstrated that schistosomula-released products (SRP-A) induced an appreciable IgE response when injected into rats in the absence of adjuvant. A high level of IgE-dependent cytotoxicity was obtained with macrophages, eosinophils and platelets as effector cells (Auriault *et al.*, 1984). Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by western blotting revealed that two major schistosomula antigens of 26 kD and 22 kD (Damonneville *et al.*, 1986) were recognized by anti-SRP-A IgE. In the present work we show that SRP-A injected without adjuvant can induce a high degree of immunity against subsequent schistosome infection in Brown Norway and Fischer rats. Moreover the same protective effect is observed *in vivo* after passive transfer of anti-SRP-A sera shown to have high cytotoxic activity *in vitro* (Auriault *et al.*, 1984). IgE antibodies appear to be partly responsible for the protection conferred.

MATERIALS AND METHODS

Parasite life cycle and preparation of schistosomula released products (SRP-A)

A Puerto-Rican strain of *Schistosoma mansoni* was maintained in *Biomphalaria glabrata* snails and golden hamsters. Schistosomula were prepared by skin penetration (Clegg & Smithers, 1972) for the cytotoxicity assay. Mechanically prepared schistosomula (Ramalho-Pinto *et al.*, 1974; Capron *et al.*, 1974) were incubated for 4 h at 37°C in Hanks' balanced solution and extensively washed in the same medium in order to eliminate most of the products of cercarial origin. The parasites (20,000/ml) were then incubated for 16 h at 37°C. The absence of bacterial contamination and the viability of the parasites were controlled at the end of the incubation. The parasite-free supernatant fraction recovered after centrifugation at 150 ***g*** for 2 min was referred to as schistosomula released products (SRP-A).

Adult antigen was prepared as previously described (Pierce *et al.*, 1983). Briefly, the worms (10,000) were repeatedly washed in 0·9% NaCl and allowed to settle before incubation with 10 ml H_2O at 37°C for 2 h. The supernatant fluid was then removed and the worms washed twice with 5 ml H_2O. The pooled supernatant fractions and washing were centrifuged at 30,000 ***g*** for 1 h at 4°C, the final supernatant fraction constituting the incubation product.

Sera

Inbred Fischer rats (Iffa Credo, L'Arbresle, France) or Brown Norway rats (CSEAL-CNRS, Orléans, France) exposed percutaneously to 1,000 *S. mansoni* cercariae 6–8 weeks previously were the source of infected rat sera used in the cytotoxicity assays. Brown Norway rats, immunized to obtain anti-SRP-A serum, were injected intraperitoneally (i.p.) (1 ml SRP-A/rat) followed by a second injection 1 month later. Sera were recovered 5 days after the second injection (day 35).

Depletion of IgE was achieved by immunoabsorption of the sera. Anti-rat IgE (5 mg) (Miles, Yeda Laboratories, Israel) were immobilized on 1 g of CNBr-activated Sepharose-4B (Pharmacia, Uppsala, Sweden) (Axen, Porath & Ernbach, 1967). The depletion of IgE from 10 ml of serum was performed by incubation with 3 ml of anti-IgE immunosorbent gel, at 4°C overnight.

Passive transfer experiments

Brown Norway or Fischer rats were infected percutaneously with 1,000 *S. mansoni* cercariae. Four hours later, each rat was injected intravenously with 1·5 ml of anti-SRP-A (day 35) or normal rat sera, previously controlled in cytotoxicity assays. Parasite burdens were evaluated by total perfusion 19 days later. The numbers of worms obtained from rats injected with anti-SRP-A sera, either complete or depleted in IgE were compared to those obtained from rats injected with 1·5 ml normal rat sera. The various experiments consisted of groups of 10 or 15 rats. The percentage protection was calculated by the formula: $A - B/A \times 100$ where A = the number of worms recovered from rats injected with normal rat serum and B = the number of worms recovered from rats injected with 1·5 ml anti-SRP-A sera, heated or IgE depleted anti-SRP-A sera.

Immunization experiments

Brown Norway or Fischer rats were injected i.p. with 1 ml of SRP-A per rat, and boosted 1 month after the first injection with 1 ml of SRP-A. Adult antigens (10 μg/rat) were first injected with *Bordetella pertussis* (5×10^9 units, IPAD, Institut Pasteur Production, France). One month later, the same amount of adult antigen was injected without adjuvant. In each case, 3 days after the second injection, rats were infected percutaneously with 1,000 *S. mansoni* cercariae. Animals were perfused 19 days later, and the degree of protection was evaluated as described for the passive transfer experiments. Control rats were injected with Hanks' Balanced salt solution (saline) or *Bordetella pertussis* diluted in this buffer.

Antibody-dependent cellular cytotoxicity (ADCC)

Macrophage ADCC. Fischer rat peritoneal macrophages were purified 95% by adherence for 2 h in plastic Petri dishes and were incubated overnight in minimal essential medium (MEM) with 10% normal heat inactivated rat sera. The cytotoxicity assay was carried out as previously described (Capron *et al.*, 1975). Effector cells were preincubated for 6 h with the serum samples (10%). After 18 h of incubation with 50 skin-prepared schistosomula, the larvae were examined and counted as dead if they were immobile, granular and opaque and surrounded by one or multiple layers of cells. Statistical analysis has indicated that a percentage of cytotoxicity greater than 25% is significant.

Platelet ADCC. The method of preparation of the rat platelets has already been described (Joseph *et al.*, 1983). Briefly, 2 ml heparinized blood was diluted with 18 ml heparinized Eagle's MEM (EMEM) and centrifuged for 15 min at 400 *g* at 4°C. The supernatant fractions containing platelets was washed with heparinized EMEM and centrifuged for 20 min at 5,000 *g* at 4°C. The platelets were counted in a haemocytometer after dilution in London staining mixture. Fifty skin-schistosomula in 80 μl EMEM, platelets ($7{\cdot}5 \times 10^7$) in 100 μl EMEM and 20 μl of the serum sample were added simultaneously and incubated for 24 h at 37°C and 5% CO_2. Dead schistosomula were counted as previously.

Radioallergosorbent (RAST)

The RAST (Alwine, Kemp & Stark, 1977; Pierce *et al.*, 1983) was used to measure the level of schistosomula specific IgE antibodies in the anti-SRP-A sera. Briefly, schistosomulum homogenate was fixed on diazobenzyloxymethyl paper (DBM paper) (100 μg proteins). The antigen-coated DBM paper discs were incubated with 100 μl of 10 times diluted anti-SRP-A sera or normal sera for 3 h at room temperature. After three washings, the discs were incubated with 50 μl (50,000 ct/min) of ^{125}I-labelled anti-rat IgE overnight at 4°C. Each sample was washed five times and counted in a gamma scintillation counter (Intertechnique, Plaisir, France). The experiments were performed in duplicate.

Prausnitz-Küstner (PK test)

The PK test (Augustin, 1967) was used to control the IgE depletion of the anti-SRP-A serum after

incubation with the anti-IgE immunosorbent. Normal male Wistar rats were given intradermally injections in a shaved area of the back with 100 μl of diluted serum to be controlled. At the same site of injection, the antigen (10 μg protein) was injected intradermally 72 h later, followed by an intravenous injection of 1 ml of 4% Evans blue in 0·15 M NaCl. Blueing of the sites was observed 30 min after the challenge. Quantification was done by measuring opposite diameters of blue sites, and results are expressed as the area in square millimeters.

Measurement of chemiluminescence

The luminol-dependent chemiluminescence of peritoneal macrophages triggered by the IgE-anti IgE reaction, arising from the production of high energy oxygen components, was measured by the Nucleotimètre 107 (Interbio, Le Thillay, France) according to the procedure described by Easmon *et al.* (1980).

Indirect immunofluorescence test

The presence of IgE on the surface of macrophages was revealed by incubation for 30 min with fluorescein isothiocyanate-labelled anti-IgE (Fc specific) (Miles Yeda Laboratories, Israel). After three washes, cells were examined under a fluorescence microscope.

RESULTS

Immunization experiments with SRP-A

Active immunization by SRP-A was investigated in two rat strains, Brown Norway and Fischer. One milliliter of SRP-A was injected i.p. without adjuvant into Brown Norway rats. Thirty days later, when the anti-schistosomula specific IgE response decreased (Fig. 1), Brown Norway rats were reinjected i.p. with SRP-A (1 ml) without adjuvant. The rats were infected percutaneously 3 days after the second injection with 1,000 cercariae, 2 days before the peak of the schistosomula

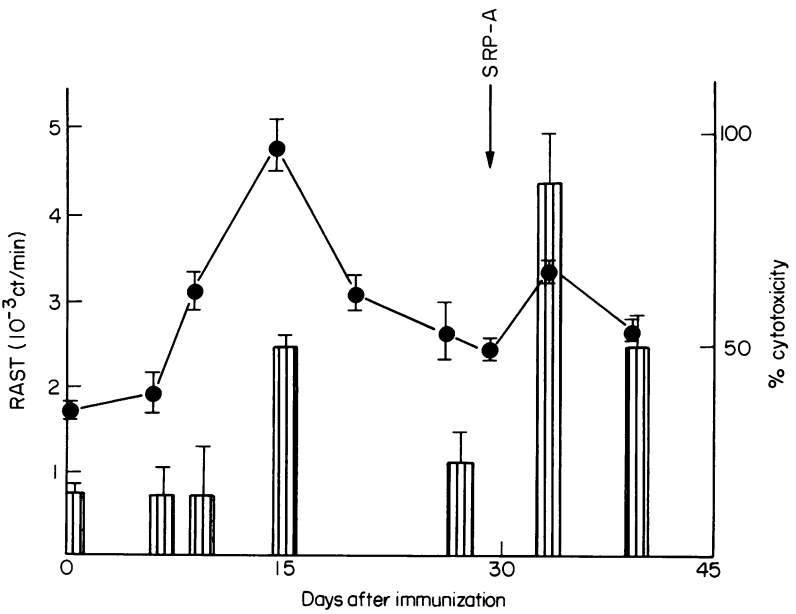

Fig. 1. Anti-schistosomula specific IgE antibodies in sera obtained by immunizing Brown Norway rats with SRP-A. The curve shows the RAST data specific for schistosomula and the histograms show (±s.e.) the % of platelet mediated cytotoxicity for schistosomula with anti-SRP-A sera.

Table 1. Immunization of rats with SRP-A: recovered worms, 19 days after the infection by *Schistosoma mansoni*

Exp. No	Rat strain	Material injected	No of worms recovered ± s.d.	% immunity	*P* value
1	Brown Norway	Saline	49 ± 13		
		SRP-A	27 ± 5·81	46	< 0·01
		B. pertussis	50 ± 4·02	0	
		Adult antigens with *B. pertussis*	59 ± 25	0	
2	Brown Norway	Saline	35 ± 7·07		
		SRP-A	18·6 ± 4·02	47	< 0·03
3	Brown Norway	Saline	30 ± 4·7		
		SRP-A	4·6 ± 1·94	83	< 0·001
4	Brown Norway	Saline	55·3 ± 9·76		
		SRP-A	28·2 ± 6·89	51	< 0·02
5	Fischer	Saline	115 ± 26·84		
		SRP-A	22 ± 6·86	81	< 0·01

The percentage of protection was evaluated by comparing the number of worms in rats injected with SRP-A or adult antigen to the number of worms in rats injected with saline or adjuvant (group of 10 rats for each experiment).

Table 2. Passive immunization of rats with anti-SRP-A sera: worms recovered 19 days after infection by *S. mansoni*

Exp No	Rat strain	Injected serum	No of worms recovered ± s.d.	% immunity	*P* value
1	Brown Norway	Anti SRP-A serum	25 ± 8·10	61	< 0·002
		Normal rat serum	63 ± 4·95		
2	Brown Norway	Anti SRP-A serum	12 ± 7·18	66	< 0·002
		Anti SRP-A serum depleted in IgE	12 ± 4·6	66	< 0·002
		Normal rat serum	27·2 ± 3·54		
3	Fischer	Anti SRP-A serum	32 ± 3·15	32	< 0·001
		Anti SRP-A serum depleted in IgE	56 ± 3·6	0	
		Normal rat serum	47 ± 3·15		
4	Fischer	Anti SRP-A serum	3·6 ± 4·9	83	< 0·017
		Heated anti-SRP-A serum	29·6 ± 18	32	
		Normal rat serum	58·0 ± 5·6		

The percentage of immunity was evaluated by comparing the numbers of worms in rats injected with anti-SRP-A sera or treated anti-SRP-A sera to the numbers of worms in rats injected with normal rat sera.

cytotoxic IgE response (Fig. 1). The recovery of worms, 19 days later, indicated a high degree of immunity (46 to 83%) against the subsequent *Schistosoma mansoni* infection (Table 1). The adult antigens, even when injected with *Bordetella pertussis* adjuvant, were unable to induce such a protective effect suggesting that schistosomula antigens, particularly those shed during the very early stages of schistosomula development, played a key role in the induction of a protective anamnestic immunity. Highly significant protection (81% and $P<0{\cdot}01$) was also obtained with Fischer rats using the same protocol of immunization (Table 1).

Passive transfer experiments with anti-SRP-A sera

In order to confirm the protective role of the humoral response towards SRP-A, passive transfer experiments were carried out by injecting Brown Norway and Fischer rats with anti-SRP-A or normal rat sera as a control. The biological activity of the anti-SRP-A sera was first tested in the rat macrophage-dependent cytotoxicity assay against schistosomula; only highly cytotoxic (80%) sera were collected. Four hours after the percutaneous infection of the rats, they were injected with 1·5 ml of sera intravenously (i.v.). With Fischer rats, transfer experiments clearly demonstrated the involvement of the heat labile antibodies in the protective immunity developed against the *S. mansoni* infection (32% compared to 83%) (Table 2). Moreover after IgE depletion by immunoabsorption of the anti-SRP-A sera, no significant protective immunity was observed (Table 2). A significant degree of protection (61–66%) was observed when Brown Norway rats were injected with anti-SRP-A. No clear decrease of the protection level was observed when the rats were passively transfered with anti-SRP-A sera depleted in IgE (Table 2).

In vitro cellular cytotoxicity

Macrophages or platelets were recovered from Brown Norway rats injected with SRP-A 5 days after the second injection. A significant level of direct cytotoxicity against the schistosomula was observed with platelets (45%) and to a lesser extent with macrophages (40%) in the absence of additional antibodies. Moreover, 40% of the peritoneal macrophages recovered from anti-SRP-A rats, compared with 15% from normal rats, expressed surface IgE as demonstrated by the immunofluorescence test (Table 3). The presence of surface IgE has been also tested by chemiluminescence, in conjunction with an anti-rat IgE serum. While a weak superoxide production was observed with control macrophages from normal rats (60 mv), the level was 6-fold higher (385 mv) when macrophages from anti-SRP-A rats were tested (Table 3).

In immunization experiments, adult antigens failed to induce a protective response against challenge infection. Interestingly, the anti-adult antigens sera obtained with *Bordetella pertussis* as adjuvant exhibited a cytotoxic activity (43·4%) in presence of macrophages as effector cells, abolished after heating of the sera (Table 4). However, the anti-adult IgE did not mediate killing of larvae as effectively as anti-SRP-A IgE (43·4% compared to 92·6%).

Table 3. Detection of surface IgE antibodies on cells recovered from anti-SRP-A rats

Cells	Source	Cytotoxic activity	Fluorescent cells with FITC anti-IgE (%)	Chemiluminescence (mV) with anti-IgE
Macrophages	Brown Norway rats anti-SRP-A	40·2±9·4	35·9±13·4	385
	Normal Brown Norway rats	23·0±9·3	15·2±2·0	60
Platelets	Brown Norway rats anti-SRP-A	45·0±11·6	ND	ND
	Normal Brown Norway rats	5·7±1·0	ND	ND

IgE-dependent cytotoxicity of macrophages and platelets recovered from anti-SRP-A (day 35) and normal Brown Norway rats. Presence of surface IgE on the macrophages was assessed by immunofluorescence test and chemiluminescence measurement with anti-IgE. The chemiluminescence results represent the values (mV) obtained with macrophages recovered from anti-SRP-A or normal rats incubated with anti-IgE serum after deduction of the values obtained by the spontaneous release of the macrophages, respectively from immune or normal rats.

Table 4. IgE dependent cytotoxic activity of various sera

	Macrophages
Anti-SRP-A serum (day 35)	92·6 ± 7·3
Anti-SRP-A serum depleted in IgE	9·4 ± 1·2
Heated anti-SRP-A serum	25·9 ± 2·4
Anti-adult antigens serum	43·4 ± 0·8
Heated anti-adult antigens serum	2·4 ± 3·4
Infected rat serum (day 42)	92·3 ± 6·6
Normal rat serum	16·1 ± 12·3

IgE-dependent cytotoxic activities of anti-schistosome sera with macrophages (IgE depletion of the sera was controlled by a negative PK test).

DISCUSSION

A highly significant level of protection (up to 83%) against *S. mansoni* infection has been observed after immunization of Brown Norway and Fischer rats with schistosomula released products (SRP-A). No significant protection was observed when the rats were immunized with soluble adult antigens, despite the fact that the anti-adult antigens IgE antibodies were able to kill the larvae *in vitro* (44% cytotoxicity). Nevertheless, James, Pearce & Sher (1985) have recently shown in the mouse model that soluble immunogens are present in both early and late developmental stages of the parasite. The experiments were carried out in parallel in two rat strains, in Brown Norway rats which are known to be IgE high responders in contrast with Fischer rats which are relatively low responders, to evaluate the involvement of IgE in protective immunity.

ADCC experiments have clearly demonstrated that incubation of schistosomula with the three classes of effector cells, macrophages, platelets and eosinophils, in the presence of IgE specific for the SRP-A antigens, results in the killing of the larvae (Auriault *et al.*, 1984). Passive transfer experiments using anti-SRP-A sera confirmed the involvement of these antibodies in the protective immunity developed by the rats, and particularly of IgE in the Fischer rat. Discrepancies concerning the role of the IgE antibody in the protective response developed by Brown Norway or Fischer rats were observed in the passive transfer results. A significant decrease of the degree of protection was obtained when the anti-SRP-A sera transferred to Fischer rats were heated or depleted in IgE, clearly suggesting the role of IgE antibodies; this was not the case in the experiments with Brown Norway rats, where probably endogenous non specific IgE saturates the cellular IgE receptors and partially inhibit the binding of the anti-SRP-A to the effector cells. The protection observed in Brown Norway rats could be partly due to the specific IgG present in the sera. Recent studies of the biological activity of the anti-SRP-A serum in the cytotoxic assay demonstrated that besides a strong cytotoxic response, SRP-A elicited the production of IgG antibodies able to kill the larvae *in vitro* in the presence of complement or eosinophils (about 50% cytotoxicity in both mechanisms) (Verwaerde *et al.*, 1985). The anti-SRP-A IgG antibodies revealed the three major schistosomula surface proteins (38, 32 and 21 kD) recognized by sera from infected rats, results which are in agreement with the previous work of Dissous, Dissous & Capron (1981) and Knight *et al.* (1984).

The recent observations by James (1985), of the induction in the C57 Bl/6 mouse of protective immunity by a preparation of nonliving parasite antigens, depending on the route of injection, support the hypothesis that the success of an effective vaccine against schistosomiasis depends not only on the identification of a parasite immunogen but also on the method of antigen presentation.

Interestingly, no adjuvant was necessary in the immunization experiments with SRP-A. Further experiments are in progress to investigate the relationship between the antigen and the adjuvant

effect of the SRP-A, and to determine more precisely how the antigens are presented to the host.

Our results have to be related to those obtained by Knopf, Mangold & Makari (1982) and Ford, Bickle & Taylor (1984) suggesting that the attrition of schistosomes in the rat mainly began to occur in the lung, at day 5. In our experiments, the site of attrition of the worms is still unknown and is now being investigated. These experiments indicate that, at least in rats, IgE is related to the protective immune response.

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