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IMMUNOGENIC AND PROTECTIVE ACTIVITY OF AN EXTRACT OF *SCHISTOSOMA MANSONI*

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The immunogenic and protective activity of an extract of S. mansoni, *obtained by incubation of viable adult worms in buffered saline, was evaluated in rabbits and mice. Animal immunization with this extract resulted in the development of both humoral and cellular immune response. All immunized rabbits developed high levels (91 to 100%) of cytotoxic antibodies as determined by* in vitro *assays of cytotoxic activity of their sera against viable schistosomules. Immunized animals challenged with* S. mansoni *cercariae showed a lower parasite load than that of normal controls. Protective activity was 88.6% and 54.0% in immunized rabbits and mice, respectively.*

Since the pioneer work of Osawa (1930) that attempted to immunize dogs through intravenous injection of either cercariae or adult *Schistosoma japonicum* worms, many authors tried different approaches for animal vaccination against *S. japonicum* or *S. mansoni* infections. In fact, different antigenic preparations from *S. mansoni* have been tested for their efficacy in stimulating resistance to subsequent infection, such as: cercarial, adult worm and egg homogenates alone or in association (Stirewalt, 1953; Oliver & Schneidermann, 1953; Meleney & Moore, 1954); metabolite products; sera of infected or immunized animals and irradiated cercariae (Murrell, Dean & Stafford, 1975; Maddison & Kagan, 1979; Murrell et al, 1979) as well as heterologous antigens or non specific stimulation of the immune system (Dean & Gadd, 1973; Civil, Warren & Mahmoud, 1978). The results so far obtained were not very encouraging leading Clegg &

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Smith (1978) to the conclusion "that very few of the attempts to vaccinate experimental animals with homogenate or extracts of larval or adult *S. mansoni* or *S. japonicum* have given more than marginal protection against challenge".

Recently, in our laboratories, preliminary studies with a *S. mansoni* saline extract (SE) showed a high degree of protection in rabbits against the challenge infection (Scapin et al, 1980).

The present paper showed further results obtained with SE as far as immunogenic and protective activity in rabbits and mice are concerned.

MATERIAL AND METHODS

S. mansoni extract (SE): Adult worms were obtained from mice after perfusion of the portal system (Pellegrino & Siqueira, 1956) with phosphate buffered solution (PBS) 0.15M, pH 6.8. The collected worms were briefly rinsed in two changes of PBS. Adult worms extract was prepared from 1g of fresh worms stored frozen in 10ml of PBS for at least 10 days. The suspension was thawed, filtered through a stainless steel wire mesh at room temperature and centrifuged at 10.000g for 60 min. at 4°C. The supernatant (saline extract, SE) was filtered through a 0.22μ Millipore disk and its protein concentration determined by Lowry's technique (Lowry et al, 1951).

Immunization schedule: Eight rabbits and 30 Swiss adult outbred mice received, with 1 week interval, 2 footpad injections each, of 0.6mg of SE emulsified in a complete Freud adjuvant (CFA) (Difco, with 1 mg/ml *M. tuberculosis*). Twenty-one days later all animals received an i.p. injections of SE containing 1 mg protein. Twelve mice were sacrified before challenge and used for immunological studies. Two rabbits and 10 mice received only CFA without antigen, and eight rabbits and 18 mice were used as normal controls.

Challenge. Three to four months after immunization the animals were infected percutaneously (abdominal region) with 700 cercariae (rabbits) and subcutaneously with 100 cercariae (mice). Forty-five days (mice) and 60 days (rabbits) later the animals were sacrified and submitted to hepatic and mesenteric perfusion (Pellegrino & Siqueira, 1956). Control animals received the same infection with cercariae and afterwards the same type of perfusion.

Schistosomula harvesting: Aliquots (10ml) containing 1500/ml intact cercariae were cooled in an ice bath for 10 min and centrifuged at low speed for 1 min. The packed cercariae were ressuspended in 2ml of Earle's saline solution containing 0.5% lactalbumin hydrolysate, 0.1% glucose, 200 units penicillin and 100 μg streptomicin (Elac) and agitated for 45 sec in Vortex. After several washings with Elac the tail-rich supernatant was decanted and the sedimented bodies resuspended in 10 ml of the solution, incubated under continuous shaking at 37°C for 90-120min and considered to be 2h schistosomula (Ramalho-Pinto et al, 1974; Ramalho-Pinto et al, 1975).

Cytotoxic assay: For the cytotoxic assay the 2h schistosomula (50 larvae/ml) were incubated at room temperature under sterile conditions with 0.1 ml of heat inactivated (30min/56°C) serum obtained from immunized and control rabbits. After 30 min the larvae were washed 3 times with Elac suspended in 0.5ml of either fresh or heat-inactivated guinea pig serum, and incubated overnight at 37°C in humid atmosphere of 5% CO_2. To determine the number of dead or damaged schistosomula, aliquots of 0.05-0.1 ml were expressed as the percentage of dead or damage schistosomula per total larvae counted. The dead schistosomula were characterized by their immobility and granulous aspect (Tavares, 1977).

Serum antibodies: Precipitating antibodies were determined by double immunodifusion tests (Ouchterlony, 1958) in agarose plates using SE containing (1 mg protein/

ml) as antigen. Hemagglutinating antibodies were determined by using human ORh-negative erythrocytes sensitized with SE by glutaraldehyde, according to Avrameas et al (Avrameas, Taudou & Chuilon, 1969).

Immunofluorescent antibodies were determined by a modification of an indirect technique (Williams et al, 1963) using slides containing fixed formalized cercariae.

Plaque forming cells (PFC): Direct PFC to sheep red blood cells (SRBC) was carried out with spleen cells obtained 4 days after i.p. injection of 10^8 SRBC. Mice were studied 90 days after immunization with SE. The results expressed as the number of PFC/10^6 spleen cells (Cunningham & Szenberg, 1968).

Footpad tests: The delayed type hypersensitivity was determined by intradermal injection of 10 μl of SE containing 10 μg protein into the footpad of immunized and control mice. An equal volume of saline was injected into the other footpad of both animals. The local swelling was measured 24h later and the results expressed as the difference in thickness between the two footpads of each animal group.

Blastogenic response: The ability of cultured lymphocytes from normal and immunized rabbits to proliferate in response to stimulation *in vitro* with the antigen SE, phytohemagglutinin (PHA, Difco) and concanavalin A (Con A, Sigma) was determined by assessing DNA incorporation of tritiumlabeled thymidine deoxyribose (^{3}H) TDR. Stimulation index calculated by dividing the counts per minute (CPM) in the presence of antigen or mitogen by CPM obtained without stimulators (Moorhead, 1978).

Inhibition of leucocyte migration: The tests were carried out according to the technique described by Rosenberg, Gray & David (1970). The leucocyte migration in the presence and absence of SE antigen was compared.

Leucocyte adherence inhibition (LAI): The test was performed following the method recomended by Powell, Sloss & Smith (1978), using lymphocytes separated by Ficoll-Hypaque. The LAI value is the difference between the pooled mean percentage of the control of adherent cells and mean percentage of adherence of the tests sample divided by the control value. It is expressed as a percentage.

Statistical analysis: For statistical analysis of the data results from groups of animals in each experiment were compared with apropriate controls by using Student's tests. The degree of protection induced by SE in rabbits, was analysed by using F test with 1–5 degree of freedom (Snedecor & Cochran, 1967).

RESULTS

Degree of protection:

The immuniztion of rabbits and mice with SE resulted in a great reduction of the parasite load induced by the challenge infection. The percentage of protection (assessed by the ratio between the number of worms recovered from immunized and control animals X 100) was 88.6% for rabbits and 54.0% for mice (Table I).

In individual rabbits the percentage of protection varied from 59.3 to 100%. The injection of CFA, without SE, in rabbits and mice, gave no significant protection. In fact, in two rabbits immunized with CFA the mean worm burden was 33.5 and 27.5 for the control rabbits.

Cytotoxic antibodies:

All rabbits immunized with SE developed serum antibodies with cytotoxic activity, as assessed by *in vitro* method, against schistosomula in the presence of complement. The results obtained showed that 91 to 100% of cytotoxic activity could be detected as early as ten days after immunization remaining at the same level until cercarial infection (90-120 days later). The sera of control unimmunized animals showed no cytotoxic activity.

Cell mediated immunity:

Six rabbits showed blastogenic transformation of lymphocytes to Con A, PHA and SE antigen (Table II). The migration inhibition of rabbit peripheral leucocytes sensitized with SE varied from 0.43 to 0.62 (Table III). The leucocytes adherence inhibition of animals sensitized to SE showed a significant reduction of the number of adherent cells in the presence of specific antigen. The percentage of adherence inhibition of immunized rabbits varied from 20 to 77% in tests performed 90-120 days after immunization (Table IV).

In mice, the delayed-type cutaneous reactions to SE indicated that vaccinated animals presented a significant increase of the footpad swelling as compared to the reactions induced with saline (Table V). Control animals gave no footpad swelling. The percentage of adherence inhibition of leucocyte adherence (LAI) with spleen cells of immunized mice showed results significantly ($p < 0.001$) below to those of control animals (Table IV).

Humoral immunity: The agarose immunoprecipitation tests, the passive hemagglutination, and the indirect immunofluorescence, performed 90 days after the immunization in 8 rabbits sensitized with SE, all gave positive results. The titers varied between 1:20 to 1:80, for passive hemagglutination and indirect immunofluorescence and 3 to 4 lines were seen in immunoprecipitation tests.

In 10 out of 30 mice immunized with SE a more intense response to SRBC in animals previously vaccinated with *S. mansoni* antigen was shown. The number of PFC was significantly greater among immunized animals (Table VI). The agarose immunoprecipitation tests and the passive hemagglutination, performed 90 days after immunization with SE both gave positive reaction with titers between 1:40 to 1:320.

Table I

Degree of protection induced in rabbits and mice by immunization with the *S. mansoni* saline extract

Animal	*Nº*	*Number of Worms Recovered*		*Protection (%)*	*Significance P*
		Immunized $\bar{x} \pm SD$	*Unimmunized* $\bar{x} \pm SD$		
Rabbit	8	8.8 ± 11	77 ± 43	88	< 0,005
Mice	18	13 ± 8	28 ± 12	54	< 0,005

Table II

Basic transformation of leucocytes from rabbits sensitized to *S. mansoni* saline extract

Rabbits	*N°*	*^3H Thymidine incorporation by 10^6 rabbit spleen cells in the presence of (stimulation index)*		
Immunized	6	*S. mansoni* (antigen 35μg/ml)	CONa (2μg/ml)	PHA (10μg/ml)
		3.23 ± 0.26	11.15 ± 2.4	3.40 ± 0.25
Unimmunized	3	1.96 ± 0.18	8.77 ± 1.70	1.20 ± 0.22

Table III

Inhibition of leucocyte migration* in rabbits and mice immunized with *S. mansoni* extract **

Animals	*N°*	*Inhibition of leucocyte migration*		*Significance P*
		*Immunized***	*Unimmunized*	
Rabbits	7	0.52 ± 0.07	0.93 ± 0.09	< 0,005
Mice	5	0.63 ± 0.08	0.95 ± 0.07	< 0,005

**S. mansoni* antigen used in a concentration of 50μg/ml

***Stimulation induced 90-120 days after immunization and before infection with *S. mansoni.*

****Immunization with SE + CFA.

Table IV

Percentage of adherence inhibition of spleen cells (mice) and blood peripheral leucocytes (rabbits)*

Animals	*N°*	*Percentage of adherence inhibition*		*Significance P*
		*Immunized***	*Unimmunized*	
Rabbir	6	49 ± 19	87 ± 9	< 0,005
Mice	6	61 ± 17	103 ± 40	< 0,001

**S. mansoni* antigen used in a concentration of 30μg/ml

***Immunization with SE + CFA; stimulation induced 90-120 days after immunization and before infection with *S. mansoni.*

Table V

Delayed-type hypersensitivity in mice immunized with *S. mansoni* extract

Mice	*Mean difference between the two footpad swelling** ($\bar{x} \pm SD$)	*Significance P*
Immunized**	0.24 ± 0.18	< 0,05
Control	0.04 ± 0.01	

*Footpad swelling measures (mm) 24h after injection of 10μg of *S. mansoni* antigen. Tests performed 90 days after immunization and before infection with *S. mansoni.*

**Immunization with SE + CFA.

Table VI

Hemolytic plaque formation (IgM) by 10^6 spleen cells of mice I.V. sensitized to 10^8 SRBC.*

The animals were previously immunized with *S. mansoni* saline extract.

Mice	*N°*	*PFC/10⁶ spleen cells*	*Significance P*
Sensitized**	4	3.968 ± 1.252	< 0,005
Controls	4	2.480 ± 246	

*Stimulation with 10^8 SRBC 90-120 days after immunization and before infection with *S. mansoni.*

**Immunization with SE + CFA.

DISCUSSION

The SE extract containing antigens of *S. mansoni* adult worms obtained by incubation of viable worms in buffered saline was capable of inducing high degree of protection in rabbits and mice against a challenge with *S. mansoni* cercariae.

The fractionation of the extract in Sephadex G-200, revelated the presence of 5 different fractions containing protein, carbohydrate, nucleic acid and other lower molecular substances. Only fractions I and II gave strong immunoprecipitations reaction with rabbit anti-SE serum (Scapin et al, 1980).

It has been demonstrated by transmission electron microscopy that the incubation of schistosoma worms in saline solution, even for short lenght of time, can induce extensive morphologic alterations in their tegument (Ernst & May, 1975). This incubation will probably provoke the realese into the storage solution of several antigenic fractions

from the worms membrane (Scapin et al, 1980). On the other hand, since the mean survival of *S. mansoni* worms in NaCl 0.15M solution varied from 260 to 340 min (Kohn et al, 1979) and taking into account the fact that in the early period of incubation (before freezing) most of the parasites are still alive and in condition to secrete many antigenic products, it seems logical to admit the presence of this material also in the SE extract.

Simultaneously with the development of a highly significant resistance against a controlled cercarial infection, the vaccinated rabbits also developed a high degree of complement dependent cytotoxic activity.

In fact the protection induced in rabbits with SE, as well as the levels of lethal antibodies developed in these animals, were significantly higher than those produced by other vaccination methods (Murrell, Dean & Stafford, 1975; Capron et al, 1977).

All other laboratory tests performed looking for cellular or humoral immunity showed that animals were highly stimulated by the SE injections.

By comparing the results obtained in rabbits immunized with SE, with those of control animals, one can see that the number of adult worms harvested in the two groups were 8.8 for immunized and 77.0 for unimmunized animals, showing that the immunization with SE indeed induced an inhibitory effect on rabbit percutaneous infection with *S. mansoni* cercariae.

The level of protection in mice vaccinated with SE was 54%. Athough less intense than that obtained in rabbits, the degree of protection in mice was also highly significant. The protection induced in mice that only received CFA was not significant (11%).

Considering that good results so far obtained with the SE immunization, further studies must be done with purified fractions aiming to increase the protective activity and to isolate specific antigen(s) that vaccinated the laboratory animals.

RESUMO

Avaliou-se em coelhos e camundongos, a atividade imunogênica e protetora de um extrato antigênico de *Schistosoma mansoni*, obtido pela estocagem de vermes adultos em solução salina tamponada (Extrato Salino). A imunização dos animais determinou o desenvolvimento de resposta imune celular e humoral, avaliada por provas específicas. Todos os coelhos imunizados com ES, desenvolveram altos níveis de anticorpo citotóxico (91 a 100%), determinados pela avaliação da atividade citotóxica *in vitro*, contra esquitossômulos. Concluiu-se que os coelhos e camundongos imunizados com o extrato salino apresentaram diminuição da carga parasitária oriunda da infecção posterior com cercárias do *S. mansoni*, em relação aos controles. Os percentuais de proteção foram de 88.6% e 54% para os animais vacinados (coelhos e camundongos respectivamente).

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