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T-cell helper response to antigens of *Schistosoma mansoni* in CBA mice

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SUMMARY

It is believed that a T-cell helper response against the schistosome surface is a necessary prerequisite for the development of protective immunity in schistosomiasis. Accordingly, the carrier effect has been used to assay eleven antigenic preparations of *Schistosoma mansoni* for their helper T-cell priming against surface components of the schistosomula. Three weeks after i.v. injection of the preparation, CBA mice were injected with schistosomula coated with trinitrophenol (TNP) and 4 days later, their spleens were assayed for plaque-forming cells (PFC) against TNP.

Formalin-fixed schistosomula and crude adult worm tegumental membrane induced the highest response; only thirty schistosomula or 10 μg of crude membrane protein were needed to generate a T-cell helper response equivalent to that induced by a living infection. All other antigenic preparations, including formalin-fixed cercariae, live miracidia, eggs and adult worm culture fluid, generated some response indicating the presence of shared carrier components.

INTRODUCTION

Although the development of immunity to reinfection with *S. mansoni* has been demonstrated in mice and other experimental animals (Sher, Mackenzie & Smithers, 1974; Smithers & Terry, 1976), it has not been possible to induce a similar level of immunity by vaccination with non-living schistosome material (Murrell, Dean & Stafford, 1975). The inability to induce protection artificially has limited progress towards identifying the schistosome antigens which stimulate protective immunity. These antigens are likely to be associated with the surface of the worm; results from *in vivo* and *in vitro* studies show that the efferent arm of the host's immunity causes initial damage to the worm's surface membrane (Hockley & Smithers, 1970; McLaren, Clegg & Smithers 1975). In mice, the mechanism of immunity depends upon antibody and non-sensitized cells (Sher, Smithers & Mackenzie, 1975; Mahmoud, Warren & Peters, 1975) and at least one antibody involved is an IgG (Sher *et al.*, 1976) and is presumably dependent on T-cell helper activity for its induction.

It seems likely therefore that a T-cell helper response against components of the schistosome surface is a necessary prerequisite for the development of protective immunity. If this supposition is true, then one approach to the problem of identifying the schistosome's 'protective' antigens would be to measure the induction of T-cell helper activity against the schistosome surface after vaccination with various antigens. Such an assay is possible by using the schistosomula as carriers for a hapten and measuring the anti-hapten response in normal and vaccinated animals. In analogous experiments using protein carriers, the anti-hapten response has been shown to be due to cooperation between T and B cells (Mitchison, 1971). Previous studies with this assay in mice exposed to infection with *S. mansoni* have demonstrated a massive early carrier response to the schistosomula which was absent in nude mice, and was assumed to be due to specific T-helper cells (Ramalho-Pinto, De Souza & Playfair, 1976).

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MATERIALS AND METHODS

Animals. A Puerto Rican strain of *S. mansoni* was used for this work; its maintenance has been described previously (Smithers & Terry, 1965). Outbred male Parkes mice weighing 18–22 g and infected 7 weeks earlier with 150 cercariae of *S. mansoni* provided the source of adult worms and eggs. Inbred female CBA mice weighing 18–24 g were used for the assay of antigens.

Antigenic Preparations.

(i) *Eggs.* These were recovered from the intestines of infected Parkes mice. The intestines were stored at 4°C for 48 hr in 1·7% saline, rinsed in 0·7% saline, finely minced with scissors and incubated at 37°C for 90 min. The tissue was then homogenized for 2 min and the suspension filtered through two sieves of apertures 300 and 180 μm (Endecott Ltd). Finally, the eggs were washed six times in saline by centrifugation at 100 *g* for 5 min.

(ii) *Soluble egg antigen (SEA).* This was prepared according to Colley (1971). Schistosome eggs in phosphate-buffered saline (PBS) at a concentration of 75,000/ml were homogenized in a Potter Elvehjem homogenizer at 4°C. The homogenate was then centrifuged at 4°C for 2 hr at 100,000 *g*. The clear supernatant fluid obtained after centrifugation was collected and stored in liquid nitrogen.

(iii) *Miracidia.* Freshly prepared schistosome eggs were placed in a petri dish containing dechlorinated tap water at 20°C and exposed to bright light for 1½ hr. The free-swimming miracidia, which were released from the eggs after hatching were poured into a 40 ml centrifuge tube and centrifuged at 250 *g* for 5 min. The supernatant containing the miracidia was then transferred into another tube and placed in an ice bath for 15 min. The drop in temperature caused the miracidia to settle to the bottom and enabled them to be washed twice with cold dechlorinated tap water. Finally, they were resuspended in 0·7% saline and used immediately for injection.

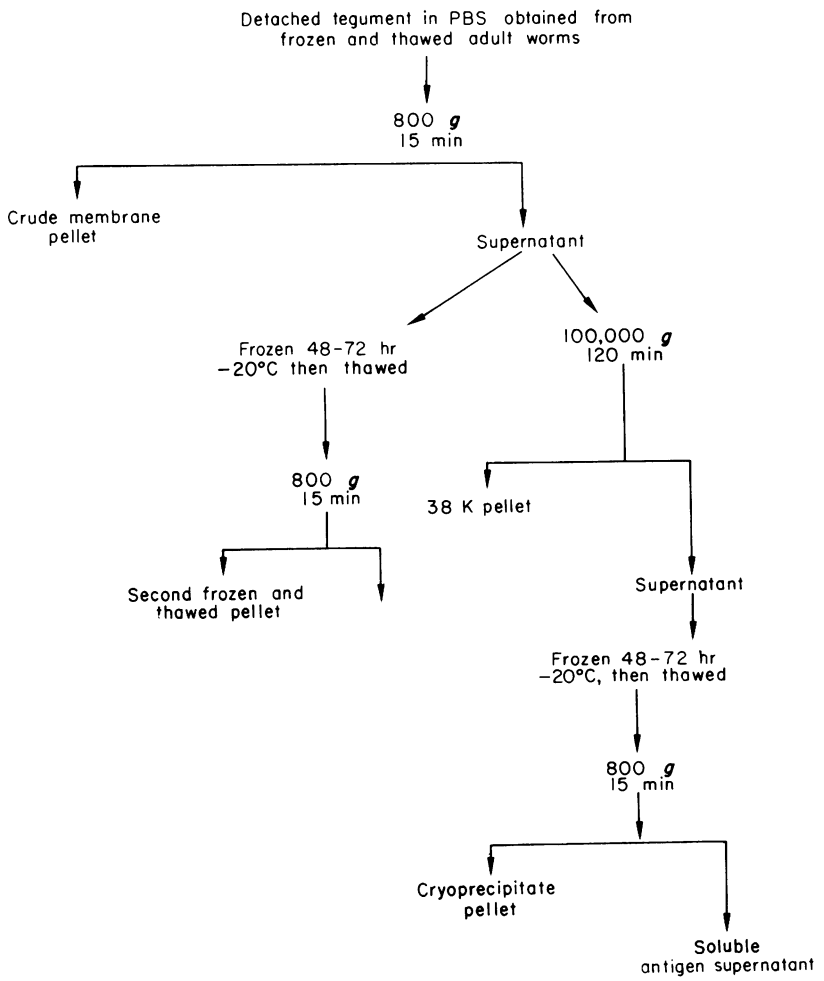

FIG. 1. Preparation of adult worm tegumental fractions.

(iv) *Formalin fixed cercariae.* Cercariae shed from snails within 2–3 hr were treated with 0·06% formaldehyde for 5 min, washed twice with phosphate-buffered saline, and finally resuspended in Hanks's balanced salt solution containing 20 mM Hepes buffer (HBSSH), pH 7·2.

(v) *Schistosomula.* These were prepared *in vitro* using a modification of the method of Ramalho-Pinto *et al.* (1974 and 1976). Cercariae were induced to concentrate at the surface of the water in which they were shed by the addition of streptomycin sulphate (2 mg/ml final concentration) and sodium benzyl-penicillin (1000 mg/ml final concentration). The concentrated cercariae were then transferred into a 15-ml centrifuge tube, chilled in an ice-bath for 10 min and centrifuged at 100 *g* for 10 sec. The pellet was rewashed, suspended in 1 ml of cold distilled water, and mechanically agitated for 60 sec using a Vortex mixer. The resulting mixture of cercarial bodies and tails was then layered onto HBSSH contained in a glass column (25 × 1 cm) and after 25 min at room temperature the cercarial bodies sedimented to the bottom of the column and the tails were removed by decanting with a pipette. The bodies which were contaminated with less than 5% tails, were transferred into Earle's balanced saline containing antibiotics (Elac) and incubated for 3 hr at 37°C in an atmosphere of 5% CO_2/95% air. This procedure caused the cercarial bodies to transform into schistosomula. Finally, the schistosomula were washed with HBSSH, fixed with 0·06% formaldehyde for 5 min, washed twice and resuspended in HBSSH.

(vi) *Adult worm tegumental fractions.* Five fractions were prepared by a modification of the method of Kusel (1972). Adult worms perfused from infected Parkes mice were frozen in PBS at −20°C and then thawed at 37°C. The worms were then placed onto a 100 mesh stainless steel screen and the detached surface tegument washed through the mesh with 100–150 ml PBS at 4°C. The washings were then subjected to differential centrifugation at 4°C to give 5 fractions as shown in Fig. 1.

Each particulate fraction was examined by electron microscopy. The crude membrane pellet contained coils and sheets of multilaminate membrane, membraneous bodies (Hockley & McLaren, 1973), small vessels bounded by trilaminate membrane and some interstitial material. The second frozen and thawed (Second FT) pellet and the 38 K pellet consisted mainly of interstitial tissue, smaller trilaminate vesicles and occasional membraneous bodies; the large multilaminate membrane sheets were rarely seen in the second frozen and thawed pellet and were absent from the 38 K pellet. The cryoprecipitate appeared to contain degenerate trilaminate membrane sheets.

(vii) *Excretion/secretion antigen (ES antigen).* Adult worms were recovered from infected Parkes mice by sterile perfusion and maintained for 72 hr in Elac at approximately 30 worms per 10 ml media. The culture fluid was dialysed for 12 hr with changes at 4 and 8 hr against 50 × volume sterile PBS at 4°C. The culture fluid was then frozen at −20°C for 48 hr, thawed at 37°C and the resulting precipitate removed by centrifuging at 400 *g* for 5 min. Appropriate dilutions in PBS of the clear supernatant were made prior to injection.

TNP conjugation. Schistosomula were coated with trinitrophenol (TNP) as described by Ramalho-Pinto *et al.* (1976).

Sheep red blood cells (SRBC) were lightly coated and horse red blood cells (HRBC) were heavily coated with TNP according to the method of Kettman & Dutton (1971).

Protein estimations. These were performed using the method of Lowry *et al.* (1951).

Assay for T-cell helper effect and carrier priming. Groups of four to six mice were injected i.v. with 0·2 ml of a solution containing the appropriate immunizing antigen. Three weeks later the mice were challenged with an i.v. inoculation of 100 TNP-schistosomula. Four days later the mice were killed by cervical dislocation and their spleens removed. Spleen-cell suspensions were prepared in RPMI 1640 containing bicarbonate and 20 mM Hepes buffer at pH 7·2 (RPMI), by gently pressing individual spleens through a 180 μm stainless steel sieve. The cells were washed once and resuspended in 10 ml RPMI. For the assay, 100 μl of the appropriate dilution of the spleen-cell suspension (to give 100–200 plaques per chamber),

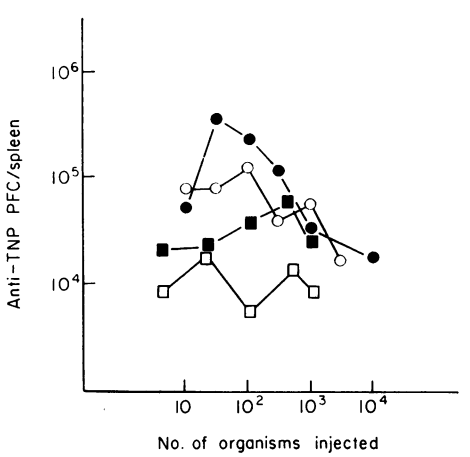

FIG. 2. Levels of carrier-priming induced after injection of different stages of *S. mansoni.* (●), Schistosomula; (○) cercariae; (■), miracidia; (□), eggs.

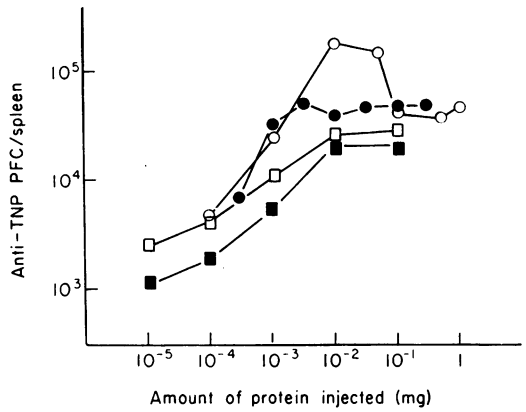

FIG. 3. Levels of carrier-priming induced after injection of particulate fractions of the tegument of *S. mansoni*. (○) Crude membrane; (●), second FT pellet; (□), cryoprecipitate; (■), 38 K pellet.

50 μl of RPMI, 25 μl of fresh guinea-pig serum and 25 μl of a 20% suspension of TNP-SRBC were mixed together and then introduced into a Cunningham chamber (Cunningham & Szenberg, 1968). The edges of the chamber were sealed with hot wax, and the chamber incubated at 37°C for 30 min. After incubation the number of haemolytic plaques was counted using a Nikon stereo microscope at × 18 magnification. The spleen-cell suspensions were also assayed against unconjugated SRBC, and this value was subtracted from the number of plaque-forming cells (PFC) obtained with TNP-SRBC to yield the number of direct (IgM) anti-TNP producing PFC. The numbers of PFC per spleen cells in Figs 2 and 3 represent the geometric mean of the anti-TNP response per group of 4–6 mice. In each experimental series, control mice which had not been immunized with antigen were injected with TNP schistosomula and their anti-TNP response assayed 4 days later; the geometric mean (± s.e.) of the response from 125 mice was 1899 (2023–1783).

RESULTS

Eleven antigenic preparations were assayed for their helper T-cell priming effect against the schistosomular surface. The results are expressed in three graphs (Figs 2–4). Fig. 2 compares the response after i.v. injection of four stages of the schistosome parasite; formalin-fixed schistosomula and cercariae and live miracidia and eggs. The highest number of plaques, up to 320,000 was obtained after injection of only thirty schistosomula; cercariae induced 116,000 plaques after injection of 100 organisms. Both schistosomula and cercariae showed evidence of a decrease in response with increasing doses.

Fig. 3 compares the response after i.v. injection of the 4 particulate fractions of the schistosome tegument. The crude membrane pellet was the best antigen in this series giving up to 180,000 plaques

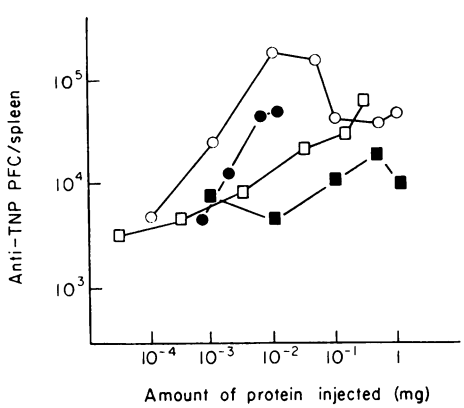

FIG. 4. Levels of carrier-priming induced after injection of soluble antigens of *S. mansoni*. (●), ES Ag; (□) Soluble antigen; (■) SEA; (○), crude membrane, as a comparison.

TABLE 1. Specificity of carrier-priming by fixed schistosomula or crude membrane pellet

Groups	Priming	Time (days before challenge)	Challenge	Anti-TNP PFC per spleen (Geometric mean ± s.e.)¶	*P* value
A	—	—	100 TNP-schistosomula	1870 (2141–1633)	
B	30 FF Schistosomula‡	21	100 TNP-schistosomula	130,105 (191,711–88,296)	< 0·001*
C	10 μg Membrane	21	100 TNP-schistosomula	89,591 (92,740–86,548)	< 0·001*
D	30 Live cercariae	10	100 TNP-schistosomula	115,812 (134,767–99,523)	< 0·001*
E	—	—	1×10^8 TNP-HRBC	12,266 (13,791–10,910)	
F	30 FF Schistosomula	21	1×10^8 TNP-HRBC	12,525 (14,083–11,140)	n.s.†
G	10 μg Membrane	21	1×10^8 TNP-HRBC	14,804 (17,674–12,401)	n.s.†
H	30 FF Schistosomula	21	Saline	217 (311–151)	
I	10 μg Membrane	21	Saline	359 (475–272)	

n.s. = Not significant.
* Compared to Group A.
† Compared to Group E.
‡ Formalin-fixed schistosomula.
¶ n = 5.

after injection of only 10 μg protein. All the particulate tegumental antigens showed evidence of a plateau response which was reached after injection of between 1–10 μg protein.

Fig. 4 shows the response after i.v. injection of three soluble antigens; the soluble fraction of the isolated tegument, SEA and ES antigen. None of these soluble antigens produced a response approaching the levels induced by crude membrane or schistosomula.

The priming induced by fixed schistosomula and crude membrane pellet appeared to be highly specific, as there was no corresponding increase in the response to TNP attached to an unrelated carrier, HRBC (Table 1).

DISCUSSION

All eleven antigenic preparations used in this study induced some degree of helper-cell response in mice against the schistosomular surface, indicating the presence of cross-reacting carrier components. It was not unexpected that the homologous antigenic preparation, formalin-fixed schistosomula, induced the highest response; the number of plaques produced was of the same order as that produced by mice 10 days after infection with thirty to forty cercariae (Table 1 and Ramalho-Pinto *et al.*, 1976). Isolated crude adult tegumental membrane induced a response almost as high, corroborating the observations of Kusel *et al.* (1975a) that the adult worm surface and the schistosomula surface contain common antigenic determinants. Kusel, Mackenzie & McLaren (1975b) have also shown that soluble common antigens of the surfaces of schistosomula and adults are present in culture media which have been used to maintain parasites *in vitro*. It was not surprising therefore to find that the ES antigen also induced a T-cell helper response.

More surprising was the high level of response induced by cercariae and miracidia. Both these stages are free-living and possess a conventional trilaminate surface membrane as seen by electron microscopy, strikingly different from the multilaminate surface membrane of the parasitic schistosomulum and adult worm stages (Hockley, 1973). In view of these structural and functional differences one might also expect immunochemical diversity of the various surface membranes; in addition, the cercariae possess a thick carbohydrate coat or glycocalyx. Nevertheless, our results indicate the presence of common surface components in all four stages, and show that the glycocalyx on formalin-fixed cercariae does not prevent sensitization of the host by underlying membrane components.

The response induced by eggs and SEA to the schistosomula surface was unexpected. A similar level

of response was produced by both preparations suggesting that with living eggs the response was due to SEA diffusing through pores in the egg shell (Race *et al.*, 1971). SEA is responsible for the delayed hypersensitivity (granuloma) reaction around eggs which are deposited in the tissues of the host (Boros & Warren, 1970). Several workers have shown that immunity to reinfection develops in the absence of egg deposition (Smithers & Terry, 1969). However, since there is a common carrier component in SEA, schistosomula and adult worm surfaces, egg production and the release of SEA may play some role in the development of immunity to reinfection. In this connection it is of interest that Sher *et al.* (1974) showed that immunity to reinfection which developed in mice with an all male infection, was 2–3 weeks later than the immunity developed by animals infected with bisexual egg-laying worms.

The object of this study was to gain insight into the antigens of the parasite which are responsible for inducing protective immunity. Assuming the surface of the parasite acts as the target of the host's immune effector response and IgG is involved, then a T-cell helper response against the worm's surface will be necessary before immunity can develop. On the assumption that all the helper effects described here are T-cell mediated, our results show that the best antigenic preparations for inducing such a response in mice are formalin-fixed schistosomula and crude adult tegumental membrane. Surprisingly small amounts of both preparations were needed to generate maximum T-cell helper response.

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