

The isolation of a 22 kDa band after SDS-PAGE of *Schistosoma mansoni* adult worms and its use to demonstrate that IgE responses against the antigen(s) it contains are associated with human resistance to reinfection

D.W.DUNNE¹, M.WEBSTER¹, P.SMITH¹, J.G.LANGLEY^{1*}, B.A.RICHARDSON^{1†}, A.J.C.FULFORD¹, A.E.BUTTERWORTH¹, R.F.STURROCK², H.C.KARIUKI³ & J.H. OUMA³

¹ Division of Microbiology and Parasitology, Department of Pathology, Cambridge University, Cambridge CB2 1QP, UK

² Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, London, UK

³ Division of Vector Borne Diseases, Ministry of Health, Nairobi, Kenya

SUMMARY

In schistosomiasis endemic areas, intensities of reinfection after treatment are greater amongst young children than amongst adults, and high levels of parasite-specific IgE are associated with resistance to reinfection in an age-dependent manner. Previously we have reported that, in Western blots, a 22 kDa band was recognized by human IgE and that the incidence and intensity of *S. mansoni* reinfection were significantly lower amongst individuals who had IgE against this band, compared with those who did not (Dunne *et al.* 1992). Here we report the isolation of a 22 kDa SDS-PAGE band, its incorporation into ELISA and the demonstration that levels of human anti-22 kDa IgE had a significant negative correlation with intensities of subsequent reinfection. Rabbit anti-22 kDa band serum recognized the outer tegument, gut tegument, and the collecting ducts and flame cells of adult worms. The 22 kDa band antigen(s) was also present in 'lung'- and 'post-lung' schistosomula stages of *S. mansoni*, and in *S. haematobium*, *S. bovis* and *S. japonicum* adult worms. Metabolic labelling of schistosomula and worms demonstrated the *in vitro* synthesis and release of 22 kDa antigens.

Keywords *Schistosoma mansoni*, IgE, human immunity, antigen, tegument

INTRODUCTION

In *Schistosoma mansoni* endemic areas of Kenya intensities of infection, measured by the number of parasite eggs excreted by individuals, rise during the first decade of life, as children accumulate worms, and then decline. The susceptibility of younger children to infection is even more apparent when intensities of reinfection are studied after the elimination of existing parasites by chemotherapy. The relatively low levels of reinfection seen amongst adults cannot be entirely explained by lack of exposure to reinfection, and may be due to the development of protective immunity which is dependent on the age of the individual and/or the length of time they have been exposed to the parasite (Butterworth *et al.* 1985, 1988). Similar observations have been reported from *S. haematobium* endemic areas in The Gambia (Wilkins *et al.* 1987), in *S. mansoni* infected populations in Brazil (Dessein *et al.* 1988) and else-where (Gryseels, 1994). This susceptibility to reinfection observed in younger children is not due to a general hypo-responsiveness to schistosome antigens. Indeed, these individuals are often found to have more vigorous anti-parasite immune responses than older, apparently resistant, individuals. However, it has been found that immune responses in human populations change with age. Younger children, for example, have higher levels of IgM and IgG2 against T-independent carbohydrate epitopes. IgG4 rises and falls with age and is the response which most closely reflects intensities of infection in human populations (Dunne *et al.* 1988), whereas levels of worm specific IgE are generally higher in older individuals (Dunne *et al.* 1992). Thus, age-dependent resistance to reinfection in these areas could be due to qualitative rather than quantitative changes in anti-parasite immune responses. Such qualitative

Correspondence: D.W.Dunne,

Received: 21 August 1996

Accepted for publication: 14 November 1996

* Present addresses: Wayne State University School of Medicine, Department of Immunology and Microbiology, Canfield Avenue, Detroit, Michigan, USA

† MRC Research Programme on AIDS in Uganda, c/o Uganda Virus Research Institute, PO Box, Entebbe, Uganda

differences could involve both the nature of the antigens responded to and the type of response itself.

In studies designed to identify particular immune responses that may be characteristic of 'reinfection-resistant' individuals, it has been reported that high levels of IgE against parasite antigens are associated with subsequent resistance to reinfection and multiple-regression analysis has shown that these responses still correlate with subsequent low intensities of reinfection even when age is treated as a confounding factor (Hagan, *et al.*, 1991, Dessein *et al.*, 1992).

In a previous study, 115 individuals aged between six and 66 years, from a *S. mansoni* endemic area of Kenya, were treated and their individual intensities of reinfection followed over the next three years. Six months after treatment, at which time no reinfection had occurred in the population, sera were taken and used to measure antibody subclass responses against different stages of the parasite life-cycle. These responses were related to the cumulative intensities of infection observed between one and three-years after treatment. This study showed that only the IgE responses against adult worm correlated negatively with subsequent reinfection intensities. In other words, high levels of anti-adult worm IgE were associated with low intensities of reinfection and were, therefore, either involved in mediating protective immunity against *S. mansoni* infections or were an immunological marker for a 'reinfection-resistant' phenotype. Adult worm antigens recognized by IgG and IgE from all the individuals in this study were examined in Western blot analysis, which showed that IgG recognized a large number of antigens, whereas the pattern of antigens recognized by IgE was much more restricted. In particular, a band of 22 kDa was strongly recognized by IgE in many, although not all, sera that showed high levels of IgE response against adult worm (Dunne *et al.* 1992). Quantitative estimates of the recognition by IgE of this 22 kDa band were not available, but the relationship between IgE recognition of this band and intensity of reinfection in this population was examined qualitatively on the basis of IgE-specific Western blots. This showed that the incidence and intensity of reinfection were significantly lower among those individuals in the population who had detectable IgE against the 22 kDa band in Western blots, compared with those individuals who had no detectable IgE against this band (Dunne *et al.* 1992).

In this paper we report the isolation of this 22 kDa band by electro-elution from SDS-PAGE and demonstrate that the antigen(s) in this isolated band are specifically recognized by IgE from schistosome infected patients. The band was incorporated into human antibody isotype ELISA assays and used to measure 22 kDa band-reactive IgE in sera from Kenyan individuals in the infection study described

above (Dunne *et al.* 1992), and show that quantitative IgE recognition of this band also correlates with resistance to reinfection following treatment. A rabbit antiserum was raised against the 22 kDa worm band and used to characterize the distribution of these antigen(s)/epitopes in different development stages of the parasite life-cycle, in different species of schistosome, and in the tissues of *S. mansoni* adult worms. In addition, we present evidence that antigens recognized by this antiserum may be secreted or shed from the migratory schistosomulum stage of the parasite's development.

MATERIALS AND METHODS

Parasite antigens and Rb antisera

A Puerto Rican isolate of *S. mansoni* was maintained in Cambridge in outbred TO strain mice (Harlan UK Ltd, Bicester, UK) and albino *Biomphalaria glabrata* as described previously (Dunne *et al.* 1981, 1988). Parasite antigen preparations used were similar to those described by Dunne *et al.* (1988, 1992). *Adult worm antigen* (Ad.Worm Ag) was prepared from *S. mansoni* worms perfused (Smithers & Terry, 1965) from mice that had been infected six weeks previously. Perfusion fluid contained Lactalbumin hydrolysate with Earl's salts (Life Technologies Ltd., Paisley, UK) plus Sodium hydrogen carbonate and trisodium citrate (Sigma, Poole, UK) to avoid damage to the worm tegument during perfusion (Simpson *et al.* 1981). Recovered worms were washed in perfusion medium, without trisodium citrate, and then snap frozen in liquid nitrogen. Frozen worms were ground into a paste, allowed to thaw and then centrifuged at 10 000 *g* at 4°C for one h. 1 mM N-Tosyl-L phenylalanine chloromethyl ketone and 1 mM Phenylmethylsulphonylfluoride (Sigma) were added, the soluble material was further centrifuged, filtered through a 0.22 µm sterile filter and then stored at -20°C until required. *Worm tegument* (W.Teg) was obtained from worms perfused and washed as above but subsequently incubated in freshly made PBS (pH 7.2) for one h at 37°C to remove the outer tegument. The resulting supernatant was centrifuged at 100 000 *g* for one h and PBS soluble material then stored at -20°C. *Soluble egg antigen* (SEA) was prepared as described previously (Dunne *et al.* 1992). *Cercarial antigen* (Cerc) was obtained from cercariae harvested within three h of being shed from snails, snap frozen in liquid nitrogen and then processed identically to the adult worms. *S. japonicum* worms were a generous gift from Dr N.A. Maloney (London School of Hygiene and Tropical Medicine); *S. haematobium* and *S. bovis* worms were kindly donated by Dr A. Agnew of Leeds University. 22 kDa PAGE band was obtained by separating Ad.Worm (200 µg) on 14% SDS-PAGE, staining with Coomassie blue and

cutting the 22 kDa band out as a gel slice. The 22 kDa band was electro-eluted from the excised gel using a Model 422 Electro-eluter (BioRad Laboratories Ltd., Hemel Hempstead, UK), using membranes with a molecular weight cut off of 3.5 kDa. The isolated material was assessed using PAGE followed by silver staining and Western blotting. Anti-22 kDa band rabbit serum was raised by giving four immunizations of electro-eluted 22 kDa band with RIBI adjuvant (RIBI Immunochem Research Inc., Hamilton, MT USA), at four weeks intervals. Rb anti-Ad.Worm serum was obtained by immunization with 40 µg of Ad.Worm Ag in Complete Freund's adjuvant, followed by three further immunizations in Incomplete Freund's adjuvant at four week intervals.

Patients and Sera

Human sera examined were from the same collection described by Dunne *et al.* (1992). Briefly, sera were taken from a 'reinfection' study cohort of 115 individuals aged six to 66 years from the Kangundo Location in Machakos District, Kenya. These individuals were from within a wider study of 1500 individuals, who were part of a comparative chemotherapy programme, described elsewhere (Butterworth *et al.* 1991). The 'reinfection' study cohort were selected as being parasite egg positive in a preliminary survey and as having high frequencies of observed contact with water that contained infected snails. Six further stool samples were collected from each of these selected individuals and examined in duplicate Kato preparations (Sturrock, Butterworth & Houbba 1976) to give an estimate of pretreatment infection intensities. The serum samples examined here were collected immediately before treatment (Bleed A) and at six months post-treatment (Bleed B). Individuals whose treatment failed or who had zero egg counts at the time of the definitive pretreatment stool survey were eliminated from the study. Further individuals, who fulfilled the selection criteria, were added to the study at this point, which meant that in Dunne *et al.* (1992) the individuals in Bleeds A and B were overlapping but not identical. Consequently, results from the pre-treatment sera were tested only against pre-treatment intensity of infection; and the post-treatment sera results were analysed only against the intensity of reinfection. In this paper, the sera used (72 individuals) comprised only those with sera from both Bleed A and Bleed B. This allowed pre- and post-treatment immune responses to be compared directly with each other as well as with pre- and post-treatment intensities of infection. The number of sera examined was further restricted because some of those used previously were no longer available. Nonetheless, the patterns of infection and reinfection with age of the 72 individuals

used in this study were representative of that found by Dunne *et al.* (1992).

ELISA

Human anti-schistosome IgM, IgG subclasses (Dunne *et al.* 1988), IgE and IgA (Dunne *et al.* 1992) ELISA assays were carried out as described previously. Serum antibodies were detected with human isotype specific MoAb as in Dunne *et al.* (1992), developed using o-phenylenediamine, read spectrophotometrically at 490 nm and the results were expressed as mean 490 nm OD for triplicate assays. Sera were assayed for specific IgE at 1/20 dilution. Other isotype assays used the conditions and reagents described by Dunne *et al.* (1992).

SDS-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed using the method of Laemmli (1970) on 14% PAG and either silver stained (Wray, Boulakis & Hancock 1981) or transferred to nitro-cellulose sheets (Towbin, Staehelin & Gordon 1979) and probed with schistosome-specific rabbit antibodies or human sera (Western blot). The binding of rabbit IgG and human IgG and IgE was visualized using the methods and reagents described in detail by Dunne *et al.* (1992). The products of immunoprecipitation experiments were separated on 8 to 20% gradient polyacrylamide gels following the procedures described by Taylor, Cordingley & Butterworth (1984).

Metabolic labelling of different *S. mansoni* developmental stages

Schistosomula were recovered from the lungs of mice using techniques modified from those described previously (Clegg 1965, Sher, Mackenzie & Smithers 1974, Lazdins, Stein, David & Sher 1982). Briefly, five to 11 days after percutaneous infection with between 5000 and 10000 cercariae, the lungs of TO strain mice were removed and minced at low speed for 15 to 20 s in a Waring blender (MSE Scientific Instruments, Crawley, UK). The lung tissue/schistosomula suspension was transferred to sterile petri dishes and incubated at 37°C for one to two h, before being sieved through graded metal riddles (down to a mesh size of 106 µm). The filtrate containing the schistosomula was centrifuged, resuspended in a small volume of medium, and layered over a 45% Percoll (Pharmacia LKB Biotechnology, Milton Keynes, UK) gradient. Tubes were centrifuged at 220 g for ten min. The pellet, containing the schistosomula, was washed several times and resuspended in sterile Minimum Essential Medium. Immature worms were recovered from mice by perfusion of the portal system at 14 and 16

days post-infection, and adult worms were recovered as 6 weeks post-infection (Smithers & Terry 1965). All these freshly isolated parasites were washed 3–4 times, and then resuspended in sterile, methionine-free medium. Newly transformed schistosomula were incubated in Minimum Essential Medium for three h and then washed and transferred to methionine-free NCTC. [^{35} S] Methionine was added to the culture medium to give a concentration of 100 mCi/ml and the parasites were cultured overnight at 37°C in a 5% CO₂ atmosphere before they, and the used culture fluid, were collected. [^{35} S] incorporation was determined by liquid β scintillation in a 1216 Racbeta liquid scintillation counter (Pharmacia LKB Biotechnology, UK). Fluorography was used to visualize the immunoprecipitated antigens after PAGE, using the techniques previously described by Taylor *et al.* (1984).

Immune-localization

Electronmicroscopy and immunogold staining with Rb anti-22 kDa band serum and control normal rabbit serum, was carried out on freshly isolated *S. mansoni* worms at the London School of Hygiene and Tropical Medicine, using standard methods of embedding in Lowicryl K4M resin, silver enhanced immunocolloidal gold labelling, and double stained with uranyl acetate and Reynold's lead citrate. Immunofluorescence localization was carried out on 5 μ m sections of formalin fixed, wax embedded adult *S. mansoni* worms, using Rb anti-22 kDa band serum, a normal Rb serum as control, and goat anti-Rb IgG-FITC (Sigma). In order to detect surface Sm22, freshly isolated worms were washed in Minimum Essential Medium, fixed in 2% formaldehyde solution, blocked with 50% goat serum and, after extensive washing, probed with Rb anti-22 kDa band serum and goat anti-Rb IgG-FITC.

Statistical analysis

Relationships between pre- and post-treatment antibody levels in the study population and intensities of infection and reinfection were examined by Spearman's rank correlation coefficient. Relationships between different antibody responses were examined by Pearson's correlation coefficient. Statistical significance was inferred as at least $P < 0.05$.

RESULTS

Isolation of a 22 kDa band recognized by human IgE

The 22 kDa band was originally identified in IgE-specific

Western blots of *S. mansoni* Ad.Worm Ag probed with sera from infected Kenyans who had high anti-Ad.Worm IgE detected by ELISA (Dunne *et al.* 1992).

Figure 1 shows antigens in *S. mansoni* adult worm recognized by IgG and IgE present in sera from selected IgE anti-Ad.Worm responding individuals. Western blot of Worm.Teg also contained a 22 kDa band recognized by human IgE (results not shown) and as this band was more discrete compared with the Ad.Worm band, after PAGE and silver staining (Figure 2a), this preparation was used for the first attempts to isolate the 22 kDa for further study. Figure 2a shows this electro-eluted band when re-run in PAGE and then silver stained. The tegumental location of 22 kDa antigen(s) was confirmed in immunolocalization experiments described below. It was found that this band could just as easily be isolated from the more complex Ad.Worm Ag and, subsequently, this was routinely done. In order to establish that this 22 kDa band was indeed the band recognized by human IgE, it was analysed alongside Ad.Worm Ag in Western blot and probed with pooled sera from either normal individuals or from infected individuals known to respond with IgE to the 22 kDa band in Ad.Worm Ag. Figure 2b shows IgG and IgE from the human 22 kDa responder serum pool specifically reacted with the single band present in the electro-eluted antigen preparation and with a number of antigens, including one of 22 kDa, in Ad.Worm Ag. This confirmed that the correct band had been isolated and all subsequent 22 kDa preparations used were verified by PAGE followed by silver staining and Western blot.

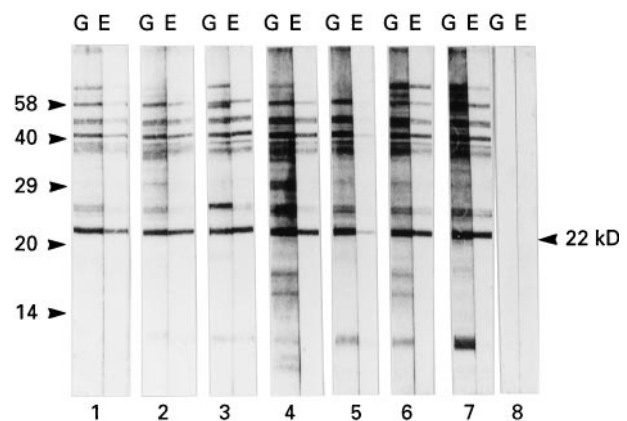


Figure 1 The recognition of a 22 kDa band in *S. mansoni* Ad.Worm Ag by human IgE and IgG. Western blot of *S. mansoni* Ad.Worm Ag probed with sera from seven *S. mansoni* infected individuals (Lanes 1 to 7) or from an uninfected human control (Lane 8). The worm antigens recognized by IgE (E) and IgG (G) present in the serum from each individual are shown, together with the molecular weight markers and the position of the 22 kDa band.

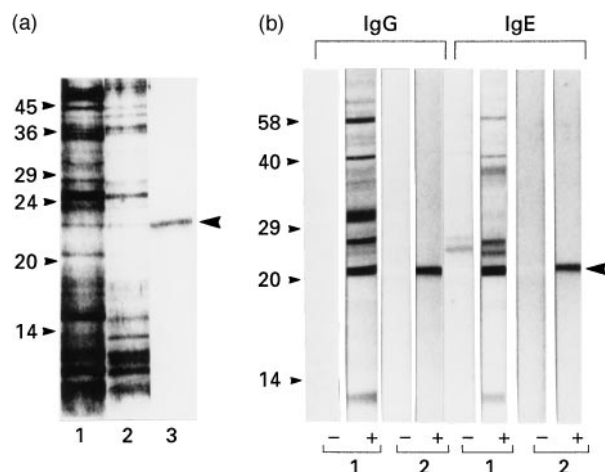


Figure 2 Purification of a 22 kDa band from *S. mansoni* Ad.Worm Ag by electro-elution after SDS-PAGE & recognition by human IgE and IgG in Western Blot. Figure 2a. Silver stain after SDS-PAGE of *S. mansoni* Ad.Worm Ag (Lane 1); Worm.Teg (Lane 2); a 22 kDa band electro-eluted from *S. mansoni* Ad.Worm Ag (Lane 3). The positions of standard molecular weight markers are shown. Figure 2b *S. mansoni* Ad.Worm Ag (Lanes 1) and an electro-eluted 22 kDa band from *S. mansoni* Ad.Worm Ag (Lanes 2) were tested against normal, uninfected, human sera (–) and a pool of human sera for *S. mansoni* infected individuals known to respond to the 22 kDa band (+); and probed for both IgG and IgE reactivity. The relative positions of standard molecular weight markers are shown.

The 22 kDa band was incorporated directly into ELISA after electro-elution by coating microtitre plates with the antigen in SDS elution buffer diluted with conventional carbonate ELISA coating buffer. When the antigen was

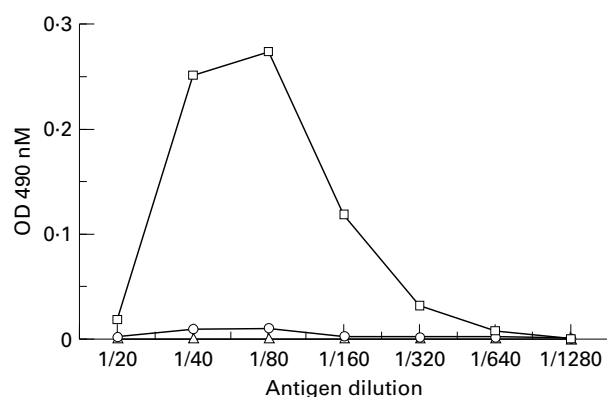


Figure 3 Human IgE response in ELISA to an electro-eluted band from *S. mansoni* adult worm. IgE reactivity against the electro-eluted 22 kDa band, serially diluted with ELISA coating buffer, and then tested against pooled sera from *S. mansoni*-infected individuals known to react against the 22 kDa band in IgE specific Western blots (□); *S. mansoni*-infected individuals who did not react against the 22 kDa band with IgE (○); or normal, uninfected individuals (△). The results are shown as mean optical densities at 490 nm for triplicate assays with each antigen dilution.

titrated in this way and tested against sera from an *S. mansoni*-infected individual who was an IgE responder against the 22 kDa band in Western blot; an infected individual who was a 22 kDa band non-responder; and an uninfected individual; and then probed for human IgE; only the serum from the infected 22 kDa responder was found to be reactive. As can be seen in Figure 3, no ELISA reactivity was found at the highest titre of antigen (high concentrations of SDS probably preventing the binding of the antigen to the microtitre plate), but specific IgE binding was detected at antigen dilutions of 1/40 and 1/80.

This 22 kDa band ELISA assay was used to test the IgM, IgA, IgE and IgG subclass reactivity of *S. mansoni* infection sera, taken before and after treatment. The antibody responses were compared with the intensities of infection before treatment and subsequent reinfection. Only the IgE responses against the 22 kDa showed statistically significant correlations. The pretreatment IgE responses against 22 kDa were not significantly correlated to pretreatment egg counts ($n = 61$, $r = -0.026$, NS), but showed a significant negative correlation with reinfection ($n = 58$, $r = -0.264$, $P < 0.05$); similarly, post-treatment IgE responses against 22 kDa did not significantly correlate with pretreatment egg counts ($n = 69$, $r = -0.163$, NS), but showed a statistically significant negative correlation with intensities of reinfection ($n = 68$, $r = -0.286$, $P < 0.02$). The pre- and post-treatment anti-22 kDa band IgE responses had a strong positive relationship ($n = 58$, $r = 0.810$, $P < 0.001$). Anti-22 kDa IgE responses were not significantly associated with the age of the population either before or after treatment. Thus, high levels of IgE specific for the 22 kDa band, either before or six months after treatment, were significantly associated with low intensities of subsequent reinfection.

Rb anti-22 kDa reactivity with different stages of the *S. mansoni* life-cycle and other schistosome species

Isolated 22 kDa band was used to immunize a rabbit and the resulting antiserum was tested in Western blot against both the immunising antigen preparation and Ad.Worm Ag. Figure 4 shows that a band of approximately 22 kDa is specifically recognized by this antiserum in both the electro-eluted preparation and Ad.Worm Ag. A band of a higher molecular weight is apparently also recognized in Ad.Worm Ag. However, this higher molecular weight band is recognized by normal rabbit serum and, therefore, is probably the result of non-specific interactions.

Rb anti-22 kDa band serum was tested in Western blot on homogenates of *S. haematobium*, *S. japonicum* and *S. bovis* adult worms. A band at approximately 22 kDa was recognized in all these species, although the intensity of the band,

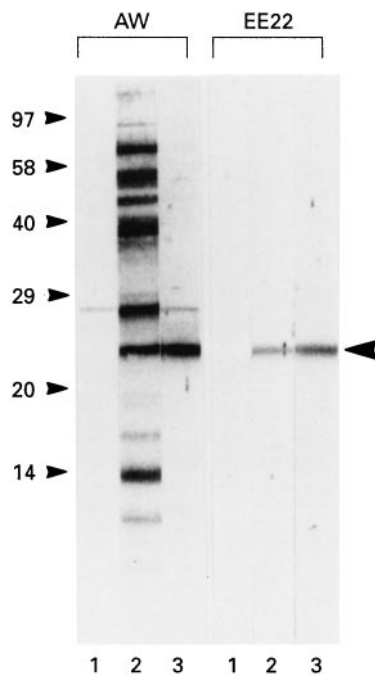


Figure 4 The anti-*S.mansoni* Ad.Worm Ag IgG reactivity of Rb antiserum raised by immunization with electro-eluted 22 kDa band. Western blot of Ad.Worm Ag and an electro-eluted 22 kDa band (EE22), probed with normal Rb serum (Lanes 1); Rb anti-*S. mansoni* Ad.Worm Ag serum (Lanes 2); and Rb anti-22 kDa band (Lanes 3).

after the development of the enzyme substrate, was weaker with the heterologous antigens, particularly *S. japonicum* (results not shown).

Similarly, a 22 kDa band was recognized in Western blots of *S. mansoni* lung stage schistosomula and 18 days post-infection, immature worms. No antigen was recognized by the anti-22 kDa band serum in *S. mansoni* SEA, while the results obtained with the cercarial stage of the life-cycle were ambiguous (results not shown).

Distribution of 22 kDa band epitopes in adult worm tissues

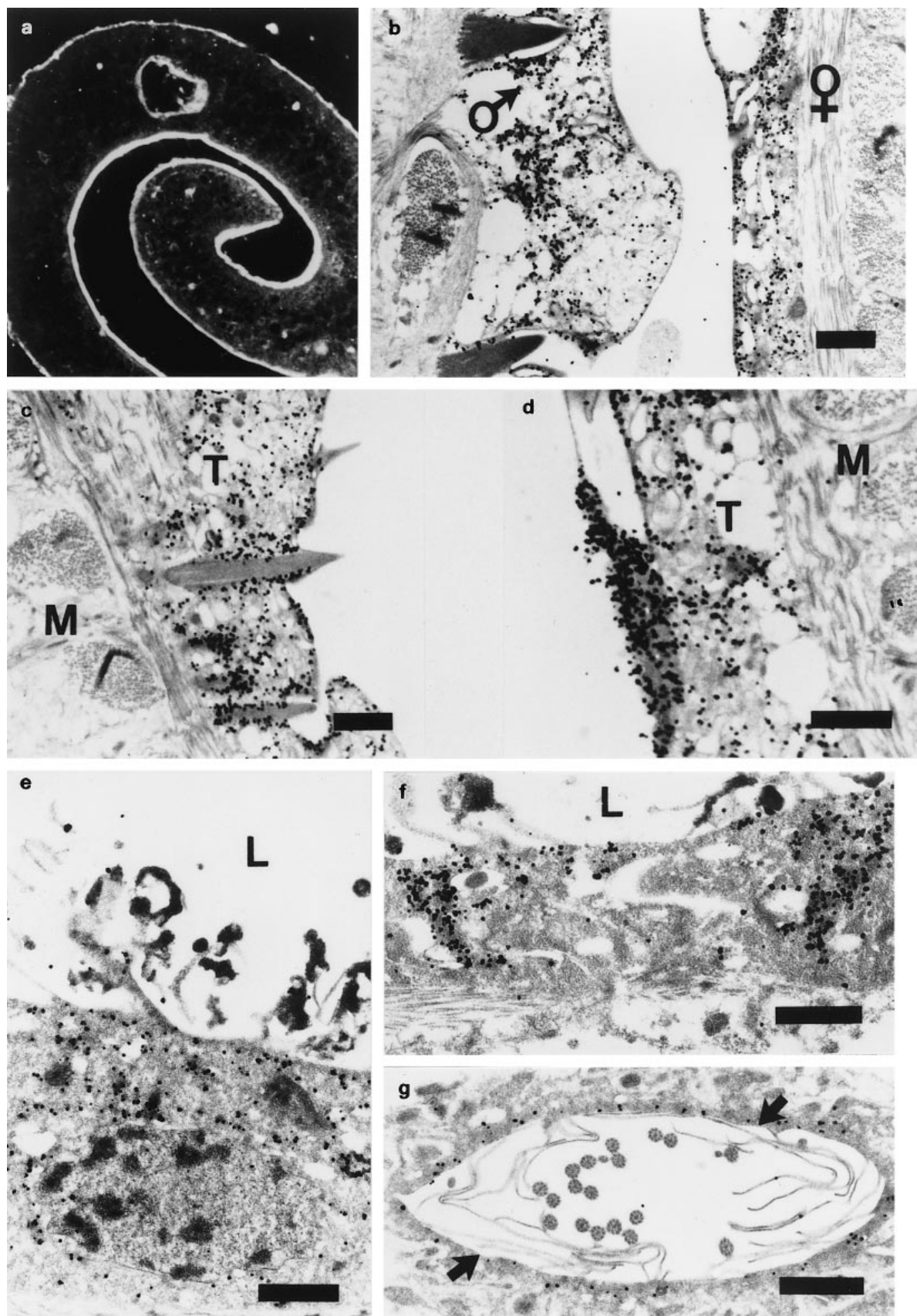
Rb anti-22 kDa band serum was used in an indirect immunofluorescence assay on sections of adult *S. mansoni* worms. As shown in Figure 5a, the epitopes recognized by this antiserum were strongly associated with the tegument of the

adult worm. In addition, some small discrete patches of fluorescence were seen within the parenchyma and gut wall. No evidence could be found for a surface localization for 22 kDa band epitopes by using the same technique on intact, fixed worms. More detailed study by immunogold staining of sections of adult worms under electronmicroscopy, showed strong localization of epitopes recognized by Rb anti-22 kDa serum throughout the tegument layer of male and female worms (Figure 5b), often associated with the outer areas of the tegumental spines (Figure 5c) and clustering close to the outer surface of the tegument (Figure 5d). Epitopes recognized by the same antiserum were also located in the syncytium of the gut tegument (Figure 5e), again sometimes in clusters (Figure 5f). Some immunostaining was also found around the flame cells and collecting ducts of the parasites excretory system (Figure 5g). The normal rabbit serum that was used for Figure 4 was also tested in these immunofluorescence and immunogold assays. As no staining was seen with control serum by either method, it was concluded that the pattern of tegument, gut and excretory system staining with Rb anti-22 kDa band serum reflected the distribution of 22 kDa band antigen(s) in adult worms.

Immunoprecipitation of metabolically labelled antigens shed or secreted/excreted during *in vitro* culture

Despite the lack of evidence for a surface localization for 22 kDa band associated epitopes, their presence in the parasite gut epithelium, outer tegument and flame cells, suggested that they may be shed or secreted/excreted by the parasite. Therefore, radioimmunoprecipitations were carried out using Rb anti-22 kDa band serum, and human infection sera from individuals who were either responders or non-responders for the 22 kDa band in Western blot and ELISA. Newly transformed schistosomula; schistosomula recovered from the lungs of mice between five and 11 days after infection; immature worms recovered from portal perfusion between 14 and 18 days after infection; and six week old, adult worms; were metabolically labelled, *in vitro*, with [³⁵S]Methionine. Immunoprecipitations were carried out using either homogenates of the whole parasite or the culture fluid after removal of the parasites and high speed centrifugation. An antigen,

Figure 5 The distribution of the epitopes recognized by Rb anti-22 kDa band serum in the adult *S. mansoni* worm. Fig. 5a. Photomicrograph showing the Indirect Fluorescent Antibody localization of epitopes recognized by rabbit anti-22 kDa band serum. An adult male *S. mansoni* worm is seen in cross-section, photographed under low power magnification (original magnification 25 times) showing strong fluorescence of both the dorsal and ventral tegument; and a cross section of the parasite gut can be seen to the centre top. Fig. 5b to 5f are electronmicrographs of *S. mansoni* adult worm, probed with Rb anti-22 kDa band and gold-labelled anti-rabbit IgG, each electronmicrograph contains a bar that represents a 2 μ . Figs. 5b, 5c & 5d show the worm tegument; Figs. 5e & 5f show worm gut tegument and Fig. 5g shows an excretory flame cell. Male (δ) and female (φ) worms can be seen in Fig. 5b.; other labels are as follows: muscle (M), tegument (T), gut lumen (L).



with an apparent molecular weight of 22 kDa, was immunoprecipitated by Rb anti-22 kDa band serum and by human 22 kDa band responder, but not 22 kDa non-responder sera, from homogenates of 11 day lung-schistosomula, immature worms and adult worms. This immunoprecipitated antigen was seen only irreproducibly when five day lung-schistosomula were used, and never seen with newly-transformed schistosomula. Figure 6a illustrates these results, showing the immunoprecipitation of a 22 kDa band with Rb anti-22 kDa serum, and two human 22 kDa responder sera, but not with two human non-responder sera. Figure 6b shows that anti-Ad.Worm Ag serum and Rb anti-22 kDa serum both immunoprecipitated a 22 kDa band from the culture fluid that was used to incubate 18 day old immature worms overnight. Similar results were obtained from 11 day old lung stage schistosomula and adult worms and indicating that, at least under *in vitro* culture conditions, 22 kDa band antigen(s) recognized by humans and rabbit are shed or secreted/excreted by the parasite.

DISCUSSION

Many studies in schistosomiasis endemic areas have shown that adults suffer much lower intensities of infection or reinfection than children. Age-dependent reductions in exposure to infection are probably insufficient to account for this. Such studies cannot distinguish between age or length of exposure as predominant factors involved in the development of resistance in the adults, because populations in these areas are exposed to infection from an early age and, therefore, age and length of exposure are co-variables, and this question is subject to an ongoing debate (Gryseels 1994). Whatever the dominant factor is, acquired immunity to reinfection remains a possible explanation for at least part of the underlying mechanism, and seroepidemiological data from studies of communities where human *S. haematobium* or *S. mansoni* infection is endemic have shown that specific IgE responses are associated with resistance to reinfection independently of age (Hagan *et al.* 1991, Rihet, Demeure, Bourgois, Prata & Dessein 1991). We have found similar general findings in *S. mansoni* infected communities in Kenya and have described the antigens recognized by IgE responding individuals in Western blots (Dunne *et al.* 1992). In that study, IgE-specific Western blots of Ad.Worm Ag demonstrated that a 22 kDa antigen band was commonly recognized by IgE, and incidence and intensities of reinfection were significantly lower among those who had detectable IgE compared with those who did not (Dunne *et al.* 1992). Here we report the isolation of the 22 kDa band by electro-elution, and the confirmation that it is recognized specifically by IgE from *S. mansoni* infected

individuals. This antigen band was incorporated directly into human antibody isotype specific ELISA assays. The 72 individuals in the present study group were the part of the study population we described previously (Dunne *et al.* 1992), but some were excluded from the current assays because either their pre- or post-treatment serum samples were missing or had been exhausted. It is worth noting that IgE responses both immediately before and six months after treatment significantly correlated with low intensities of subsequent reinfection, and that the IgE anti-22 kDa responses did not have statistically significant correlation with age. Thus, whether or not this response is directly involved as an immune effector mechanism in human immunity, IgE responses against this 22 kDa band are a predictive marker for a resistant human phenotype.

The Rb antiserum raised against the electro-eluted material was specific for the 22 kDa band recognized by human IgE, and recognized epitopes present in the outer tegument, the gut tegument and around the collecting duct and flame cells of the adult worm excretory system. Thus, although this antiserum was not necessarily monospecific, in that a number of antigens of a molecular weight of around 22 kDa might be contained within the immunizing material, the epitopes that it recognized had a sharply restricted distribution within the tissues of the parasite. The epitopes recognized by anti-22 kDa band serum are also present on antigens of the same molecular weight in the 'lung'- and 'post-lung' schistosomular stage of the parasite life-cycle and in *S. haematobium*, *S. bovis* and *S. japonicum*. 22 kDa antigens recognized by this antiserum were only weakly recognized in Western blots of pre-lung stage *S. mansoni* schistosomula and cercariae, and no antigen recognition was evident in the egg stage. The absence or relatively weak reactivity of 22 kDa antigen(s) in eggs and cercariae/early schistosomula, respectively, may be due to developmentally regulated variation in these antigen(s), or due to qualitative reduction in their expression. Greater amounts of the antigen(s) would be expected in the adult worm because the parasite's gut does not start to develop until the lung-stage of migration. It is not known whether the same antigen is being recognized in the outer and gut tegument and the excretory system. Nonetheless, localization of the antigens recognized by the Rb anti-22 kDa serum to these sites suggests that they may be shed or secreted/excreted by the parasite during an active infection. This might be important in the development of potentially protective immune responses directed against these antigens and their availability as targets for immune effector mechanisms. Antigen(s) carrying anti-22 kDa band epitopes were found, often clustered, in close proximity to the outside of the surface tegument (see Figure 5d). However, no evi-

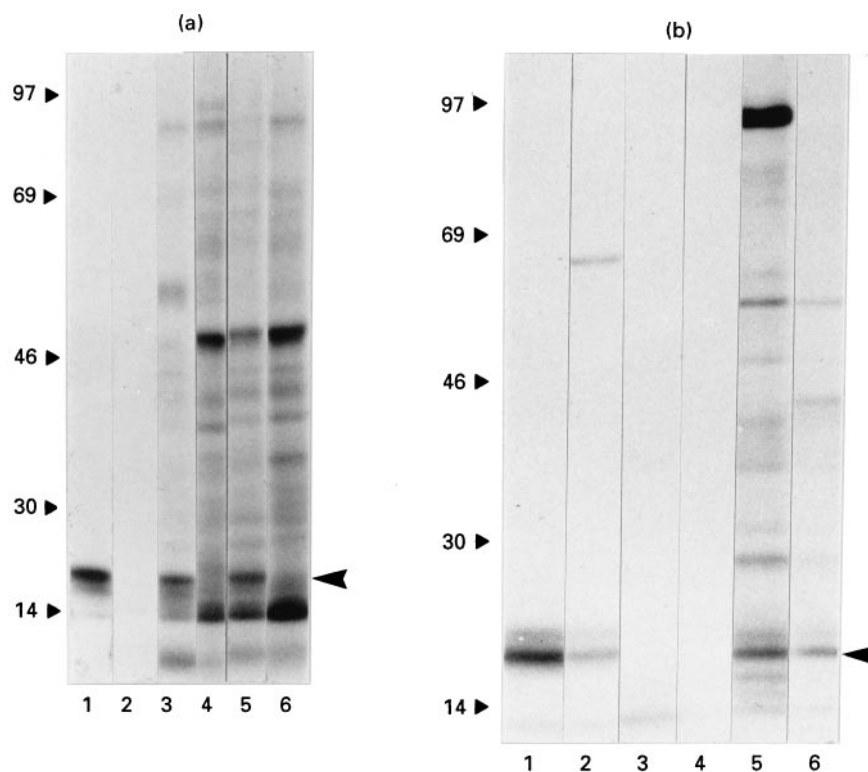


Figure 6 Radioimmunoprecipitation of metabolically labelled antigens from 'lung-stage' *S. mansoni* schistosomula by Rb and human anti-22 kDa band sera & the release Ags from immature worms during *in vitro* culture. Figure 6a. Homogenates of schistosomula, isolated from the lungs of mice 11 days after infection and metabolically-labelled, *in vitro*, with [^{35} S] Methionine, were immunoprecipitated with Rb. anti-22 kDa band serum (Lane 1), normal uninfected human serum (Lane 2), human sera from two individuals infected with *S. mansoni* and known to respond to the 22 kDa band in Western blot and ELISA (Lanes 3 & 5); human sera from two individuals infected with *S. mansoni*, but non-responders to the 22 kDa band (Lanes 4 & 6). The immunoprecipitates were run on SDS-PAGE alongside radiolabelled molecular weight markers (relative position shown) and fluorographed. Figure 6b. 18 day old *S. mansoni* worms were recovered from the portal system of mice, metabolically labelled *in vitro* with [^{35} S] Methionine and homogenized after culture. Both labelled 18 day old worm Ag (Lanes 1, 3 & 5) and the 'used' culture fluid (Lanes 2, 4 & 6) were immunoprecipitated with Rb anti-22 kDa band serum (Lanes 1 & 2); Normal Rb serum (Lanes 3 & 4); and Rb anti-*S. mansoni* Ad.Worm Ag (lanes 5 & 6), then run on SDS-PAGE alongside radiolabelled molecular weight markers (relative position shown) and fluorographed. The position of the 22 kDa band is arrowed in both 6a and 6b.

dence was found for a surface location for these epitopes. Metabolic labelling of schistosomula and worms did demonstrate the synthesis and release of 22 kDa antigens recognized by Rb anti-22 kDa band serum and human infection sera, at least, under the *in vitro* culture conditions used here. It is possible that such antigens released from the surface tegument and gut, or from the excretory system, during the course of migration, could trigger specific IgE mediated effector mechanisms creating a local environment which might be damaging to the parasite.

A number of *S. mansoni* antigens with molecular weights in the 22 kDa region have been described (Auriault *et al.* 1984, Frances & Bickle 1992, Jeffs *et al.* 1991, Knight *et al.* 1984, Lodes & Yoshino 1989, Oligino, Percy & Harn 1988, Suama & Strand 1990, Simpson, James & Sher 1983, Stein

& David 1986) and the 22 kDa SDS band described there could contain a mixture of any of these antigens. We have used the Rb anti-22 kDa band to isolate clones expressing a 22.6 kDa antigen from cDNA libraries derived from *S. mansoni* adult worms, and IgE responses against this recombinant antigen are also significantly correlated with resistance to reinfection after treatment (Webster *et al.* 1996).

ACKNOWLEDGEMENTS

We wish to thank Frances Jones, Hazel Ebling and Andrew Hammersley for their technical assistance and Dr Q.D. Bickle for providing the freshly isolated *S. mansoni* worms for the electronmicroscope studies. Financial support for this work was provided by the European Commission,

the Rockefeller Foundation, the Medical Research Council and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

REFERENCES

- Auriault C., Damonville M., Verwaerde C., Pierce R., Joseph M., Capron M. & Capron, A. (1984) Rat IgE directed against schistosomular-released products is cytotoxic for *Schistosoma mansoni* schistosomular *in vitro*. *European Journal of Immunology*, **14**, 132–138
- Butterworth A.E., Capron M., Cordingley J.S. *et al.* (1985) Immunity after treatment of human schistosomiasis mansoni. II. Identification of resistant individuals, and analysis of their immune responses. *Transactions of the Royal Society for Tropical Medicine and Hygiene*, **79**, 393–408
- Butterworth A.E., Fulford A.J., Dunne D.W., Ouma J.H. & Sturrock R.F. (1988) Longitudinal studies on human schistosomiasis. *Philosophical Transactions of the Royal Society, London, Biology*, **321**, 495–511
- Butterworth A.E., Sturrock R.F., Ouma J.H., Mbugua G.G., Fulford A.J.C., Kariuki H.C. & Koech D. (1991) Comparison of different chemotherapy strategies against *Schistosoma mansoni* in Machakos District, Kenya: effects on human infection and morbidity. *Parasitology*, **103**, 339–355
- Clegg J.A. (1965) *In vitro* cultivation of *Schistosoma mansoni*. *Experimental Parasitology*, **16**, 133–147
- Dessein A.J., Begley M., Demeure C., Calliol D., Fueri J., dos Reis M.B., Andrade Z.A., Prata A. & Bina J.C. (1988) Human resistance to *Schistosoma mansoni* is associated with IgG reactivity to a 37-kDa larval surface antigen. *Journal of Immunology*, **140**, 2727–2736
- Dessein A.J., Couissinier P., Demeure C. *et al.* (1992) Environmental, genetic and immunological factors in human resistance to *Schistosoma mansoni*. *Immunological Investigations*, **21**, 423–454
- Dunne D.W., Lucas S., Bickle Q.D., Pearson S., Madgwick L., Bain J., & Doenhoff M.J. (1981) Identification and partial purification of an antigen (ω_1) from *Schistosoma mansoni* eggs which is putatively hepatotoxic in T-cell deprived mice. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **75**, 54–71
- Dunne D.W., Grabowska A.M., Fulford A.J.C., Butterworth A.E., Sturrock R.F., Koech D. & Ouma J.H. (1988) Human antibody responses to *Schistosoma mansoni*: the influence of epitopes shared between different life-cycle stages on the response to the schistosomulum. *European Journal of Immunology*, **18**, 123–131
- Dunne D.W., Butterworth A.E., Fulford A.J.C. *et al.* (1992) Immunity after treatment of human schistosomiasis: association between IgE antibodies to adult worm antigens and resistance to reinfection. *European Journal of Immunology*, **22**, 1483–1494
- Frances P. & Bickle Q. (1992) Cloning of a 21.7 kDa vaccine-dominant antigen gene of *Schistosoma mansoni* reveals an EF hand-like motif. *Molecular and Biochemical Parasitology*, **50**, 215–224
- Gryseels B. (1994) Human resistance to *Schistosoma* infections: Age or experience? *Parasitology Today*, **10**, 380–384
- Hagan P., Blumenthal U., Dunn D., Simpson A. and Wilkins H. (1991) Human IgE, IgG4 and resistance to reinfection with *Schistosoma haematobium*. *Nature*, **349**, 243–245
- Jeffs S.A., Hagen P., Allen R., Correa-Oliveira R., Smithers S.R. & Simpson A.J.G. (1991) Molecular cloning and characterization of the 22-kilodalton adult *Schistosoma mansoni* antigen recognized by antibodies from protectively vaccinated with isolated tegumental surface membranes. *Molecular and Biochemical Parasitology*, **46**, 159–168
- Knight M., Simpson A.J.G., Payares G., Chaudri M. & Smithers S.R. (1984) Cell-free synthesis of *Schistosoma mansoni* surface antigens: stage specificity of their expression. *EMBO*, **3**, 213–219
- Laemmli U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685
- Lazdins J.K., Stein M., David J.R. & Sher A. (1982) *Schistosoma mansoni*: rapid isolation and purification of schistosomula of different development stages by centrifugation on discontinuous density gradients of Percoll. *Experimental Parasitology*, **53**, 39–44
- Lodes M.J. & Yoshino T.P. (1989) Characterization of excretory-secretory proteins synthesized *in vitro* by *Schistosoma mansoni* primary sporocysts. *Journal of Parasitology*, **75**, 853–862
- Oligino L.D., Percy A.J. & Ham D.A. (1988) Purification and immunochemical characterization of a 22 kilodalton surface antigen from *Schistosoma mansoni*. *Molecular and Biochemical Parasitology*, **28**, 95–104
- Rihet P., Demeure C.E., Bourgois A., Prata A. & Dessein A. (1991) Evidence for an association between human resistance to *Schistosoma mansoni* and high anti-larval IgE levels. *European Journal of Immunology*, **21**, 2679–2686
- Sauma S.Y. & Strand M. (1990) Identification and characterization of glycoposphatidylinositol-linked *Schistosoma mansoni* adult worm immunogens. *Molecular and Biochemical Parasitology*, **38**, 199–210
- Sher A., Mackenzie P. & Smithers S.R. (1974) Decreased recovery of invading parasites from the lungs as a parameter of acquired immunity to schistosomiasis in the mouse. *Journal of Infectious Diseases*, **130**, 626–633
- Simpson A.J.G., James S.L. & Sher A. (1983) Identification of surface antigens of schistosomula of *Schistosoma mansoni* recognized by antibodies from mice immunized by chronic infection and by exposure to highly irradiated cercariae. *Infection and Immunity*, **41**, 591–597
- Simpson A.J.G., Schryer M.D., Cesari I.M., Evans W.H. & Smithers S.R. (1981) Isolation and partial characterization of the tegumental outer membrane of adult *Schistosoma mansoni*. *Parasitology*, **83**, 163–177
- Smithers S.R. & Terry R.J. (1965) The infection of laboratory hosts with cercariae of *Schistosoma mansoni* and the recovery of the adult worms. *Parasitol.*, **55**, 695–670
- Stein L.D. & David J.R. (1986) Cloning of a developmentally regulated tegument antigen of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology*, **20**, 253–264
- Sturrock R.F., Butterworth A.E. & Houba V. (1976) *Schistosoma mansoni* in the baboon (*Papio anubis*): parasitological responses of Kenyan baboons to different exposures of a local parasite strain. *Parasitology*, **73**, 239–252
- Taylor D.W., Cordingley J.S. & Butterworth A.E. (1984) Immuno-precipitation of surface antigen precursors from *Schistosoma mansoni* messenger RNA *in vitro* translation products. *Molecular and Biochemical Parasitology*, **10**, 305–318
- Towbin H., Staehelin T. & Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, USA*, **76**, 4350–4354
- Webster M., Fulford A.J.C., Braun G. *et al.* (1996) Human Immuno-

- globulin E responses to a Recombinant 22.6-Kilodalton antigen from *Schistosoma mansoni* adult worms are associated with low intensities of reinfection after treatment. *Infection and Immunity*, **64**, 4042–4046
- Wilkins H.A., Blumenthal U.J., Hagan P., Hayes R.J. & Tulloch S. (1987) Resistance to reinfection after treatment of urinary schistosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **81**, 29–35
- Wray W.T., Bouliskas V.P. & Hancock R. (1981) Staining of proteins in polyacrylamide gels. *Journal of Analytical Biochemistry*, **30**, 148–152.