Characterization, cloning and immunogenicity of antigens released by transforming cercariae of *Schistosoma mansoni*

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SUMMARY

A schistosome infection is initiated when the parasite penetrates the skin of a susceptible host. Relatively large quantities of protein are released by transforming cercariae compared to later larval stages. This represents the first parasite material to which the host's immune system is exposed, yet little is known about the proteins which are released during the first few hours post-transformation. We have shown that antiserum raised against such molecules was capable of imparting protection against a schistosome challenge infection upon passive transfer to naïve mice. By screening a cercarial cDNA library with this serum, 38 positive clones were identified. Sequence analysis showed these to represent 8 different molecules which included *Schistosoma mansoni* 21·7 kDa antigen, calcium-binding-protein and the vaccine candidate glutathione S-transferase (Sm28GST). In addition, 5 clones were isolated, 1 of which had significant homology to many cytochrome C proteins, another with leukocyte elastase inhibitors and 3 which represented novel molecules. Four clones were expressed in a prokaryotic high-level expression vector, sera produced against each purified recombinant protein and used subsequently to probe Western blots and parasite sections. The leukocyte elastase inhibitor homologue and 2 unknowns induced significant proliferation by lymph node cells recovered from mice vaccinated with irradiated cercariae. More strikingly, the 2 novel proteins stimulated very high levels of interferon γ (IFNγ) secretion both by lymph node cells and those recovered by broncho-alveolar lavage from the lungs of vaccinated mice. Such results will be discussed in the context of vaccine development.

Key words: *Schistosoma mansoni*, released proteins, sequence analysis, high-level expression, immunolocalization.

INTRODUCTION

Despite coordinated international control programmes, schistosomiasis remains a major health problem in many tropical and subtropical countries. Approximately 200 million people are infected worldwide (Bergquist & Colley, 1998) and, despite a low mortality rate and the existence of effective chemotherapy, 1 million deaths occur annually which can be attributed to the direct or indirect effects of this disease. For this reason, and the recent reports of low drug efficacy (Ismail *et al*. 1996; Stelma *et al*. 1995), a vaccine against schistosomiasis remains highly desirable and is the ultimate goal of many research projects.

A schistosome infection is initiated when the water-borne cercarial stage encounters the skin of a susceptible host. Mucous secretions enable the parasite to adhere to the surface of the host while proteases released from the acetabular glands digest through the stratum corneum of the skin. In addition, spines present on the surface of the cercaria aid penetration. At this point, the cercarial tail is lost and transformation into the next larval stage, the schistosomulum begins. This process involves profound biochemical and morphological changes which are vital for parasite survival within the mammalian host. The material released by cercariae undergoing transformation has been investigated previously at the biochemical level by SDS–PAGE and autoradiography (Harrop & Wilson, 1993). This study showed that radio-isotope labelled cercariae released protein at a much greater rate during the first few hours post-transformation than at later stages. Some of the material lost at this early stage has been characterized in more detail. For example, the carbohydrate-rich glycocalyx which serves to waterproof the cercaria has been analysed biochemically (Xu *et al*. 1994), and the serine protease which facilitates penetration through the epidermis and dermis of the skin has been cloned, sequenced (McKerrow *et al*. 1985) and immunolocalized (Marikovsky, Fishelson & Arnon, 1988). In addition to the protease, it has been demonstrated that approximately 12 major proteins are released by schistosomula during the first 3 h of transformation (Mountford & Harrop, 1998), but the identity of the majority of these remains unknown.

The molecules released during skin penetration represent the first parasite proteins to which the host's immune system is exposed. An appropriate effector response, induced by vaccination, against one or more of these released proteins might interfere

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with the process of transformation/maturation or facilitate clearance of the parasite at an early stage. Following Francis & Bickle (1992), we have screened expression libraries with rabbit antiserum raised against the proteins released by transforming cercariae and used it to confer passive protection to mice challenged with *S. mansoni*. In addition, we describe the cloning and sequence analysis of cDNAs encoding proteins released by schistosomula during the first 3 h post-transformation. A number of these molecules have been subcloned into a high-level expression vector and the purified proteins used subsequently to produce sera. Such sera were used to identify the full-length proteins in Western blot analysis and to immunolocalize the molecules in parasite sections. This study provides further insight into the developmental biology of the parasite at a highly critical stage of its life-cycle and details 4 molecules which have not been described previously in *S. mansoni*. More importantly, in the context of vaccine design and development, the ability of the expressed recombinant proteins to induce lymphocyte proliferation and cytokine production has been determined.

MATERIALS AND METHODS

Preparation of parasite antigens

The life-cycle of *S. mansoni* was maintained in the laboratory by routine passage through the intermediate snail host *Biomphalaria glabrata* and MF1 mice. Soluble protein preparations of cercariae (SCAP), lung-stage schistosomula (SLAP) and adult worms (SWAP) and proteins released by skin-stage schistosomula cultured *in vitro* during the first 3 h post-transformation (0–3 hRPs) were produced as described previously (Harrop & Wilson, 1993). The released material was concentrated by centrifugation in Ultrafree centrifugal filter devices (Millipore, Watford, UK) containing 5 kDa cut-off membranes, and the protein concentration determined subsequently by the Bradford assay.

Production of antisera to schistosomula-released proteins and recombinant proteins

Antiserum against 0–3 hRPs was produced by immunizing a rabbit subcutaneously with approximately 100 μg of the heterogeneous antigen preparation emulsified in complete Freund's adjuvant for the primary inoculation and in incomplete Freund's adjuvant for the 2 subsequent boosts. The antibody titre against 0–3 hRPs was assessed by ELISA (data not shown) and deemed to be sufficient after 2 boosts. Antisera against purified recombinant proteins were produced in a similar manner with the exception that mice with a disrupted IFNγ receptor gene (129 IFNγ R$^{-/-}$) were used. This mouse strain was chosen because of the strong antibody response induced upon vaccination. Approximately 10 μg purified recombinant protein was administered per mouse in both primary inoculations and in subsequent boosts.

Western blotting

Proteins were separated electrophoretically through 12 % polyacrylamide gels under reducing conditions according to the method of Laemmli (1970). Resolved fractions were electroblotted onto Immobilon-P membrane (Millipore) and probed with sera as described by Cutts & Wilson (1997).

Passive transfer

Prior to transfer, both normal rabbit serum (NRS) and serum raised in rabbits against 0–3 hRPs (0–3 hS) were heat inactivated at 56 °C for 30 min. Subsequently, the sera were incubated with 2 ml of packed mouse red blood cells for 2 h at 37 °C to adsorb potentially damaging antibodies directed against murine antigens. For all passive transfer experiments, groups of 5 C57BL/6 mice received either NRS or test serum. In each case, mice were exposed to a mean of 120 hand-counted cercariae via the tail on day 0. Mice were divided into 4 groups which received either NRS on day 0 (Group 1), 0–3 hS on day 0 (Group 2), 0–3 hS on day 1 (Group 3) or 0–3 hS on days 4 and 7 (Group 4). Groups 1 and 2, which received serum on day 0, were injected several hours prior to challenge infection. For each inoculation, 0·5 ml of serum was administered intravenously. Portal perfusion of the mice was carried out 5 weeks after challenge infection and the adult worm burden determined. Resistance was expressed as the percentage reduction in the numbers of adult worms recovered from mice receiving the test serum compared to that of mice receiving NRS (Group 1), and was calculated from the formula:

$$\%R = (C - T)/C \times 100,$$

where R = level of resistance, C = number of worms recovered from the control group and T = number of worms recovered from the test group. Differences in worm burdens were tested for statistical significance using Student's t-test.

Screening of cDNA libraries and sequence analysis

A cercarial cDNA library was screened essentially as described by Huynh, Young & Davis (1986). The primary serum (0–3 hS) was used at 1:1000 for screening. Positive clones were isolated and purified to homogeneity by several rounds of re-screening. Plasmid DNA preparation and sequence analysis was undertaken as detailed by Harrop, Coulson & Wilson (1999).

Table 1. Resistance of mice following a cercarial challenge on day 0 plus passive transfer of 0·5 ml of 0–3 hS on the days listed

(Worm burdens were determined following portal perfusion 5 weeks after challenge. Resistance compared to the NRS control group is shown and statistical significance indicated in parentheses.)

Treatment	Mean worm burden ± S.E.	Resistance compared to NRS control group
NRS on day 0	51·7 ± 4.1	–
0–3 hS on day 0	36·6 ± 2·7	29 % ($P < 0·05$)
0–3 hS on day 1	27·4 ± 2·1	47 % ($P < 0·01$)
0–3 hS on days 4 and 7	25·6 ± 3·7	51 % ($P < 0·01$)

Table 2. Summary of the predicted identity of cDNA clones following homology searches of the GenBank and SwissProt databases

Clone identity	Number	Percentage
S. mansoni 21·7 kDa antigen	21	55·3
S. mansoni calcium-binding protein	11	29
S. mansoni glutathione-S-transferase	1	2·6
Homology to non-*S. mansoni* genes	2	5·3
No homology	3	7·8

High-level expression

A number of clones were selected for expression in a prokaryotic high-level expression system. The cDNA inserts were subcloned into the pQe expression vector and then used to transform competent *E. coli* M15 cells. In addition to the schistosome molecules, β-galactosidase cDNA was ligated into the pQe vector. Expression and subsequent purification of recombinant proteins was undertaken according to the protocols provided by Qiagen. The histidine-tagged proteins were purified under denaturing conditions by Ni-NTA resin chromatography. Where required, purified proteins were dialysed extensively against decreasing concentrations of urea and finally against phosphate-buffered saline, pH 7·2.

Immunocytochemistry

Cercariae, lung-stage schistosomula and adult worms were prepared for immunocytochemistry based on the methodology developed by Riengrojpitak *et al*. (1989) and as detailed by Harrop *et al*. (1999).

Assays of lymphocyte proliferation and cytokine production

Assays were undertaken as described by Mountford, Harrop & Wilson (1995) and more specifically by Harrop *et al*. (1999). Results illustrated are representative of 2 (lung) or 3 (lymph node) individual experiments. Where appropriate, the mean ± S.D. is plotted.

RESULTS

Passive transfer

0–3 hS was transferred to naïve mice on various days to assess the importance of skin- and lung-stage parasites as targets of immune elimination by antibodies reacting with antigens released during transformation. The mean worm burdens and calculated resistance values compared to the NRS control group are illustrated in Table 1. Mice in all 3 test groups showed statistically significant levels of protection compared to controls (a single control group was used in order to reduce the numbers of animals). Mice receiving serum on day 1 or days 4 and 7 showed the most significant reduction in worm burdens (47 % and 51 % respectively).

cDNA library screening

By screening approximately 3×10^5 plaques in the cercarial cDNA library, 38 positive clones were identified. Each was picked and subsequently purified to homogeneity after several rounds of re-screening. Plasmid preparations of each clone provided the template for sequence analysis. A summary of the clones identified following single-pass

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                    1                                                  50
Human LEI                        MEQLSSA NTRFALDLFL ALSENNPAGN IFISPFSISS
Horse LEI                        MEQLSTA NTHFAVDLFR ALNESDPTGN IFISPLSISS
Pig LEI                          MEQLSAA NTRFALDLFR ALNESNPAGN IFISPFSISS
Clone 8             ILGKLPDYKQ MDVLQSLKNF SGRFYGDIVE ..EKQSHSEN TFLSPFNVYT
Consensus           ---------- ------L--- ---F--D--- ---------N -F-SP-----

                    51                                                 100
Human LEI           AMAMVFLGTR GNTAAQLSKT FHFNTVEE.. .VHSRFQSLN ADINKRGASY
Horse LEI           ALAMIFLGTR GNTAAQVSKA LYFDTVED.. .IHSRFQSLN ADINKPGAPY
Pig LEI             ALAMILLGTR GNTEAQMSKA LHFDTVKD.. .IHSRFQSLN ADINKCGASY
Clone 8             ALGMILSGSE MNTKAEIMKV MHLSNCLEHH AIHYGISGLL FDCSERGEGV
Consensus           A--M---G-- -NT-A---K- ---------- --H-----L- -D----G---

                    101                                                150
Human LEI           ILKLANRLYG EKTYNFLPEF LVSTQKTYGA DLASVDFQHA SEDARKTINQ
Horse LEI           ILKLANRLYG EKTYNFLADF LASTQKMYGA ELASVDFQQA PEDARKEINE
Pig LEI             ILKLANRLFG EKTYHFLPEF LASTQKTYGA ELASVDFLRA SEEARKAINE
Clone 8             EIMFGNGLFT AEDVNVKEDY QNTLKSYYNA QTESVAFQMD PEDAGKRINQ
Consensus           -----N-L-- ---------- -------Y-A ---SV-F--- -E-A-K-IN-

                    151                                                200
Human LEI           WVKGQTEGKI PELLASGMVD NMTKLVLVNA IYFKGNWKDK FMKEATTNAP
Horse LEI           WVKGQTEGKI PELLVKGMVD NMTKLVLVNA IYFKGNWQEK FMKEATRDAP
Pig LEI             WVKEQTEGKI PELLASGVVD SATKLVLVNA IYFKGSWQEK FMTEATKDAP
Clone 8             WASSLTKGKI QELFSSQSLS TDTSVLVITT TYFEXMWDLP FLQGSSHESD
Consensus           W----T-GKI -EL------- --T------- -YF---W--- F---------

                    201                                                250
Human LEI           FRLNKKDRKT VKMMYQKKKF AYGYIEDLKC RVLELPYQGE ELSMVILLPD
Horse LEI           FRLNKKDTKT VKMMYQKKKF PYNYIEDLKC RVLELPYQGK ELSMIILLPD
Pig LEI             FRLNKKDSKT VKMMYQKKKF PFGYIKELKC RVLELPYQGK DLSMVILLPD
Clone 8             FFKLDGSTMN VKLMYMNSSF DMTSLPDLKS RAIKIPFKNP KFSLLIVLPT
Consensus           F--------- VK-MY----F -------LK- R----P---- --S--I-LP-

                    251                                                300
Human LEI           DIEDESTGLK KIEEQLTLEK LHEWTKPENL DFIEVNVSLP RFKLEESYTL
Horse LEI           DIEDESTGLE KIEKQLTLEK LREWTKPENL YLAEVNVHLP RFKLEESYDL
Pig LEI             SIEDESTGLR KIEQHLTLEK LNEWTKPDNL ELLEVNVHLP RFRLEESYDL
Clone 8             PMMDYLSY.. .......... .......... .......... ..........
Consensus           ---D------ ---------- ---------- ---------- ----------
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Fig. 1. Lineup of Clone 8 and the leukocyte elastase inhibitors of humans, horses and pigs. Identities are illustrated in the consensus line and gaps introduced (.) to optimize the alignment.

sequencing from the 5′ end, is given in Table 2. Previously identified schistosome genes, including the 21·7 kDa antigen (U30663), calcium-binding protein (J04641; Ram *et al*. (1989)), and glutathione-S-transferase (S71584; Pierce *et al*. (1989), accounted for the vast majority of positive clones identified (approximately 85%). The remaining positive clones included 2 which had homology to non-schistosome genes and 3 which had no homology to anything present in the databases.

Full-length sequence analysis

Full-length sequence analysis of all 5 novel schistosome clones was undertaken by primer walking from both 5′ and 3′ ends.

(*a*) *Clones 13, 19 and 24* (*Accession numbers: AF030971, AF030973 and AF030974 respectively*). Despite obtaining complete sequences of the partial cDNA fragments present in clones 13, 19 and 24, no

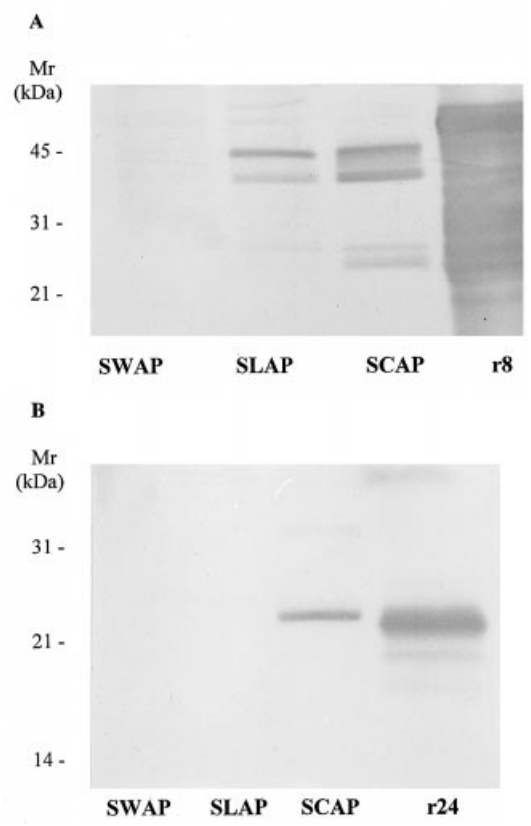

Fig. 2. Western blots of the target recombinant protein alongside SCAP, SLAP and SWAP probed with sera against (A) r8, and (B) r24. Five μg soluble protein preparations and 0·5 μg recombinant protein were loaded per lane. Molecular weights are illustrated.

homologies were identified to any molecule sequenced to date. The clones had cDNA inserts of 446, 409 and 601 bp respectively which contained open reading frames of 37, 68 and 127 aa.

(*b*) *Clone 8* (*Accession number : AF030970*). Clone 8 contains an insert of 866 bp which was sequenced from both 5′ and 3′ ends and the fragments aligned to yield the complete cDNA sequence. The single open reading frame of 256 aa encodes a protein of predicted molecular weight 29 kDa and pl of 5·05. Searches of SwissProt showed the molecule to have significant homology to many proteinase inhibitors, but particularly with the leukocyte elastase inhibitors of horses (P05619), humans (P30740) and pigs (P80229). Identities of 29, 28 and 26 % occur over a 173 aa overlap in the 3 proteins respectively. If conservative substitutions are taken into consideration, the homology reaches 49 %. A lineup of clone 8 and these 3 elastase inhibitors is illustrated in Fig. 1. The homology occurs at the N-terminal end of the proteins and within this region, only 3 gaps in the mammalian enzymes and 2 gaps in clone 8 are required for optimal alignment. No poly A tail is evident in the nucleic acid sequence suggesting that clone 8 is incomplete at the 3′ end. The 5′ end contains a methionine at amino acid position 11, but it is difficult to determine whether this represents the start codon of the parasite molecule. The sequence of clone 8 appears to be distinct from those of the serpin family.

(*c*) *Clone 16* (*Accession number : AF030972*). Clone 16 contains an insert of 285 bp and a deduced open reading frame of 52 aa which ends at 158 with a UGA stop codon. The molecule has a predicted molecular weight of 6·5 kDa and a pI of 9·2. Searches of SwissProt showed the clone to have significant homology with many cytochrome C molecules. Identities of 71 % over a 28 aa overlap occurred in all proteins, predominantly at the C-terminal end. In addition, clone 16 showed very high homologies to many schistosome ESTs (including a number derived from cercariae) listed as being homologous to cytochrome C molecular species.

Identification of the full-length proteins in different life-cycle stages

Clones 8, 13, 19 and 24 were expressed in the pQe31 expression vector. Sera produced against each recombinant protein (r8, r13, r19 and r24) were used subsequently to probe Western blots of the recombinant protein as well as soluble preparations of cercariae, lung-stage schistosomula and adult worms (SCAP, SLAP and SWAP respectively). Sera against r13 and r19 were capable of detecting the recombinant against which they were raised, but did not bind to any other proteins in the soluble preparations (data not shown). Western blots probed with anti-r8 and anti-r24 are illustrated in Fig. 2(A and B). Serum against r8 (Fig. 2A) appeared to bind a range of molecules in the recombinant protein fraction purified from a bacterial lysate. Presumably, these represent multimers and breakdown products of the recombinant. However, when used to probe blots of SCAP, SLAP and SWAP, the serum bound strongly to a doublet at 43 and 47 kDa in the 2 earlier life-cycle stages. Serum against r24 (Fig. 2B) bound to a single protein of 25 kDa in both the recombinant and SCAP fractions. No proteins were detectable in SLAP or SWAP.

Immunolocalization

Sera against the 4 recombinant proteins were used to probe sections of cercariae, lung worms and adult worms. The results are shown in Fig. 3(A–F). Proteins 13 and 24 appeared to be most abundant in muscle while 8 and 19 showed a more diffuse distribution, being present in muscle but also many other tissues. Clone 19 also appeared to be located in discrete, intensely staining foci in the lung worm; it is possible that these are flame cells of the parasite's excretory/secretory system.

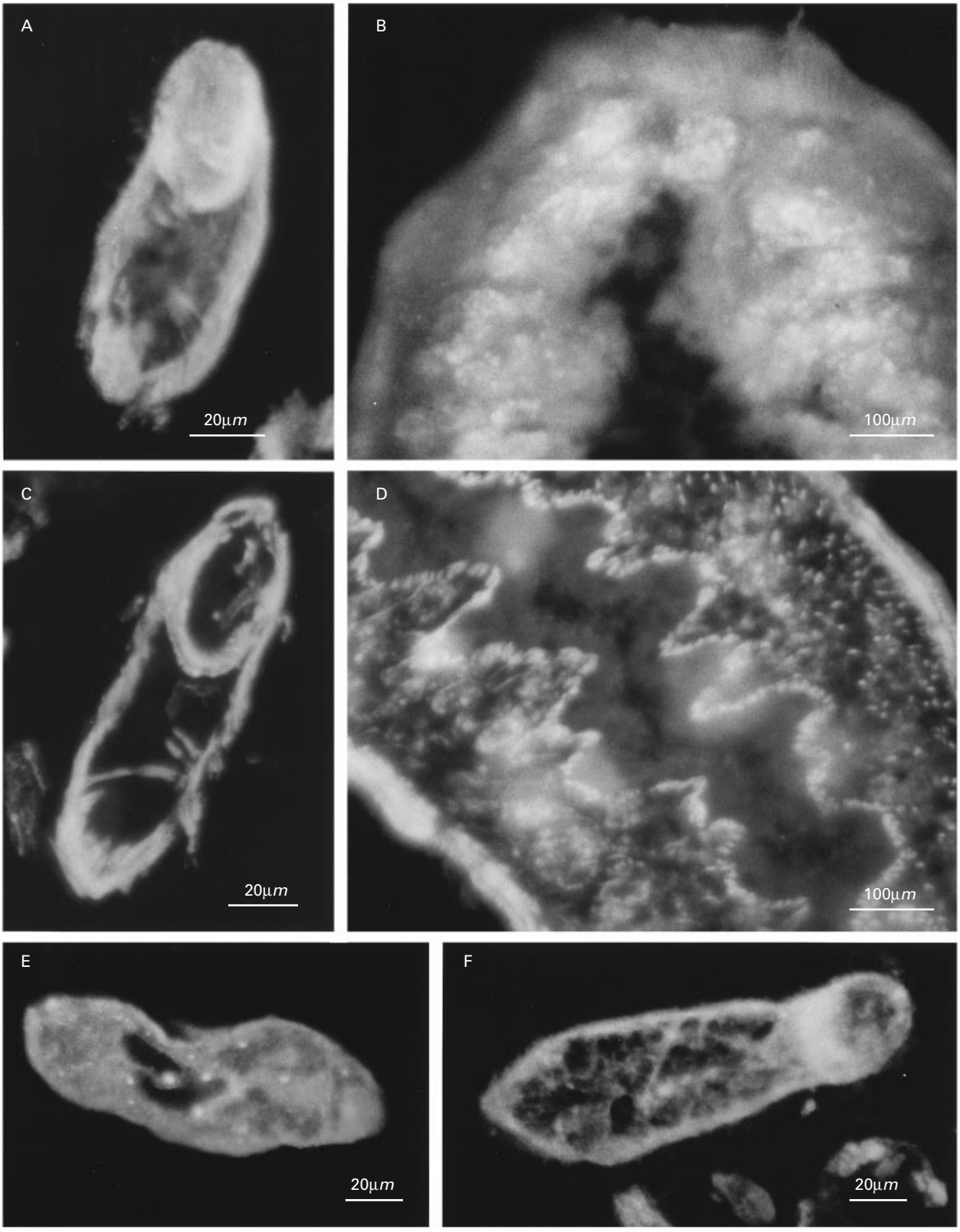

Fig. 3. Immunolocalization of clones 8 (A and B), 13 (C and D), 19 (E) and 24 (F) in sections of cercariae (A and E), lung-stage schistosomula (C and F) and adult worms (B and D).

Lymphocyte proliferation and cytokine production

The ability of recombinant proteins 8, 13, 19 and 24 to induce lymphocyte proliferation and cytokine production by cells recovered from mice at day 5 (lymph node) or day 21 (lung) post-vaccination was determined. Comparisons were made to 2 pure, non-schistosome proteins (BSA and β-galactosidase), which served as negative controls and a soluble schistosome preparation (SCAP) which was used as

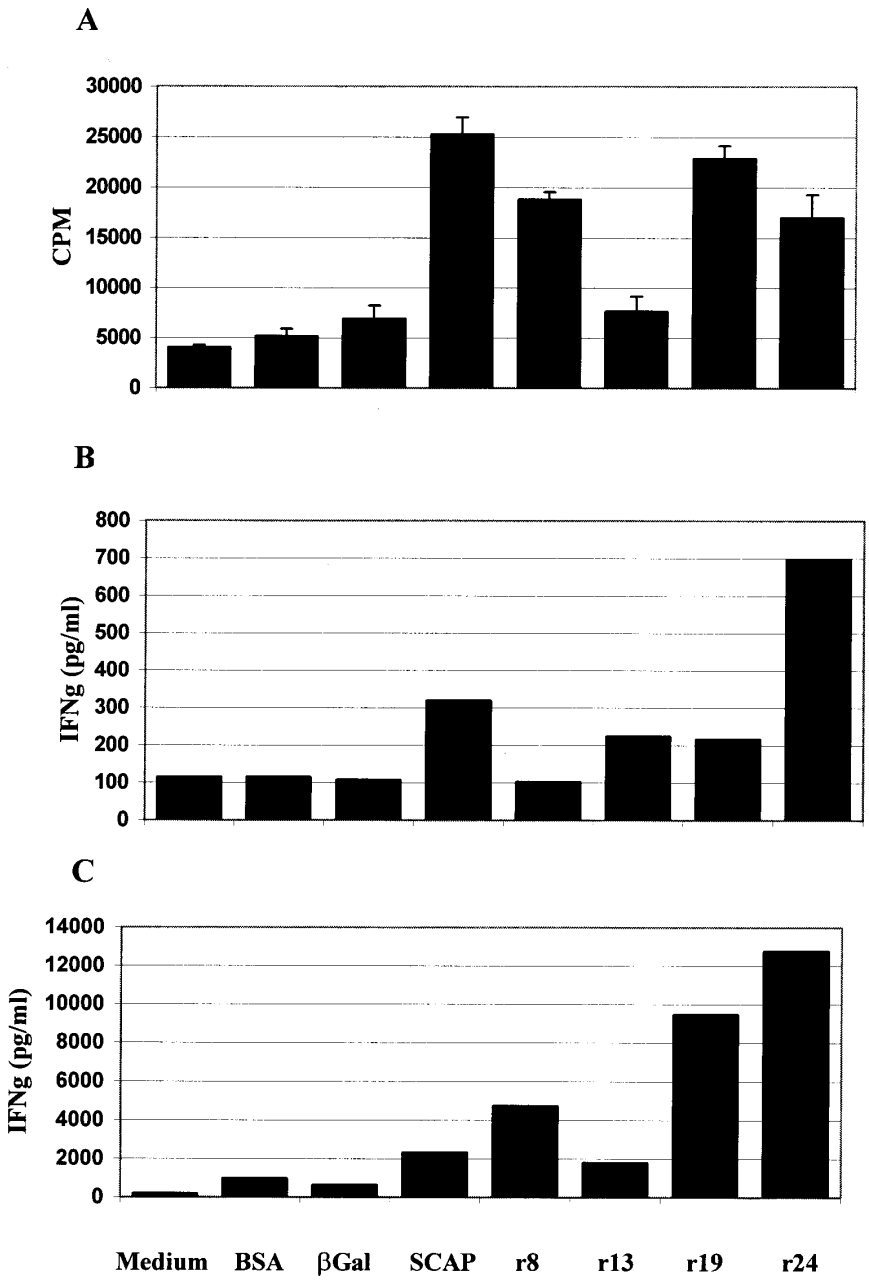

Fig. 4. Proliferative responses (A) and IFNγ production (B and C) by cells recovered from the axillary lymph nodes of mice 5 days after vaccination with irradiated cercariae (A and B) and from the lungs of mice 21 days post-vaccination. Cells were used at a concentration of 1×10^5 per well and antigen at 5 μg/ml. Proliferative responses are expressed as cpm $\pm$ S.D. from triplicate wells. These are representative results from 3 separate experiments. IFNγ production is expressed as pg/ml following extrapolation from a standard curve.

a positive control (Fig. 4A, B and C). All 4 recombinant proteins induced greater levels of proliferation than that seen with either medium alone, BSA or purified recombinant β-gal (Fig. 4A). Recombinants 8, 24 and particularly 19 caused proliferation approaching that seen with SCAP. Interferon γ production by the same lymph node cells on day 3 is illustrated in Fig. 4B. Cytokine production following stimulation with r8 was no different from that induced by BSA or β-galactosidase. In contrast, r13 and r19 caused higher levels of IFNγ secretion while r24 yielded > 700 pg IFNγ/ml. No IL-4 secretion was detectable. When cells recovered from the lungs of mice 21 days post-vaccination were restimulated *in vitro* with the same antigens (Fig. 4C), all caused greater IFNγ secretion than the negative control antigen β-gal. Recombinants 8, 19 and 24 induced significantly more IFNγ production than did the heterogeneous protein preparation SCAP. Indeed, r24 caused IFNγ to be secreted at a concentration approaching 13000 pg/ml.

DISCUSSION

By using serum raised against 0–3 hRPs, we have been able to transfer resistance against a cercarial challenge to naïve mice. Although the use of rabbit serum in a murine model represents a heterologous system, the binding of antibodies present in the serum to parasite-derived molecule(s) renders the schistosomulum sensitive to immune-mediated clearance. Paradoxically, the serum, which was raised against skin-stage released proteins, was most efficient at conferring passive immunity when transferred at a time coincident with parasite residence in the lungs. This suggests that some of the proteins released by transforming cercariae are still expressed by lung worms. Likewise, sera from rabbits (Bickle *et al*. 1985; Mangold & Dean, 1992) and mice (Mangold & Dean, 1986) which had been multiply vaccinated with irradiated cercariae were equally, or more highly protective when serum transfer coincided with parasite residence in the lungs, rather than the skin stage of migration. It is possible that the lungs may simply represent a site in which the schistosomulum is stationary for several days while it undergoes adaptation necessary for further migration and is therefore more prone to immune attack. We cannot discount the possibility that mice receiving serum on day 0 or day 1 are clearing a proportion of the parasite challenge at the skin-stage. However, this is clearly not essential for the transfer of optimal resistance since when serum was transferred on days 4 and 7, few parasites would still be present in the skin (Wilson, Coulson & Dixon, 1986). Although the mechanism of schistosome elimination is not known, we must assume that antibodies are binding to accessible proteins on the surface of the challenge parasite or generating immune complexes with released proteins leading to inflammation. Inhibition of essential parasite processes or binding of cells bearing Fc receptors could result in killing. It is impossible to determine which of the 0–3 hRPs is the target of the protective antibodies transferred to naïve mice. However, we have shown here that the vaccine antigen GST was detected following screening of a cercarial cDNA library with serum raised against 0–3 hRPs. In this respect, it has been demonstrated previously that monospecific serum against GST could transfer resistance against a cercarial challenge in mice (Balloul *et al*. 1987). We do not know if this also occurs in our experimental system and therefore could be partially or wholly responsible for the protection observed. The importance of antibodies in protective immune responses to *S. mansoni* remains a matter of conjecture. In the irradiated cercaria vaccine model, antibodies are thought to have little importance in 1 time vaccinated mice (Anderson *et al*. 1999). However, in mice capable of making a strong Th2 type response (e.g. IFNγR$^{-/-}$ mice), antibodies do play a protective role following multiple vaccination (Coulson & Wilson, unpublished data).

By screening a cercarial library with serum raised against 0–3 hRPs, we sought to identify cDNA clones encoding secretory molecules involved in the processes of skin penetration and parasite transformation. Several criteria were applied to determine whether this goal had been achieved, including the presence of a putative signal sequence, homology to known secretory proteins and immunolocalization of the molecule. In total, 38 clones encoding 8 different molecules were identified. By far the 2 most abundant molecules detected by library screening were the 21·7 kDa antigen and calcium-binding protein which together represented almost 85% of the positive clones identified. This may be due to either the presence of high titre and/or affinity antibodies in the serum or a high frequency of the specific cDNAs in the library. In this respect, by using the same cercarial library as a source of ESTs, Franco *et al*. (1997) showed that the calcium-binding protein represented 10% of the total useful clones.

Clone 8 appears to encode a schistosome homologue of mammalian leukocyte elastase inhibitors (LEIs). The cDNA insert is not complete since there is no poly A tail and serum against r8 binds 2 proteins of 43 and 47 kDa in SCAP and SLAP, although the predicted molecular weight of the 0·86 kBa insert is 29 kDa, further suggesting that only a fragment is present. The LEIs of humans, horses and pigs are of molecular weight 42·7, 43 and 42·6 kDa respectively. We can only speculate on the role this molecule may play in the parasite. It is possible that the protein regulates the serine elastase which is released during skin penetration. This potent protease may need to be tightly controlled to avoid damaging the parasite itself. However, if this were the case, why the molecule has greatest homology to mammalian molecules is difficult to explain. Another possibility is that the protein has a defensive role and acts to inhibit enzymes released by host leukocytes.

Clones 13, 19 and 24 show no homology to any molecule sequenced previously. Each seems to have an intact poly A tail, but an incomplete 5′ end. Thus, it is not possible to search for putative signal sequences which might provide circumstantial evidence that the molecule was secreted. In addition, we can say little about the size and distribution of the 'native' proteins representing clones 13 and 19 in different parasite life-cycle stages as Western blotting with each serum was not successful. Since each serum was capable of detecting the recombinant against which it was raised, we presume that the inability to bind proteins in soluble parasite fractions is due to either low abundance of the molecule in these preparations or poor sensitivity of the serum. Using the sera to probe blots of 0–3 hRPs may prove

more successful, although the highly glycosylated nature of this preparation makes clean Western blots difficult to achieve. Serum against r24 binds a single protein which is only detected in SCAP by Western blotting, suggesting that it either exhibits stage-specific expression, or is highly represented in cercariae compared to later life-cycle stages. However, immunofluorescence studies suggest that the protein is also present in adult worms (data not shown). Since the molecular weight of the recombinant and the 'native' protein are identical, this would suggest that clone 24 is full-length although, from the nucleotide sequence, it is difficult to predict where the start codon might be. It is possible that the clone is missing the extreme 5′ end which includes the start methionine.

Immunofluorescence studies do not provide compelling evidence that the molecules we have cloned, sequenced and expressed encode secretory proteins since none of the sera stained the acetabular glands of the cercaria. However, the process by which proteins are released during cercarial transformation must be considered. Vesicles, originating from the acetabular glands, are released in large numbers on to the surface of the host; this process is readily observed *in vitro* by light microscopy. Upon contact with skin (or *in vitro* with the plastic tissue culture flask), the vesicles burst releasing their contents. The majority of molecules described here are likely to include contents of such vesicles released by the process of holocrine secretion. This does not diminish the importance of these proteins, since they are the first molecules to which the host's immune system is exposed. We may have limited the number of different clones identified in this study by screening only a cercarial library. It is likely that a number of the proteins expressed at this transitional point of the parasite life-cycle are only required at this specific stage. For example, the cercarial elastase which is essential for parasite transformation and skin penetration, is only expressed for a few days in the schistosomulum, and the gene only transcribed in the sporocyst. Thus, mRNA encoding this enzyme would not be represented in the cercarial library. Other constituents of the acetabular glands may also fall into this category.

The degree of proliferation and IFNγ secretion by cells recovered from lymph nodes and lungs of vaccinated mice following restimulation with 3 of the purified recombinant antigens was very high. Secretion of the Th1-type cytokine IFNγ by cells, recovered from the lungs of vaccinated mice, after stimulation with r8, r19 and r24 greatly exceeded that observed in response to the heterogeneous soluble preparation. Considering the number of antigens present in SCAP and therefore the number of T cell epitopes compared to a single recombinant protein, this is perhaps surprising. In addition, we have demonstrated previously that the heterogeneous mixture of proteins released during the first 3 h after cercarial transformation induced the lowest level of IFNγ secretion by cells recovered from vaccinated mice when compared to other parasite preparations (Mountford, Harrop & Wilson, 1995). When compared to recombinant proteins representative of a subset of lung-stage schistosomula released proteins (Harrop, Coulson & Wilson, 1999), r19 and r24 induce greater levels of IFNγ secretion by cells recovered from the lungs of vaccinated mice. We have shown r24 to be highly represented in cercariae compared to later life-cycle stages as determined by Western blotting. This would suggest that its release from the parasite soon after penetration results in large-scale priming and expansion of specific Th1 cells which are recruited subsequently to the lungs. The much greater levels of IFNγ secretion by cells recovered from the lungs, compared to the lymph nodes, of vaccinated mice could be due to several factors such as the preferential expansion or recruitment of schistosome-specific Th1 cells to the lungs. Since we have shown that little or no proliferation of parasite-specific T cells occurs in the lungs (Smythies & Wilson, unpublished observations), we would suggest that their preferential recruitment to this site is responsible for the enhanced IFNγ secretion.

The cloning and sequence analysis of the proteins released by transforming cercariae provides further insights into the developmental biology of the parasite at a crucial stage in its life-cycle. More importantly, we have shown that a number of the novel molecules are potent immunogens, inducing a strong Th1-type response. Since the secretion of high levels of IFNγ in the lungs of 1 time vaccinated mice is essential for the expression of resistance following cercarial challenge (Smythies, Coulson & Wilson, 1992), these results are encouraging when considering vaccine development. Further work will assess the potential of these proteins as vaccine candidates.

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REFERENCES

ANDERSON, S., COULSON, P. S., LJUBOJEVIC, S., MOUNTFORD, A. P. & WILSON, R. A. (1999). The radiation-attenuated schistosome vaccine induces high levels of protective immunity in the absence of B cells. *Immunology* **96**, 22–28.

BALLOUL, J. M., GRZYCH, J. M., PIERCE, R. J. & CAPRON, A. (1987). A purified 28,000 dalton protein from

Schistosoma mansoni adult worms protects rats and mice against experimental schistosomiasis. *Journal of Immunology* **138**, 3448–3453.

BERGQUIST, N. R. & COLLEY, D. G. (1998). Schistosomiasis vaccines: research to development. *Parasitology Today* **14**, 99–104.

BICKLE, Q. D., ANDREWS, B. J., DOENHOFF, M. J., FORD, M. J. & TAYLOR, M. G. (1985). Resistance against *Schistosoma mansoni* induced by highly irradiated infections: studies on species specificity of immunization and attempts to transfer resistance. *Parasitology* **90**, 301–312.

CUTTS, L. & WILSON, R. A. (1997). The protein antigens secreted *in vivo* by adult male *Schistosoma mansoni*. *Parasitology* **114**, 245–255.

FRANCIS, 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.

FRANCO, G. R., RABELO, E. M. L., AZEVEDO, V., PENA, H. B., ORTEGA, J. M., SANTOS, T. M., MEIRA, W. S. F., RODRIGUES, N. A., DIAS, C. M. M., HARROP, R., WILSON, A., SABER, M., ABDEL-HAMID, H., FARIA, M. S. C., MARGUTTIA, M. E. B., PARRA, J. C. & PENA, S. D. J. (1997). Evaluation of cDNA libraries from different developmental stages of *Schistosoma mansoni* for production of expressed sequence tags (ESTs). *DNA Research* **4**, 231–240.

HARROP, R., COULSON, P. S. & WILSON, R. A. (1999). Characterization, cloning and immunogenicity of antigens released by lung-stage larvae of *Schistosoma mansoni*. *Parasitology* **118**, 583–594.

HARROP, R. & WILSON, R. A. (1993). Protein synthesis and release by cultured schistosomula of *Schistosoma mansoni*. *Parasitology* **107**, 265–274.

HUYNH, T. V., YOUNG, R. A. & DAVIS, R. W. (1986). Constructing and screening cDNA libraries in lambda gt10 and lambda gt11. In *DNA Cloning. A Practical Approach* (ed. Glove, D. M.), pp. 187–209. IRL Press Limited, Oxford.

ISMAIL, M., METWALLY, A., FARGHALY, A., BRUCE, J., TAO, L. E. & BENNETT, J. L. (1996). Characterization of isolates of *Schistosoma mansoni* from Egyptian villagers that tolerate high doses of praziquantel. *American Journal of Tropical Medicine and Hygiene* **55**, 214–218.

LAEMMLI, U. K. (1970). Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

MCKERROW, J. H., PINO-HEISS, S., LINDQUIST, R. & WERB, Z. (1985). Purification and characterization of an elastinolytic proteinase secreted by cercariae of *Schistosoma mansoni*. *The Journal of Biological Chemistry* **260**, 3703–3707.

MANGOLD, B. L. & DEAN, D. A. (1986). Passive transfer with serum and IgG antibodies of irradiated cercaria-induced resistance against *Schistosoma mansoni* in mice. *Journal of Immunology* **136**, 2644–2647.

MANGOLD, B. & DEAN, D. A. (1992). The role of IgG antibodies from irradiated cercaria-immunized rabbits in the passive transfer of immunity to *Schistosoma mansoni*-infected mice. *American Journal of Tropical Medicine and Hygiene* **47**, 821–829.

MARIKOVSKY, M., FISHELSON, Z. & ARNON, R. (1988). Purification and characterization of proteases secreted by transforming schistosomula of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **30**, 45–54.

MOUNTFORD, A. P. & HARROP, R. (1998). Vaccination against schistosomiasis: the case for lung-stage antigens. *Parasitology Today* **14**, 109–114.

MOUNTFORD, A. P., HARROP, R. & WILSON, R. A. (1995). Antigens derived from lung-stage larvae of *Schistosoma mansoni* are efficient stimulators of proliferation and gamma interferon secretion by lymphocytes from mice vaccinated with attenuated larvae. *Infection and Immunity* **63**, 1980–1986.

PIERCE, R. J., KHALIFE, J., WILLIAMS, D. L., KANNO, R., TROTTEIN, F., LEPRESLE, T., SABATIER, J., ACHSTETTER, T. & CAPRON, A. (1989). *Schistosoma mansoni*: characterization of sequence variants of the 28-kDa glutathione S-transferase. *Experimental Parasitology* **79**, 81–84.

RAM, D., GROSSMAN, Z., MARKOVICS, A., AVIVA, A., ZIV, E., LANTNER, F. & SCHECHTER, I. (1989). Rapid changes in the expression of a gene encoding a calcium binding protein in *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **34**, 167–175.

RIENGROJPITAK, S., VOJVODIC, M., BOOT, C. & WILSON, R. A. (1989). Reactivity of anti-tegument monoclonal antibodies with target epitopes in different worm tissues and developmental stages of *Schistosoma mansoni*. *Parasitology* **98**, 213–225.

SMYTHIES, L. E., COULSON, P. S. & WILSON, R. A. (1992). Monoclonal antibody to interferon-gamma modifies pulmonary inflammatory responses and abrogates immunity to *Schistosoma mansoni* in mice vaccinated with attenuated cercariae. *Journal of Immunology* **149**, 3654–3658.

STELMA, F. F., TALLA, I., SOW, S., KONGS, A., NIANG, M., POLMAN, K., DEELDER, A. M. & GRYSEELS, B. (1995). Efficacy and side effects of praziquantel in an endemic focus of *Schistosoma mansoni*. *American Journal of Tropical Medicine and Hygiene* **53**, 167–170.

WILSON, R. A., COULSON, P. S. & DIXON, B. (1986). Migration of the schistosomula of *Schistosoma mansoni* in mice vaccinated with radiation-attenuated cercariae, and normal mice: an attempt to identify the timing and site of parasite death. *Parasitology* **92**, 101–116.

XU, X., STACK, R. J., RAO, N. & CAULFIELD, J. P. (1994). *Schistosoma mansoni:* fractionation and characterization of the glycocalyx and glycogen-like material from cercariae. *Experimental Parasitology* **79**, 399–409.