

Immunogenicity and immunolocalization of the 22·6 kDa antigen of *Schistosoma japonicum*

YUESHENG LI^{1,2}, ALYSON AULIFF^{3,4}, MALCOLM K. JONES³, XINYUAN YI⁵ & DONALD P. McMANUS¹

¹Molecular Parasitology Unit, Tropical Health Program, Australian Centre for International and Tropical Health and Nutrition, The Queensland Institute of Medical Research and the University of Queensland, 300 Herston Road, Herston, Brisbane, Queensland, 4006, Australia, ²Hunan Institute of Parasitic Diseases, Hunan, China, ³Centre for Microscopy and Microanalysis and ⁴Department of Parasitology, The University of Queensland, Queensland 4072, Australia and ⁵Hunan Medical University, Changsha, Hunan, China

SUMMARY

The 22·6 kDa tegumental-associated antigens of *Schistosoma mansoni* (Sm22·6) and *Schistosoma japonicum* (Sj22·6) are of recognized interest in schistosomiasis vaccine development, although no direct vaccination/challenge experiments using either Sm22·6 or Sj22·6 had been previously described. We report that *Escherichia coli*-expressed reSj22·6 failed to protect mice or water buffaloes against subsequent challenge with *S. japonicum* cercariae. This was despite the fact that specific IgG (buffaloes) and IgG and IgE (CBA mice) antibodies were generated against the 22·6 kDa molecule, observations consistent with some of our earlier findings. We could find no evidence from immunolocalization studies that Sj22·6 is expressed or exposed on the surface of the adult parasite since it appears to be restricted to the apical cytoplasm of the tegument and is not associated with the apical or basal membrane or any membrane-bound structures in the apical cytoplasm. Nevertheless, Sj22·6 must be released to the immune system during the course of infection because specific anti-Sj22·6 IgG antibodies were present in the sera of nonvaccinated but challenged mice. We conclude that it may be necessary to produce reSj22·6 in a more relevant expression system, such as baculovirus, to further establish its vaccine potential and that detailed immunochemical and immunolocalization studies of early developmental stages may be necessary to determine how Sj22·6 is released or shed in *S. japonicum* infections.

Keywords Schistosomiasis, *Schistosoma japonicum*, immunoglobulin-E (IgE), Sj22·6, vaccine, buffalo, immunofluorescence, immunoelectron microscopy

INTRODUCTION

There has been much recent interest in defining the molecular targets of IgE antibodies from subjects resistant to schistosomiasis as these may provide important candidates for a vaccine. In schistosomiasis mansoni, individuals whose IgE antibodies recognized antigen(s) in the 22-kDa range were reinfected less frequently and had lower intensities of reinfection (Dunne *et al.* 1997, Webster *et al.* 1998). The molecule responsible was cloned and shown to correspond to a previously identified tegumental protein, Sm22·6 (Webster *et al.* 1996). In schistosomiasis-endemic communities, recombinant Sm22·6-specific IgE increases linearly with age (Webster *et al.* 1996) but, in contrast, a recently established Senegalese focus showed that IgE responses were associated with infection intensity rather than age *per se* (Webster *et al.* 1998). A significant negative correlation between IgE responses to recombinant (re) Sm22·6 and intensity of reinfection after treatment indicates that this antigen either induces a protective response against *S. mansoni* or is a marker for schistosomiasis mansoni resistant individuals (Webster *et al.* 1996).

It has been proposed that IgE antibodies play a significant role in immunity to human schistosomiasis japonica with antigen(s) in the 22-kDa range, especially the homologue (Sj22·6) of Sm22·6, also appearing to be major targets of this response, at least in studies carried out in The Philippines (Webster *et al.* 1997, Santiago *et al.* 1998, Hafalla *et al.* 1999). In China, we recently reported that a subset of subjects living in an area of the Dongting Lake region endemic for schistosomiasis japonica were high IgE responders to the reSj22 antigen (Li *et al.* 1999). B-cell epitopes have recently been mapped on Sj22·6 (Waine *et al.* 1997a, Zhou *et al.* 1999), but there have been no reports on the direct vaccine efficacy of the 22·6 kDa tegumental antigen of schistosomes in animal models. Here, we describe the results of vaccination/challenge experiments in mice and buffaloes using purified reSj22·6 (Waine *et al.* 1994).

Correspondence: Donald P. McManus, Queensland Institute of Medical Research, PO Royal Brisbane Hospital, Queensland 4029, Australia

Received: 21 December 1999

Accepted for publication: 23 March 2000

Immunoprecipitation and Western blotting experiments have shown the Sm22-6 is present in all postsnail life cycle stages, except the egg (Stein & David 1986, Jeffs *et al.* 1991) and indirect immunofluorescence of adult worm sections indicated strong association with the tegument but not a surface localization (Stein & David 1986). More detailed study was carried out by immunoelectron microscopy using a rabbit antiserum raised by immunization with an electro-eluted 22 kDa band cut from an sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel slice of separated *S. mansoni* adult worm components (Dunne *et al.* 1997). This study indicated that Sm22-6 is expressed in lung- and postlung schistosomula and present in the outer tegument (but was not exposed on the surface), the gut tegument and the collecting ducts and flame cells of adult worms. Other than a recent immunohistochemical light microscopy study which indicated the presence of Sj22-6 in the tegument of adult *S. japonicum* worms (Chen *et al.* 1998), no indepth localization studies had been carried out previously on this antigen. In addition, we report on the immunolocalization of Sj22-6 using immunofluorescence and immunoelectron microscopy.

MATERIALS AND METHODS

Animals and parasites

Six-week-old CBA or BALB/c female mice were obtained from the Animal Resource Centre, Western Australia. *Oncomelania hupensis hupensis* harbouring *S. japonicum* were collected from Anhui province, China and transported to Brisbane one week before they were required for cercarial shed. Twenty, 9-month-old, helminth-free, male buffaloes (*Bos buffelus*), each weighing approximately 150 kg, were purchased from Longhui County (a nonendemic area for schistosomiasis, Hunan, China). The buffaloes were held in the Hunan Institute of Veterinary Medicine for the duration of the experiment.

Preparation of native and reSj22-6

Whole parasite extracts were prepared by harvesting the soluble fraction obtained from sonicated *S. japonicum* adult worms (Li *et al.* 1999). Briefly, adult *S. japonicum* worms (Chinese strain) were collected from rabbits by perfusion of the hepatic portal system with a balanced salt solution 7 weeks after initial infection with 2000 cercariae. The perfused worms were sonicated in distilled water and centrifuged at 14 000 *g* for 30 min at 4°C. The protein concentration of the supernatant, as assessed by the BCA Protein Assay Regent Kit (Pierce, Rockford, IL, USA), was 2.17 mg/ml. The native adult worm antigen preparation (AWA) was aliquoted and stored at -20°C until use.

Sj22-6 was expressed and affinity purified under non-denaturing conditions using imidazole for elution as previously described by Li *et al.* (1999). Imidazole was removed using Centriprep-10 concentrators (Amicon Inc., Beverly, MA, USA), according to the manufacturers' instructions. The protein concentration of the supernatants containing reSj22-6 was assessed by the BCA Protein Assay Regent Kit (Pierce) and the protein (final concentration 1 mg/ml phosphate-buffered saline, PBS) was aliquoted and stored at -70°C until use. A hyperimmune rabbit serum was prepared against the purified reSj22-6 as described previously (Yang *et al.* 1999). Briefly, an outbred rabbit (Lop Hybrids) was injected s.c. three times with reSj22-6 G at 2-week intervals. ReSj22-6 (0.5 ml, 0.075 mg protein) was mixed with an equal volume of Freund's Complete Adjuvant (FCA) for the first injection and with Incomplete Freund's Adjuvant (IFA) for the second injection. The final injection comprised reSj22-6 only (1 ml, 0.125 mg). Ten days after the final boost, the rabbit was bled and the serum separated and stored at -20°C until required.

Vaccination experiments

Mice

In a pilot experiment, 12 BALB/c (group 1A) and 12 CBA mice (group 1B) were immunized subcutaneously with 20 µg reSj22-6 in an equal volume (50 µl) of Freund's Complete Adjuvant (FCA) at day 1 and then with 20 µg reSj22-6 in an equal volume of Freund's Incomplete Adjuvant (IFA) on day 14. An equal number of control mice (groups 2A, 2B) were injected with FCA or IFA on days 1 and 14, respectively. In a subsequent trial, CBA mice were divided into four groups (A-D) and injected with equal volumes (100 µl) by subcutaneous injection on the back. On days 1 and 14 of the experiment, mice in group A (*n* = 13) were immunized with 25 µg reSj22-6 only, those in group B (*n* = 11) received 25 µg reSj22-6 together with 15 µg Quil A (Superfos, Baedbak, Denmark) as adjuvant, those in group C (*n* = 12) were injected with 15 µg Quil A and animals in group D (10) were injected with 100 µl PBS. Three weeks later, groups A and B received 50 µg reSj22-6 without (A) or with 15 µg Quil A (B) while groups C and D received 15 µg Quil A and PBS, respectively.

Two weeks after the second (pilot experiment) or third (second experiment) injections, each of the mice was challenged subcutaneously with 40 freshly shed *S. japonicum* (Anhui) cercariae. Six weeks after challenge, mice were sacrificed, perfused with saline containing heparin, and worms were collected and counted. Blood samples for serum analysis were collected in the second experiment

just prior to the first inoculation (prebleed), immediately prior to challenge (second-bleed) and 1 day prior to perfusion (third-bleed).

Buffaloes

The 20 buffaloes were randomly divided into two groups and ear-tagged. The buffaloes were carefully monitored for gastrointestinal parasitic infections before vaccination and were treated as necessary. One group was immunized with purified reSj22-6 (vaccinated group) and the other acted as an adjuvant control group. All the buffaloes were immunized with equal volumes (2 ml) by intramuscular injection. The neck was divided into three equal areas (longitudinal) and injections made into the middle portion. The same side was used for every injection (i.e. right hand or left hand). On day 1, the vaccinated group was immunized with 1 ml (1 mg) of reSj22-6 mixed with 1 ml (2 mg) of Quil A adjuvant (Superfos) while the adjuvant control group was injected with 1 ml of normal saline mixed with 1 ml (2 mg) of Quil A. These initial injections were followed by two sets of booster injections administered at 14 day intervals on day 15 and day 29. The buffaloes were bled for serum samples from the ear vein prior to the commencement of immunization and on day 43 when the animals were challenged with 1000 cercariae. Fifty-three days postchallenge, the buffaloes were perfused, the adult worms, and liver and faecal eggs counted, and the hatchability of miracidia in faecal eggs determined and compared in the vaccinated and control groups as described previously (Liu *et al.* 1997). The sera collected were used in Western blot analysis (Waine *et al.* 1997) and in the definition of B cell epitopes on the 22-6 kDa antigen by IgG-ELISA (Zhou *et al.* 1999). The trial was carried out semiblind in that only the scientist in charge (X.Z.) was aware which animals were vaccinated with reSj22-6.

ELISA

Standard microwell enzyme-linked immunosorbent assays (ELISA) were performed by using AWA and reSj22-6 as antigen source. Optimum incubation conditions and reagent dilutions were determined by checkerboard titration (Waine *et al.* 1997). Anti-mouse IgG and IgE antibodies were used to quantify the AWA and reSj22-6-reactive antibodies present in the individual mouse sera. In brief, ELISA plates (Titertek^R Immuno Assay-Plate, ICN Biomedicals, Inc., Costa Mesa, CA, USA) were coated with AWA (5 µg/ml), or reSj22-6 (1 µg/ml) in 50 mM carbonate buffer (pH 9.6), and stored overnight at 4°C. Each plate was washed three times with phosphate-buffered saline (pH 7.6) containing 0.05% Tween 20^R (PBST), and blocked with 0.3% (w/v) bovine serum albumin (BSA) in PBS for 1 h at 37°C. The

plates were further washed three times with PBST, and the sera diluted with 0.3% (w/v) BSA to 1 : 100 for the detection of IgG and 1 : 20 for IgE, and then incubated at 37°C for either 1 h (IgG) or overnight (IgE) at 4°C. The plates were washed four times with PBST, followed by addition of goat anti-mouse IgG at 1 : 3000, IgE at 1 : 2500 (Binding Site Limited, Cambridge, UK), then incubated for 1 h at 37°C (IgG) or overnight at 4°C (IgE). The plates were washed five times with PBST. The assays were developed with *O*-phenylene diamine dihydrochloride (OPD peroxidase substrate, Sigma, St Louis, MO, USA) for 30 min. The optical density (OD) of the colour developed in the plate was read at 450 nm using a BioRad (Hercules, CA, USA) ELISA reader.

Electrophoresis and Western blotting

AWA (40 µg) or reSj22-6 (15 µg) were separated in 10% SDS-PAGE, transferred to nitrocellulose, then probed for IgG and IgE recognition. Briefly, the protein blots were blocked in PBS-0.3% Tween 20 for 1 h and subsequently incubated with buffalo or mouse sera (1 : 100 for IgG) at room temperature for 3 h and mouse sera (1 : 20 for IgE) for 24 h in a flat rotary shaker at 4°C. After four washings, the blots were incubated with peroxidase conjugated sheep antimouse IgG (diluted 1 : 3000) or rabbit antibovine IgG (1 : 5000, Sigma) for 1 h at room temperature, or with 1 : 1000 sheep antimouse IgE for 24 h. Bands were revealed by 4-chloro-1-naphthol (Sigma).

Immunolocalization

Adult male and female *S. japonicum*, perfused from CBA mice 6–7 weeks postinfection, were fixed in 3% paraformaldehyde (w/v) in 0.1 M cacodylate buffer pH 7.2 for at least 24 h and transferred to 0.1 M cacodylate buffer and then PBS. Cercariae were shed from oncomelanid snails and transferred by pipette into fixative (above). Miracidia were obtained from mouse livers using the methods of Dalton *et al.* (1997) and pipetted into fixative (above). Adult worms were cut into smaller blocks approximately 2–3 mm long and were transferred into a cryoprotection buffer consisting of 2.0 M sucrose and 20% polyvinylpyrrolidone (w/v) for at least 24 h. Fixed miracidia and cercariae were placed into molten 10% gelatin in PBS and centrifuged. The gelatin was then solidified on ice and small blocks containing worms were cut from the larger mass and placed in cryoprotection buffer. Blocks were mounted onto pins and plunge-frozen in liquid nitrogen. Transverse semithin sections (0.3 µm) were cut and collected onto slides previously coated with 1% (w/v) aqueous Poly L-lysine (Sigma) for immunofluorescence experiments. Transverse ultrathin sections of males and

females were cut at 60–100 nm thickness using a Leica Ultracut S/FCS ultramicrotome (Leica Microsystems, Vienna, Austria) and glass knives in the presence of a Diatome antistatic line (Diatome Ltd, Bienne Switzerland) and collected onto formvar/carbon coated copper grids.

Samples of adults were prepared for resin embedment using the cryo-substitution method. Thus, small cryo-protected blocks were mounted into pins, frozen in liquid nitrogen and transferred to a precooled substitution solvent consisting of 0.5% (w/v) uranyl acetate in anhydrous methanol. Samples were cryo-substituted for 36 h at -85°C , followed by 12 h at -35°C . The samples were infiltrated and polymerized under UV lamps with Lowicryl K11M resin (Chemische Werke Lowi, GmbH, Waldkraiburg, Germany) at -35°C . Ultrathin sections were cut at room temperature using a Leica Ultracut T ultramicrotome and diamond knife.

For immunofluorescence, semithin sections were immunolabelled using the indirect labelling method with Cy3- (Jackson Immuno Research Laboratories, West Grove, PA, USA) or FITC-labelled goat anti rabbit antibodies (Sigma) as secondary sera. All incubations were performed in blocking buffer, consisting of 0.1% (v/v) fish skin gelatin (Sigma) and 0.1% (w/v) BSA (Fraction V, Sigma) in PBS. Primary serum was diluted 1:200 in blocking buffer and secondary sera were diluted in blocking buffer according to manufacturer's instructions. Pre-immune rabbit serum, diluted 1:200 in blocking buffer was used as negative control. Sections were viewed and photographed using an Olympus BX-60 microscope fitted with phase contrast and fluorescence optics and a Dage MTI digital camera (Dage-MTI Inc., Michigan, IN, USA). The fluorescence plate was constructed using Adobe Photoshop version 5.0. For immunoelectron microscopy, an indirect labelling method described previously (Jones *et al.* 1996) was used. The primary antiserum (RAreSj22-6) was diluted at 1:120 in blocking buffer and the reaction was visualized using goat antirabbit secondary antibodies (Biocell, Cardiff, UK) conjugated to 10 nm colloidal gold particles diluted at 1:60 in blocking buffer. Pre-immune rabbit serum, diluted 1:120 in blocking buffer was used as a negative control. Sections were examined using a JEM 1010 transmission electron microscope (JEOL Ltd., Akishima, Tokyo, Japan) operating at 80 kV.

Statistical analysis

Analysis was conducted using Epi-Info 6.01 (Centers for Disease Control and prevention, Atlanta, GA and WHO, Geneva, Switzerland). $P < 0.05$ was considered statistically significant. Student's *t*-test was used to evaluate differences between means.

RESULTS

IgG and IgE antibodies raised in reSj22-6 inoculated mice

Figure 1 shows the ELISA levels of IgG and IgE antibodies raised to the 22-6 kDa antigen in CBA mice inoculated with reSj22-6 in the second experiment. Pre-challenge, 2 weeks after the third inoculation, the mice had produced relatively high levels of specific IgG (mean OD value $\pm$ SD = 1.0 ± 0.13) and IgE (mean OD value $\pm$ SD = 0.38 ± 0.08) antibodies to reSj22-6 compared with the Quil A adjuvant control group (mean 0.11 ± 0.03 for IgG and 0.16 ± 0.03 for IgE). There was no significant difference in antibody levels produced by mice inoculated with reSj22-6 alone (group A) or with reSj22-6 and Quil A adjuvant (group B). Sera taken prior to challenge from mice inoculated with reSj22-6 (group A) or reSj22-6 plus adjuvant (group B) induced IgG and IgE antibodies capable of recognizing native antigens in AWA but the levels were significantly less than those reacting against reSj22-6. Weak levels of IgG and IgE antibodies to AWA were detectable in sera (third bleed) from the Quil A (group C) and PBS (group D) control mice obtained 6 weeks after cercarial challenge.

Western blot analysis (Figure 2) confirmed the ELISA data. All of the CBA mice inoculated with reSj22-6, with or without Quil A adjuvant, generated an IgE antibody response and this response, in prechallenged animals, was directed exclusively at a single parasite antigen with a molecular mass of approximately 22 kDa. In contrast, none of the sera from the control mice showed IgE reactivity to any schistosome antigen. Mice inoculated with reSj22-6, but not control mice, also produced IgG antibodies specific for the 22 kDa antigen. To confirm that the native antigen with a molecular mass of approximately 22 kDa recognized by IgE and IgG antibodies in whole parasite extracts was indeed Sj22-6, sera were reacted with reSj22-6 in Western blots. The pattern of IgG and IgE recognition was identical to that shown in Figure 2 (not shown).

Figure 2 shows also that all CBA mice inoculated with reSj22-6 and challenged with *S. japonicum* cercariae generated IgG and IgE antibodies which recognized strongly a native molecule of approximately 22 kDa in AWA; the mice recognized also a band at approximately 36 kDa. IgG antibodies in sera from 6-week old-challenged mice injected only with Quil-A adjuvant or PBS bound a native molecule of approximately 22 kDa and the band at approximately 36 kDa in AWA, whereas IgE antibodies in sera from the same control mice bound weakly only to the 36 kDa band (Figure 2). The native molecules of approximately 22 kDa (IgG) and 36 kDa (IgG and IgE) were clearly the

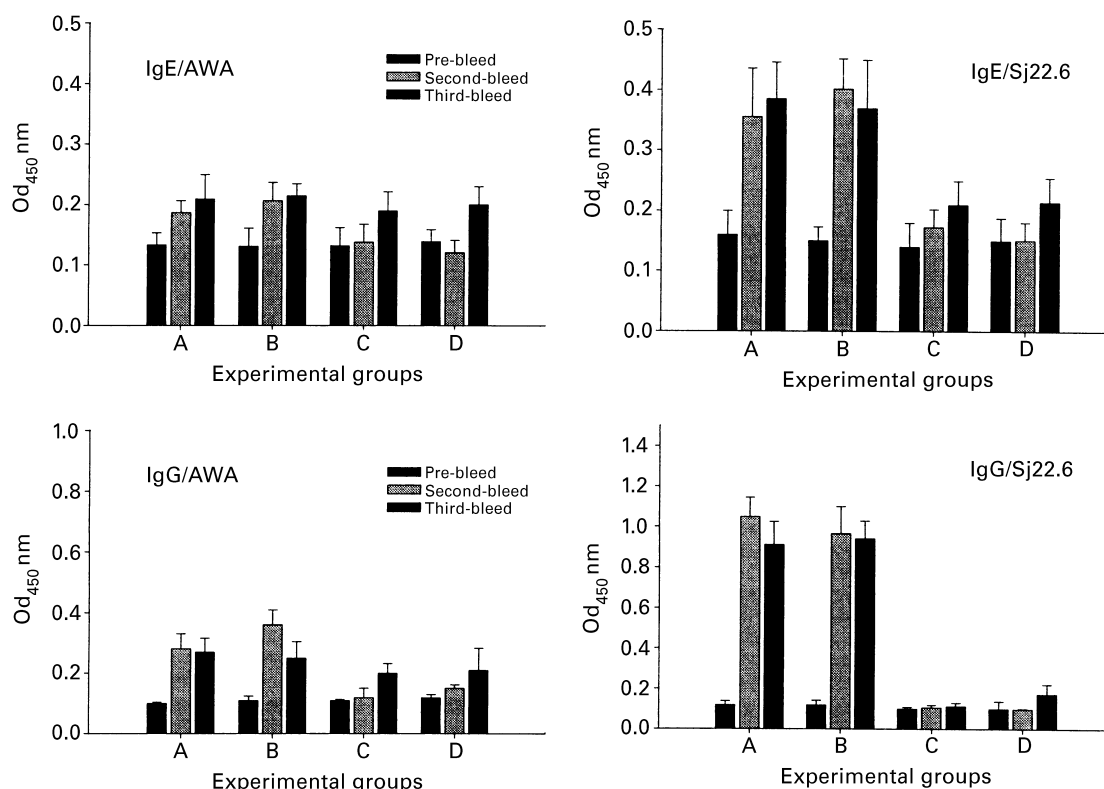


Figure 1 Total IgG and IgE antibodies in sera of reSj22-6-immunized CBA mice against *Schistosoma japonicum* adult worm antigen (AWA) and reSj22-6 determined by ELISA. Experimental groups: (A) reSj22-6; (B) reSj22-6 + Quil A; (C) Quil A; (D) PBS. Pre-bleed, prior to first inoculation; second bleed, immediately prior to challenge; third-bleed, 1 day prior to perfusion.

dominant antigens recognized by antibodies in sera of the nonvaccinated, *S. japonicum*-infected mice. To again confirm that the native antigen with a molecular mass of approximately 22 kDa recognized by IgG antibodies in whole parasite extracts was indeed Sj22-6, sera were reacted with reSj22-6 in Western blots. The pattern of IgG recognition was identical to that shown in Figure 2 (not shown).

IgG antibodies raised in reSj22-6 inoculated buffaloes

Each of the buffalo sera taken after the third immunization (day 43) were tested individually in Western blot analysis for the presence of anti-Sj22-6 IgG antibodies only as we were unable to obtain any antiovine IgE secondary antibodies commercially; all sera from the 10 reSj22-6-inoculated animals reacted specifically with a component of approximately 22 kDa when either reSj22-6 or a AWA were used as antigen source (data not shown). The sera were also tested individually with reSj22-6 by ELISA. All of the 10 buffaloes immunized with the recombinant antigen reacted with the recombinant antigen (mean OD₄₅₀ ± SD = 0.99 ± 0.13) and there was a significant difference ($P < 0.01$) compared with

the adjuvant control group (mean OD₄₅₀ ± SD = 0.36 ± 0.11).

Lack of protective efficacy of reSj22-6

There were no significant differences in worm burden (male and females) (mean ± SD) among BALB/c (group 1A, 12.4 ± 3.6; group 1B, 13.6 ± 2.9) or CBA mice (group 2A, 13.3 ± 4.1; group 2B, 11.8 ± 3.6) (pilot experiment) nor between the four groups of CBA mice (subsequent experiment) after cercarial challenge and perfusion. Worm burdens (male and females) in groups A, B, C, D were 9.2 ± 5.4, 7.6 ± 2.8, 9.3 ± 4.3, 10.7 ± 6.3.

The reSj22-6 plus Quil A (vaccinees; group 1) and Quil A (adjuvant controls; group 2) were well tolerated by the buffaloes, all of which survived the trial. All control animals were perfused successfully but perfusion of three of the 10 vaccinated animals proved problematical and the results were not recorded. There were no significant differences (mean ± SD) between the vaccinated group and the adjuvant control group in worm burden [group 1, 237.9 ± 99.6 ($n = 7$); group 2, 260.0 ± 89.7 ($n = 10$)], total liver egg counts per female worm [group 1, 9779.9 ± 2261 ($n = 7$);

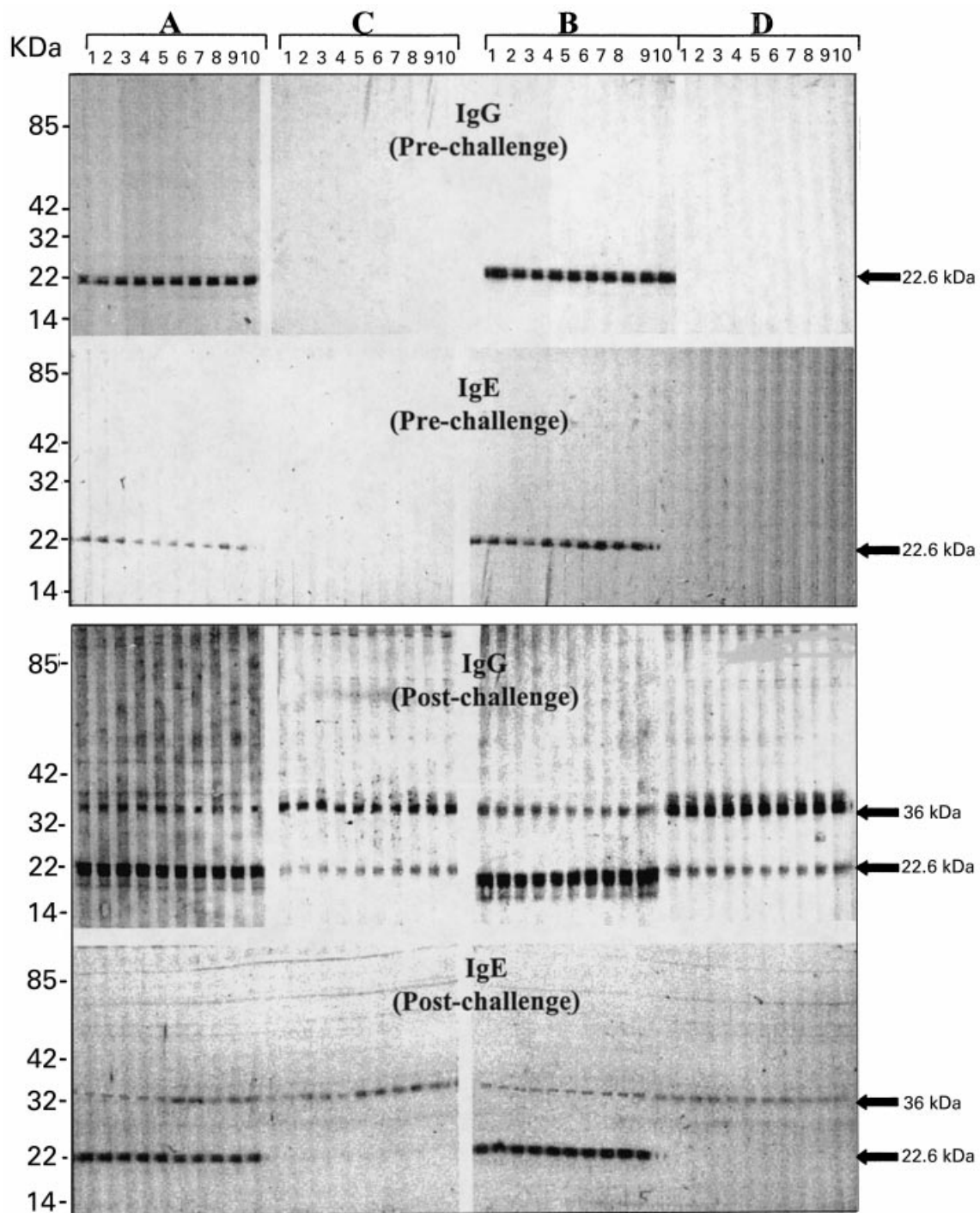


Figure 2 IgG- and IgE-specific immunoblots of individual sera from preand postchallenged reSj22-6-immunized CBA mice and controls against *Schistosoma japonicum* adult worm antigen (AWA). Experimental groups (results of 10 animals in each group are shown): (A) reSj22-6; (B) reSj22-6 + Quil A; (C) Quil A; (D) PBS. Pre-challenge – second bleed, immediately prior to challenge; postchallenge – third bleed, one day prior to perfusion. The 22.6 and 36 kDa antigens are arrowed.

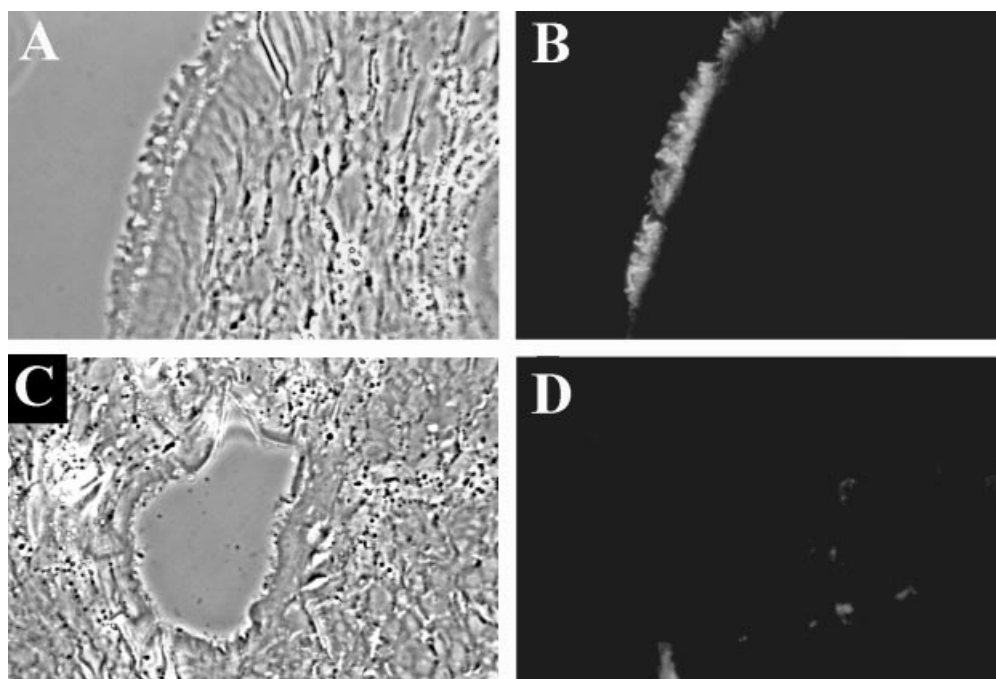


Figure 3 Immunolocalization of Sj22-6 in tissues of male *S. japonicum* by immunofluorescence. (A) Tegument and underlying tissues, phase contrast optics. (B) Fluorescence micrograph of the same region as in (A), labelled with antisera to reSj22-6 and Cy3-conjugated secondary antisera. (C) Gut and surrounding tissues, phase contrast optics. (D) Fluorescence micrograph of the same region as in (A), labelled with reSj22-6 and Cy3-conjugated secondary antisera. Fluorescence label is not apparent in the gastrodermal lining.

group 2, 10205 ± 2638 ($n = 10$)], eggs per gram of faeces [group 1, 2.24 ± 0.34 ($n = 7$); group 2, 3.06 ± 1.44 ($n = 10$)] or miracidial hatching rate (%) (group 1, 32.9 ± 13.4 ; group 2, 34.1 ± 21.6).

Immunolocalization of Sj22-6

Sj22-6 was detected in the apical cytoplasm of the tegument of males (Figure 3a,b) and females (data not shown) by immunofluorescence. The native protein was not detected in other tissues and, significantly, was not apparent in the gastrodermal lining of males (Figure 3c,d) or females (data not shown). No immunofluorescence labelling was detected in control sections, for which native rabbit serum was used in place of primary serum (data not shown).

Immunoelectron microscopy of thawed cryo-sections revealed high levels of gold label in the tegument of males and females (Figure 4). Label was not observed in other tissues of adults, nor was it apparent in any tissues of the cercariae or miracidia. Immunoreactivity was not detected in sections exposed to preimmune rabbit serum as primary antiserum (Figure 4a). In sections labelled with reSj22-6 antiserum, the gold particles were localized specifically to the apical cytoplasm of the tegument in adult males and females (Figure 4b–d) and to a lesser extent in

subtegumental cell bodies (not shown). Within the apical cytoplasm, the gold particles appeared to localize components of the cytoplasmic matrix of the apical cytoplasm, and were not preferentially located in apical or basal domains of the apical cytoplasm (Figure 4b–d). Gold was present in the cytoplasmic matrix immediately beneath the apical membrane but did not appear to be expressed on the surface of the worm (Figure 4b–d). The apical cytoplasm is rich in inclusion bodies, including discoid bodies, multilamellar bodies and mitochondria (Figure 4a–d). Although gold particles were detected within, or at the surface of some of these inclusion bodies, particularly the multilamellar bodies (Figure 4b), this label was inconsistent and did not suggest that Sj22-6 occurs within any of these bodies. In most cases, the protein was localized to cytoplasm in the immediate vicinity of the inclusion bodies.

The immunolocalization of Sj22-6 in Lowicryl K11M sections was similar to that observed on cryosections, although the intensity of label was much less. However, a curious incidence of background labelling was noticed on L11M resin in which the secondary colloidal gold probe labelled the ribs of flames cells intensely. This label was also observed in negative controls labelled with normal rabbit serum as primary sera, but was not observed in any cryosections labelled for immunoelectron microscopy. Taken

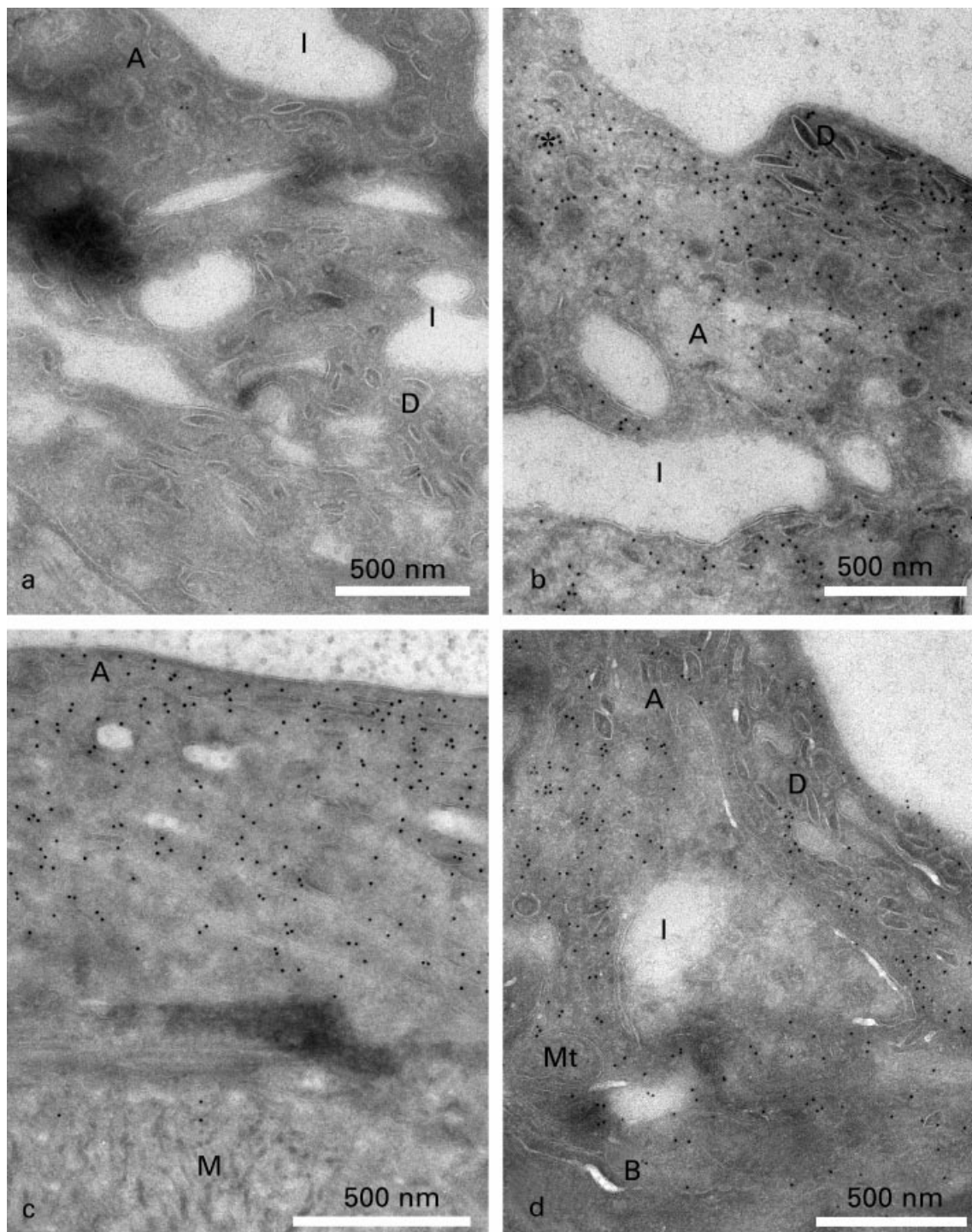


Figure 4 Immunolocalization of Sj22-6 in tissues of adult *S. japonicum* by immunoelectron microscopy. (A) Control labelled with preimmune serum. (B) Male worm, apical cytoplasm of dorsal tegument. Some label was detected in some, but not all, multilamellar bodies (asterisks). Most gold particles were found in amorphous regions of the cytoplasm. (C) Male worm, apical cytoplasm of ventral tegument. (D) Female worm. A, apical cytoplasm; Bm, basal matrix; D, discoid bodies; I, surface invaginations; M, muscle; Mt, mitochondria.

together, these observations suggest that secondary colloidal gold conjugates used in this study recognize nonspecifically a component of the flame cells that is exposed after embedding in K11M resin.

DISCUSSION

There are a number of reports of studies in schistosomendemic populations that show that resistance to reinfection following parasitological cure correlates with older age and elevated IgE titres against worm antigens (Hagan *et al.* 1991, Rihet *et al.* 1991, Dunne *et al.* 1992). In both *S. mansoni* (Dunne *et al.* 1997, Webster *et al.* 1996) and *S. japonicum* (Santiago *et al.* 1998), low levels of reinfection following chemotherapy are associated with the recognition of Sm22-6 and Sj22-6 by IgE antibodies. Earlier, it had been shown that native and recombinant forms of Sm22-6 were recognized by sera from mice protectively vaccinated with adult *S. mansoni* surface membrane preparations (Jeffs *et al.* 1991). As a result of this evidence, the potential of the 22-6 kDa tegumental antigen as a vaccine target in schistosomiasis has been well documented (McManus 1999), although no direct vaccination/challenge experiments using Sm22-6 or Sj22-6 had been previously reported.

The results of the experiments described here indicated that *E. coli*-expressed reSj22-6 failed to protect mice or water buffaloes against subsequent challenge with *S. japonicum* cercariae. This was despite the fact that specific IgG (buffaloes) and IgG and IgE (CBA mice) antibodies were generated against the 22-6 kDa molecule, observations consistent with some of our earlier findings following inoculation of CBA, BALB/c and C57BL/6 mice with reSj22-6 (Waite *et al.* 1997b). We can only conclude that the titres of antibodies produced may have been insufficient to induce protection against challenge, the particular antibodies generated were of the incorrect isotype or that conformational epitopes important for inducing protective immunity were not expressed in the bacterial system which we employed to produce the reSj22-6. We are currently investigating the use of the recombinant baculovirus expression system (Kost & Condreay 1999) to produce Sj22-6 with more relevant physicochemical properties and biological activity similar to those of the native molecule as a prelude to further vaccination/challenge trials.

Other explanations for the apparent lack of protective efficacy of Sj22-6 are that the molecule was presented incorrectly to the immune system or that it may be an inappropriate vaccine target. We could find no evidence from the immunolocalization studies that Sj22-6 is expressed or exposed on the surface of the adult parasite, although we have not investigated other stages of *S. japonicum* including schistosomula. Sj22-6 appears to be restricted in adults to

the apical cytoplasm of the tegument and is not associated with the apical or basal membrane or any membrane-bound structures in the apical cytoplasm. Similarly, Dunne *et al.* (1997) demonstrated that components of the 22 kDa band of *S. mansoni* eluted from SDS-PAGE were abundant in the cytoplasm surrounding tegumentary spines and in clusters beneath the apical plasma membrane, but not specifically bound to any membrane-associated structure in the tegument. In contrast to our observations, Dunne *et al.* (1997) detected Sm22-6 in the gastrodermis and protonephridial system, as well as the tegument, in *S. mansoni*. Dunne and colleagues acknowledge that their antiserum, raised against the 22 kDa band may recognize multiple antigens. The different localization of Sj22-6 and Sm22-6 may, in part, be due to this or suggest that a number of isoforms of Sm22-6 of similar molecular weight exist in *S. mansoni*, or that the single form of Sm22-6 is expressed in a wider range of tissues than its homologue in *S. japonicum*.

At present, there is limited functional information available on the 22-6 kDa antigen. A potential EF-hand Ca^{2+} binding motif has been identified in Sm22-6 and Sj22-6 (Waite *et al.* 1994, 1997a), but it now appears that these molecules and the other tegument-associated antigens comprising the 20–22-6 kDa family are unable to bind calcium experimentally (Hoffman & Strand 1997, Mohamed *et al.* 1998, Hafalla *et al.* 1999). Furthermore, recent studies have shown that the 20–22-6 kDa family have sequence similarity with the cytoplasmic dynein light chains (Hafalla *et al.* 1999). Further functional characterization of Sj22-6 may provide a better understanding of its role in tegument structure and dynamic organization and, more importantly, may give clues on the mechanisms by which this antigen is released from the parasite. The Sj22-6 antigen must be released to the immune system during the course of infection as specific anti-Sj22-6 IgG antibodies were present in the sera of all the control, nonvaccinated but challenged mice (Figure 2) analysed in the current study. Radioimmuno-precipitation assays indicated that schistosomula and immature worms of *S. mansoni* release 22 kDa band antigens *in vitro* (Dunne *et al.* 1997). Detailed immunolocalization studies of early developmental stages may be necessary to determine how Sj22-6 is released or shed in *S. japonicum* infections.

ACKNOWLEDGEMENTS

We would like to thank Mary Duke for technical assistance with the mouse studies and Professors Xianfang Zeng and Qingren Zeng and Ms Jinchun Zhou (Hunan Medical University, Hunan, P.R. China) who were involved in the buffalo trial. Financial support from the UNDP/World Bank/WHO Special programme for Research and

Training in Tropical Diseases, and the National Health and Medical Research Council of Australia is gratefully acknowledged.

REFERENCES

- Chen L., Gao P., Yamashita T. *et al.* (1998) Molecular cloning and expression of a *Schistosoma japonicum* tegumental membrane-associated antigen from Japanese strain. *Parasitology International* **47**, 289–300
- Dalton J.P., Day S.R., Drew A.C. & Brindley P.J. (1997) A method for the isolation of schistosome eggs and miracidia free of contaminating host tissues. *Parasitology* **115**, 29–32
- Dunne D.W., Butterworth A., Fulford A.J. *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
- Dunne D.W., Webster M., Smith P. *et al.* (1997) 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. *Parasite Immunology* **19**, 79–89
- Hafalla J.C.R., Alamares J.G. II, Acosta L.P. *et al.* (1999) Molecular identification of a 21.7 kDa *Schistosoma japonicum* antigen as a target of human IgE response. *Molecular and Biochemical Parasitology* **98**, 157–161
- Hagan P., Blumenthal U.J., Dunne D., Simpson A.J.G. & Wilkins H.A. (1991) Human IgE, IgG4 and resistance to reinfection with *Schistosoma haematobium*. *Nature* **349**, 243–245
- Hoffman K.F. & Strand M. (1997) Molecular characterization of a 20.8-kDa *Schistosoma mansoni* antigen. *Journal of Biological Chemistry* **272**, 14509–14514
- Jeffs S.A., Hagan P., Allen R. *et al.* (1991) Molecular cloning and characterization of the 22-kilodalton adult *S. mansoni* antigen recognised by antibodies from mice protectively vaccinated with isolated tegumental surface membranes. *Molecular and Biochemical Parasitology* **46**, 159–167
- Jones M.K., Zhang L.H., Leggett G.R., Stenzel D.J. & McManus D.P. (1996) The ultrastructural localization of *Echinococcus granulosus* antigen 5. *Parasitology* **113**, 213–222
- Kost T.A. & Condreay J.P. (1999) Recombinant baculovirus vectors for insect and mammalian cells. *Current Opinion in Biotechnology* **10**, 428–433
- Liu S.X., He Y., Song G. *et al.* (1997) Anti-fecundity immunity to *Schistosoma japonicum* induced in Chinese water buffaloes (*Bos bufelus*) after vaccination with recombinant 26kDa glutathione-S-transferase (reSjc26GST). *Veterinary Parasitology* **69**, 39–47
- Li Y.S., Ross A.G.P., Sleight A.C. *et al.* (1999) Antibody isotype responses, infection and reinfection for *Schistosoma japonicum* in a marshland area of China. *Acta Tropica* **73**, 79–92
- McManus D.P. (1999). The search for a vaccine against schistosomiasis – a difficult path but an achievable goal. *Immunological Reviews* **171**, 149–161
- Mohamed M.M., Shalaby K.A., LoVerde P.T. & Karim A.M. (1998) Characterization of Sm20.8, a member of a family of schistosome tegumental antigens. *Molecular and Biochemical Parasitology* **96**, 15–25
- Rihet P., Demeure C., Preta 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
- Santiago M.L., Hafalla J.C.R., Kurtis J.D. *et al.* (1998) Identification of the *Schistosoma japonicum* 22.6-kDa antigen as a major target of the human IgE response: similarity of IgE-binding epitopes to allergen peptides. *Allergy Immunology* **117**, 94–104
- Stein L.D. & David J.R. (1986) Cloning of a developmentally regulated tegument antigen of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **20**, 253–264
- Waine G.J., Becker M.M., Scott J.C. *et al.* (1994) Purification of a recombinant *Schistosoma japonicum* antigen homologous to the 22-kDa membrane-associated antigen of *S. mansoni*, a putative vaccine candidate against schistosomiasis. *Gene* **142**, 259–263
- Waine G.J., Mazzer D.R., Brandt E.R. & McManus D.P. (1997a) A dominant B-cell epitope on the 22 kDa tegumental membrane-associated antigen of *Schistosoma japonicum* maps to an EF-hand calcium binding domain. *Parasite Immunology* **19**, 337–345
- Waine G.J., Mazzer D.R. & McManus D.P. (1997b) Production of IgE antibodies against the 22 kDa tegumental membrane-associated antigen of schistosomes is directed by the antigen itself. *Parasite Immunology* **19**, 531–533
- Webster M., Fulford A.J.C., Braun G. *et al.* (1996) Human immunoglobulin E responses to a recombinant 22.6-kilodalton antigen from schistosomiasis mansoni adult worms are associated with low intensities of reinfection after treatment. *Infection and Immunity* **64**, 4042–4046
- Webster M., Libranda-Ramirez B.L.D., Aligui G.D. *et al.* (1997) The influence of sex and age on antibody isotype responses to *Schistosoma mansoni* and *Schistosoma japonicum* in human populations in Kenya and the Philippines. *Parasitology* **114**, 383–393
- Webster M., Roberts M., Fulford A.J.C. *et al.* (1998) Human IgE responses to rSm22.6 are associated with infection intensity rather than age *per se*, in a recently established focus of schistosomiasis mansoni. *Tropical Medicine and International Health* **3**, 318–326
- Yang W., Jones M.K., Fan J., Hughes-Stamm S.R. & McManus D.P. (1999) Characterization of a family of *Schistosoma japonicum* proteins related to dynein light chains. *Biochimica et Biophysica Acta* **1432**, 13–26
- Zhou J., Waine G.J., Zeng Q. *et al.* (1999) B-cell epitopes recognized by Chinese water buffaloes (*Bos bufelus*) on the 22 kDa tegumental membrane-associated antigen (Sj-22) of the Asiatic bloodfluke, *Schistosoma japonicum*. *Veterinary Research* **30**, 427–432