16 September 2010

Differential recognition patterns of *Schistosoma haematobium* adult worm antigens by the human antibodies IgA, IgE, IgG1 and IgG4

Francisca Mutapi[1], Claire Bourke[1], Yvonne Harcus[1], Nicholas Midzi[3], Takafira Mduluza[4], C. Michael Turner[5], Richard Burchmore[2], Rick M. Maizels[1]

1. Institute of Immunology & Infection Research, School of Biological Sciences, University of Edinburgh, Ashworth Laboratories, King's Buildings, West Mains Rd, Edinburgh, EH9 3J, UK.

2. Institute of Biomedical Life Sciences, Division of Infection and Immunity, Joseph Black Building, University of Glasgow, G12 8QQ, UK.
3. National Institute of Health Research, Box CY 570, Causeway, Harare, Zimbabwe.
4. Department of Biochemistry, University of Zimbabwe, P.O. Box 167, Mount Pleasant, Harare, Zimbabwe.
5. Faculty of Biomedical and Life Sciences, Glasgow Biomedical Research Centre, University of Glasgow, G12 8QQ, UK.

Running title: Isotype-specific reactivity of helminth antigens

Keywords: schistosomiasis, isotype, proteomics, recognition patterns

Footnotes
Corresponding author: Francisca Mutapi, Institute for Immunology & Infection Research, School of Biological Sciences, University of Edinburgh, Ashworth Laboratories, King's Buildings, West Mains Rd, Edinburgh, EH93JT, UK. Tel + 44 131 650 8662, Fax + 44 131 650 5054, email address; f.mutapi@ed.ac.uk

Disclosures: None

Abstract

Schistosoma haematobium antigen recognition profiles of the human isotypes IgA, IgE, IgG1 and IgG4 were compared by image analysis of western blots**.** Adult worm antigens separated by 2-dimensional gel electrophoresis were probed with pooled sera from Zimbabweans resident in a *S. haematobium* endemic area, followed by identification of individual antigenic parasite proteins using mass spectrometry. Overall, IgG1 reacted with the largest number of antigens, followed by IgE and IgA which detected the same number, while IgG4 detected the fewest antigens. IgE recognised all the antigens reactive with IgG4 as well as an additional four antigens; an isoform of 28kDaGST, phosphoglycerate kinase, actin 1 and calreticulin. IgG1 additionally recognised fatty acid binding protein, triose-phosphate isomerase, and heat shock protein 70, which were not recognised by IgA. Recognition patterns varied between some isoforms e.g. the 2 fructose 1-6-bis-phosphate aldolase isoforms differentially recognised by IgA and IgG1. Although the majority of *S. haematobium* adult worm antigens are recognised by all the four isotypes there are clear restrictions in antibody recognition for some antigens. This may partly explain differences observed in isotype dynamics at a population level. Differential recognition patterns for some isoforms indicated in the study have potential importance for vaccine development.

Introduction

Schistosomiasis is a major human parasitic disease in tropical and subtropical countries in Africa, the Middle East and South America (1). Urinary schistosomiasis caused by *Schistosoma haematobium* affects over 100 million people and a recent survey in sub-Saharan Africa indicated that 70 million individuals had experienced haematuria and 32 million dysuria associated with *S. haematobium* infection (2). Furthermore, it was estimated that 18 million people suffered schistosome-related bladder wall pathology and 10 million suffered hydronephrosis.

Epidemiological studies in endemic human populations have shown that schistosome prevalence and intensity levels rise to peak in childhood (around ages 9-14) (3) and decline thereafter, so that in any endemic population, children carry the heaviest infection levels while adults carry little or no infection. This pattern has been taken to reflect the development of immune-meditated resistance to infection/re-infection (3, 4), and suggests that protective immune responses develop slowly as a result of cumulative exposure to parasite antigens (5). Early serum transfer studies in the mouse model (6-9) as well as human immuno-epidemiological studies have shown that schistosome-specific antibody responses are associated with protection to infection and re-infection (10-14). Despite the demonstration that antibody-mediated responses can protect against schistosome infection in experimental models, current human schistosome vaccine research based on antibody-mediated protection has stalled with the **failure of many of the** vaccine candidate antigens to enter Phase III clinical trials (15). Limitations in our current understanding of the development of protective anti-schistosome responses against specific antigenic proteins may be contributing to the slow development of effective anti-schistosome vaccines. Previous studies characterising immune responses to schistosome proteins have relied on recombinant proteins expressed in bacterial systems (16-18). However, there are restrictions associated with this approach; for example, it cannot detect immunogenic epitopes arising from post-translational modifications. Recent developments in 2-dimensional gel electrophoresis and proteomics have the potential to overcome some of these restrictions because crude parasite antigen preparations can be separated into individual protein spots which can then be identified by mass spectrometry analysis and matching to newly-available genomic databases.

The nature of the anti-schistosome antibody response to crude antigen preparations has been studied in terms of the different antibody isotypes involved and their relationship to

each other, to host age and to schistosome infection level by ourselves and others (for example see (5, 12, 19-21, 22). Relatively few individual target antigens have been analysed in the context of selective antibody isotype recognition (23). IgA, IgE, IgG1 and IgG4 are of particular interest as they have been implicated in the development of protective human anti-schistosome immunity. Hagan *et al* demonstrated a significant relationship between anti-schistosome IgE and IgG4 responses in *S. haematobium* infections. Furthermore, they reported a decrease in IgG4 accompanied by an increase in IgE with host age, which was associated with resistance to re-infection after treatment (11). We have previously reported that children exposed to *S. haematobium* produced a predominantly IgA response against egg and adult worm antigens which was gradually replaced by an IgG1 response in adulthood (5). These studies were based on immune responses to crude antigens containing a heterogeneous mixture of proteins and thus did not provide any information on the recognition patterns of individual antigens. Therefore the antigenic source of variation in isotype-specific responses to *S. haematobium* is unknown. Differences may arise from the recognition of the same antigen by different antibody classes, from the recognition of different antigens, or a combination of both. Identifying the individual antigens recognised by the different antibody isotypes in crude adult worm antigens would help resolve the cause(s) of some of these differences. .In this study, we used proteomic approaches in combination with 2-dimensional western blotting to determine which adult worm antigens are recognised by specific antibody isotypes/subclasses from schistosome infected/exposed individuals. Thus we identify the isotype-specific recognition patterns of individual parasite proteins within the crude adult *S. haematobium* proteome for the first time. We identified antigens recognised by IgA, IgE, IgG1 and IgG4, focussing our attention on WHO vaccine candidate antigens (24). Characterising the antigens recognised by each antibody class will both improve our understanding of naturally acquired schistosome-specific immunity and inform vaccine development where the aim may be to stimulate an isotype-specific antibody response to a single recombinant antigen rather than a heterogeneous mixture of peptides. **In this study we determined the reactivity of sera from 7-18 year olds the age group where infection levels are changing from rising, peaking and declining in this population (25, 26) to capture the range or immune reactivities as the dynamics of infection change. The analyses were conducted on pre-treatment sera to study natural immune reactivities before enhancement of antibody reactivity with anti-helminthic treatment which we have previously demonstrated in a different subgroup from the same population (27).**

Materials and Methods

Study area

The study was conducted in two rural villages in the Mashonaland East Province of Zimbabwe (31^{o}30'E; 17^{o}45'S) where *S. haematobium* is endemic. The study area is described in detail elsewhere (27). The study received ethical and institutional approval from the Medical Research Council of Zimbabwe and the University of Zimbabwe respectively. Permission to conduct the work in this province was obtained from the Provincial Medical Director. The villages were selected because health surveys conducted regularly by the Ministry of Health and Child Welfare in the region showed little or no infection with other helminths and a low *S. mansoni* prevalence (<5%). The selected villages had not been included in the National Schistosome Control Programme and therefore had not received treatment for schistosomiasis or other helminth infections. The main activity in these villages is subsistence farming. Drinking water is collected from open wells while bathing and washing is conducted in two main rivers in the villages. Most families maintain a garden located near the river where water is collected for watering the crops and the schools surveyed were all in close proximity to rivers.

Study subjects

The study participants are described in detail elsewhere (27). Briefly, only permanent inhabitants of the villages who had never been treated for any helminth infection were eligible for inclusion in the study. Informed consent was obtained from all participants prior to enrolment into the study. Following explanation of the project aims and procedures to the community, school children and their teachers, an initial parasitology (stool and urine samples) and serology (blood sample) survey was conducted amongst all compliant participants. A questionnaire survey confirmed that on average, all participants frequented water contact sites at least four times per week and that frequency of water contacts at the various sites was not significantly different within the age range included in this study (data not shown).

Parasitology samples (at least 2 urine and 2 stool samples collected on 3 three consecutive days) and 20mls of venous blood were collected from each participant. Stool samples were processed following the Kato-Katz procedure (28) to detect *S. mansoni* eggs and other intestinal helminths, while the urine filtration method (29) was used to detect *S. haematobium* eggs in urine samples. After collection of the samples,

all participants were offered treatment with the recommended dose of praziquantel, 40mg/kg body weight. In order to be included in the cohort, participants had to meet all the following criteria: 1) have provided at least 2 urine and 2 stool samples on consecutive days for detection of helminth parasites; 2) have given a blood sample for serological assays; 3) be negative for intestinal helminths and *S. mansoni.* In practice, all people meeting criteria 1 and 2 were egg negative for intestinal helminths and *S. mansoni* and so no participants were excluded on this third criterion. 215 people (110 male and 105 female) aged from 5 to 18 years met all criteria. Serum samples obtained from 20mls of venous blood from each participant were frozen and stored in duplicate at –20^{o}C in the field and transferred to a –80 ^{o}C freezer in the laboratory. One complete set of the samples was subsequently transported frozen from Zimbabwe to the UK, stored at -80 ^{o}C and defrosted for the first time for use in this study. A serum pool for use as a positive control in the western blot assays was made using equal volumes (50ul) of sera from each of the 215 participants irrespective of their schistosome infection status and frozen in aliquots which were defrosted only once for the immunoassays.

Parasite antigens

Adult worm antigens were used in this study as previous studies have shown that adult *S. haematobium* parasites suffer immune attrition and are targeted by host antibodies (30). Soluble *S. haematobium* adult worm antigens (SWAP) were obtained freeze dried, from the Theodor Bilharz Institute in Egypt and reconstituted as previously described (27). These were used in the subsequent gel-electrophoresis and immuno-assays.

Antibody enzyme linked immunosorbent assays

Circulating levels of IgA, IgE, IgG1, and IgG4 directed against the *S. haematobium* adult worm antigens were detected by indirect enzyme linked immunosorbent assays (ELISA) as previously described [5] using 5ug/ml of antigen to coat plates, sera diluted at 1:50 and the secondary horseradish peroxidase conjugated secondary antibodies IgE (Sigma A9667), IgG1(The Binding Site, AP006) and IgG4 (Ap009), all at 1:1000 dilution, and IgA (Dako,P0216) which was diluted at 1:2000. Negative control sera from 5 European volunteers' who have never travelled to tropical countries and therefore presumed not previously exposed to schistosomiasis were also included in the assays to generate reactivity cut-off points

Gel electrophoresis

The soluble adult worm antigen preparation was separated into constituent proteins by two dimensional gel electrophoresis on 13cm gels as previously described (27) to generate a reference gel used for identifying proteins (200 µg of SWAP used) and the second (100 µg of SWAP used) for western blotting to determine which antigens were recognised by the sera following a previously established protocol [5]

Determining sera and secondary antibody dilutions for western blotting

To minimise differences in sensitivity and specificity of the different assays arising from technical differences, assays were optimised by titration assays. A pool of sera from all the Zimbabwean participants was used as a positive control while a pool of sera from 5 European volunteers (same as the ones used in the ELISA assays above) was used as a negative control. The dilutions used for the sera and secondary antibodies (IgA, IgE, IgG1 and IgG4) were determined by a combination of Enzyme Linked Immunosorbent Assay (ELISA) titration assays and trial western blots. First, ELISAs were conducted (varying both secondary antibody (1:500-1:2000) and sera dilutions (1:10-1:11280; see example in Supplementary Figure 1) with 5ug/ml of adult worm antigen. This informed the subsequent trial western blots conducted using sera diluted at 1: 200 or 1:400 to allow enough sample for all replicate assays and to test a range of potential secondary antibody dilutions (1:1000, 1:2000, 1: 4000) on a blot of 100 ug of 2d-electrophoresis-seperated SWAP. From these data, we determined dilutions that gave the most consistent replicates and optimal sensitivity and specificity for each antibody isotype. Higher dilutions of secondary antibodies resulted in the detection of fewer antigenic spots (see supplementary Figure 2) while reducing the secondary antibody dilution factor from 1:1000 (IgE, IgG1, IgG4) or 1:2000 (IgA) resulted in higher background rather than an increase in the number of spots detected. Furthermore, sera and secondary antibody dilutions used were consistent with those we have previously used in ELISA (25, 31) and western blot assays [17] enabling our results to be related to previous studies. The finalised protocol is described below.

Western Blotting

Western blotting was used to determine the proteins reactive with the isotypes IgE, IgG4, IgA and IgG1 in sera from the participants. To achieve this, proteins were transferred from the gel onto nitrocellulose membrane as previously described (27). Blots were run in pairs (one gel/membrane) in order to probe for two antibody isotypes at a time in parallel assays. To verify efficient transfer of SWAP proteins, membranes were stained with Ponceau S solution (Sigma) to and then blocked for 1

hr at room temperature in TBS blocking buffer (Pierce) with 0.05% Tween 20. This was followed by 2 x 10 min washes with TBS / 0.05% Tween 20 / 0.5% Triton-X 100 (TBS/TT) (used for all washes). The pool of positive control sera (diluted at 1:200 in TBS blocking buffer with 0.02% Tween 20) was added to each of the membranes and incubated overnight at 4^{o}C followed by 3 x 10 min washes. Horseradish peroxidise-conjugated secondary antibodies diluted in TBS blocking buffer / 0.05% Tween 20 were then added to respective membranes diluted at 1:1000 for IgE and 1: 2000 for IgA. Membranes were subsequently incubated at room temperature for 1 hr, followed by 4 x 10 min washes in TBS/TT and 1 x 10min wash in TBS alone. The proteins were visualized using the chemiluminescence product ECL Plus (GE Healthcare) according to the manufacturer's instructions. Films exposed to the blots for 5 sec were developed and spots matched to the Coomassie blue stained reference gel using the image analysis software Image master. Following visualisation, the membranes were stripped of the ECL reagent, secondary antibody and sera following the manufacturer's protocol. The same membranes were then re-probed using the same pool of sera, The IgE membrane was re-probed with IgG4 diluted at 1:1000 while the IgA membrane was re-probed with IgG1 diluted at 1:1000. A previous assay showed that the stripping procedure removed all proteins not directly bound to the nitrocellulose membrane as indicated by the lack of ECL reactivity with a stripped membrane. This procedure did not remove any of the parasite proteins as evidenced by probing the same membrane with three serum samples successively, i.e. sera from the endemic population, followed by negative control sera (a pool from 5 Europeans who had never travelled to tropical regions) then the same sera from the endemic population. An example of a membrane blotted with negative control sera and the IgG4-specific secondary antibody is shown in Supplementary Figure 3.. Gel electrophoresis and western blotting was repeated twice using the secondary antibodies in different order on the same membrane i.e. IgG1 followed by IgA and IgG4 followed by IgE to confirm the recognition patterns obtained.

Image Analysis and Mass Spectrometry

Images from the western blots were electronically scanned for image analysis. Spots on these blots were detected by pixel analysis and matched across the different blots for each isotype using the 2-d gel image analysis software ImageMaster from GE Healthcare.. All electronic spot identifications and predicted matches were manually verified via independent visualisation by two researchers. Spots on the Coomassie blue stained gel, which matched to those on the western blots, were excised,

digested with trypsin and analysed by mass spectrometry analysis as previously described (27). Data thus obtained were- submitted to an MSMS ion search via the Mascot search engine (Matrix Science), searching both locally established databases for *S. mansoni* EST sequence and the current non-redundant NCBI database.

Data analysis

The ratios of IgG1/IgA and IgE/IgG4 were calculated for each individual from ELISA data for each individual. Partial correlations were conducted to determine the association between the ratios and schistosome infection intensity allowing for residential village, age and sex. Significance level for the statistics tests was set at p <0.05. For all analyses of antigenic spots, if the recognition intensity of the spot after subtraction of the background intensity was 0 pixels then the spot was designated 'not recognised' by the isotype, if it was greater than 0, then the antigenic spot was designated as 'recognised'. To allow comparisons of the intensity of antigen recognition by the different isotypes, the detected spots were categorised by their recognition intensity into 5 groups; Group 1 comprised all antigens not recognised by the pooled sera and groups 2-5 were based on the interquartile ranges for each isotype using the mean intensity from the 3 replicate blots per isotype. The interquartile ranges were calculated from the antigens using a spot detected from pixel analysis and manual verification. These ranges were calculated for each isotype separately so that the spot intensities were standardised within the gel. The intensity categories rather than absolute values could then be compared across the different gels. This approach was used as it does not require any spots to be recognised equally by the 4 isotypes for calibrating recognition intensity across all isotypes.

Results

Levels of IgA, IgE, IgG1 and IgG4 detected in individuals.

The *S. haematobium* infection prevalence in the participants was 57% and the corresponding mean infection intensity was 36 eggs/ 10ml urine (SE of the mean = 5.7), with individual egg counts ranging from 0 to 676 eggs/10ml urine. Individual infection intensity followed the typical age-infection profile for schistosome infections, rising with age to peak in childhood and then declining thereafter as shown in Figure 1A. The levels of the four antibody isotypes IgA, IgE, IgG1 and IgG4 directed against adult worm antigen were measured for each individual who was included in the serum pool. A participant was considered reactive for an isotype if their titre was above the cut-off point of mean +2 standard deviations of the negative control sera. Based on this cut-off point, the least prevalent schistosome-specific isotype was IgA being detected in 31 % of the participants, then IgE detected in 50 % of the participants. IgG1 was detected in 58% of the participants while IgG4 was the most prevalent response, detected in 74% of the participants**. Levels of the isotypes were plotted by age groups in Figure 1B.**

Earlier studies have shown that higher ratios of IgG1/IgA and IgE/IgG4 are associated with lower infection levels (11, 32). Therefore to determine whether the relationship between *S. haematobium* infection and antibody responses in this study was consistent with those reported from other earlier studies the ratios of IgG1/IgA and IgE/IgG4 were calculated and plotted against schistosome infection intensities. The ratio of IgE/IgG4 showed a significant inverse relationship with schistosome infection intensity ($r= -0.21$, $p =0.002$, $df = 210$), so that people with high values for these ratios carried little or no schistosome infection (Figure 1C). The IgA/IgG1 ratio did not show a significant association with infection intensity ($r= 0.11$, $p =0.10$, $df = 210$). The subsequent Western blotting analyses were performed to determine if these different isotypes recognised the same individual native antigens.

Antigens recognised by the different isotypes

The potential repertoire of schistosome antigens recognised on 2-dimensional blots of adult parasite extract was first defined with a highly reactive positive post-treatment serum pool made from a subgroup of this population previously used to investigate the effects of praziquantel treatment on schistosome-specific responses (27), which was probed with a secondary antibody combination reactive to IgA/IgG/IgM. This serum pool reacted with a total of 71 spots compared to negative non-endemic sera which reacted with none of the protein spots. The 71 proteins were

identified and characterised in our previous study (27) which gives their predicted molecular weights, isoelectric point (p*I*), Mascot output statistic, and accession numbers from NCBI.

Compared to the IgA/IgG/IgM positive control, no single isotype in the serum pool recognised all the 71 antigenic spots. The differences in recognition of the antigenic spots between the isotypes were both qualitative and quantitative. IgG1 recognised the largest number of spots (n=45) followed by IgA and IgE which both recognised 39 spots, with IgG4 recognising the fewest antigenic spots (n=35) as shown in Figure 2 and Table 1. The most strongly recognised antigens by all the subclasses were the two isoforms of glyceraldehyde-3-phosphate, some isoforms of heat shock protein (HSP) 70 as well as some proteins still to be identified (spots 60 and 61). The antigenic spots 2 (protein yet to be indentified), 6 (triose-phosphate isomerase) and 15 (yet to be identified) were strongly recognised by IgG1 but were not detected by any of the other isotypes. There were 2 isoforms of fructose-1-6-bis-phosphate aldolase, one reactive with IgA (antigenic spot 25 in Figure 2A) and one reactive with IgG1 (antigenic spot 23 in Figure 2A). Some antigens were not recognised at all by any of the 4 isotypes tested; these included the muscle proteins myosin (heavy and light chain), tropomyosin and paramyosin, HSP 60 and some isoforms of enolase, and immunophilin. Some antigenic spots such as those between spots 22 and 52 in Figure 2 were strongly recognised by the sera but were in such low abundance in the Coomassie reference gel that they could not be isolated and identified by mass spectrometry as reported in previous studies (27, 33).

To quantify the relationships between protein spot recognition by different isotypes, the intensity of recognition of each antigenic spot by each isotype was categorised by interquartile range (to reduce the effects of differences arising due to intra-isotype variation in detection thresholds) as shown in Table 1. The recognition intensity of the spots was positively correlated so that for most spots, those intensely recognised by IgA were also intensity recognised by IgG1 (Figure 3A). 36 of the 39 antigenic spots recognised by IgA were also recognised by IgG1 (Table1). However, IgG1 recognised an additional nine spots indicated in Figure 3A (numbered spots on the y axis). These resolved to the antigens fatty acid binding protein (Sm14), phosphoglycerate kinase, triose-phosphate isomerase, an isoform of fructose 1-6-bis-phosphate aldolase, HSP 70, calreticulin, and two proteins yet to be identified (spots 2 and 15) . Conversely IgA recognised three antigenic spots not recognised by IgG1 (numbered spots on the x-axis of Figure 3A). These were an isoform of enolase

an isoform of fructose 1-6-bis-phosphate aldolase and a protein (spot 50) yet to be identified. These were also not recognised by IgG4 (Figure 3D) or IgE (Figure 3F).

Similar patterns of correlations between isotypes, but with some antigens being exclusively or largely recognised by only one isotype, were seen in all other pairwise comparisons (Fig 3). IgE recognised all 34 antigenic spots recognised by IgG4 as shown in Table 1 and Figure 3B. In addition it also recognised an isoform of 28kDa glutathione-S-transferase, phosphoglycerate kinase, actin 1, calreticulin and one protein (spot 16) still to be identified.

Of all the recognised antigens, only triose-phosphate isomerase (spot 6 in Figure 2 and Table 1), isoforms of fructose 1-6-bis-phosphate aldolase (spots 23 and 25), enolase (spot 26), HSP70 (spot 29) and spots 2 and 15 which are proteins still to be identified were recognised by a single isotype. The isoforms of fructose-1-6-bis-phosphate aldolase, were reactive with either IgA (antigenic spot 25 in Figure 2A) or IgG1 (antigenic spot 23 in Figure 2A) but not both. IgG1 recognised all the antigenic spots recognised by IgG4 (Table 1, Figure 3C) and IgE (Table 1, Figure 3E).

Of the current World Health Organisation's 10 schistosome vaccine candidates, only three were recognised by all the isotypes assayed. These were an isoform of 28kDa glutathione-S-transferase (spot 8), both isoforms of glyceladeyde-3 –phosphate (spots 21 and 22) and several isoforms of actin-binding/filamin-like protein (spots 66-69). The vaccine candidate paramyosin was not recognised by any of the antibodies assayed.

Discussion

Passive transfer studies in animal models show that antibodies can protect against schistosomiasis (6-9, 34). In parallel, human immuno-epidemiology studies have shown that some antibody classes are associated with low levels of infection in endemic populations as well as low re-infection rates following treatment with anti-helminthic drugs (11, 13, 14, 35-37). However, all previous studies to date have focused on describing antibody responses either to individual recombinant antigens or to uncharacterised 'whole worm' antigen preparations. We have previously defined native schistosome adult proteins recognised by the total IgG fraction of human serum from samples collected from this same population (27). In this study, we compare the reactivity of different isotypes to the soluble component of adult worm crude antigen, separated into its constituent defined antigens, to answer two questions. Firstly, which antigens are recognised by the different antibody iostypes IgA, IgE, IgG1 and IgG4? Second, do different isotypes recognise the same antigens? It is possible that observed differences in recognition intensity reflect not only the biological (functional) differences between the isotypes but also differences in the sensitivity and specificity of the isotypes, so that some antigens would be below the detection limit of the assays. However, we have carefully calibrated dilutions of sera and secondary antibodies to enable optimal comparison and minimise this possibility. We have used 3 replicates of each blot to verify the patterns and ratios of recognition intensity of each protein spot that we observed for each of the 4 isotypes. Thus we have shown that some antigens from within the adult *S. haematobium* proteome are preferentially recognised by certain isotypes. For example, one isoform of the antigen fructose-1-bis-phosphate aldolase was intensely recognised by IgA but not detected by IgE, IgG1 or IgG4. Furthermore comparing recognition intensity between isotypes using quartiles calculated within each subclass (Table 1), rather than absolute values (Figure 3), corrected for some intra-isotype variation in sensitivity. The study was designed to extend our understanding of the individual antigens detected in immunoassays routinely conducted by ELISA using crude antigens. In these former assays, protocols are designed to optimise sensitivity and specificity as done here to reduce the effects of differences in detection thresholds of the different isotypes.

The 4 isotypes we investigated detected a total of 48 antigenic spots out of a possible 71 spots detected with total IgG antibodies previously described by screening adult worm antigens with post-treatment sera (27). The sera used in this current study were from 7-18 year olds before enhancement of antibody reactivity with anti-helminthic treatment (27). Our previous study involved older people i.e. up to 42 years old and

included the reactivities of the additional IgG subclasses IgG2, IgG3 which are reactive against some of the schistosome vaccine candidates (24). Therefore, it is not surprising that the number of spots recognised by the IgG subclasses (IgG1 and IgG4) in this study would be fewer than in previous studies. Comparing the number of antigenic spots detected and the prevalence of the response in the study population, it is interesting that while IgG4 was the most prevalent response in the study population (present in 74% of the population), this isotype detected the least number of antigenic spots suggesting that there are few dominant antigens stimulating the IgG4 response. Conversely, IgG1 detected in 58% of the participants, recognised the largest number of spots suggesting that the IgG1 response is directed against a larger repertoire of antigens than the other isotypes.

We have previously shown a dichotomous association between IgA and IgG1 responses directed against adult worm antigens in a different Zimbabwean population (14) and the spot identification in Figure 3A shows that there are some antigens recognised either by IgG1 or IgA but not by both antibodies. An inverse relationship has also been reported in a case of idiopathic arthritis where patients deficient in IgG1 had high titres of IgA (38) suggesting that the expression of these two subclasses is differentially regulated. IgG1 recognised the largest number of antigenic spots: 45 including 8 of the 10 of the WHO's schistosome vaccine candidates. Furthermore, in this study, IgG1 levels rose with age to peak in the oldest age group with the least infection suggesting that the antibody is associated with protection against infection. This is consistent with our previous studies showing an association between anti-worm IgG1 responses and protection to *S. haematobium* infection (14). IgA recognised fewer antigenic spots than IgG1, reacting with only 3 of the 10 schistosome vaccine candidates including the leading vaccine candidate 28kDa glutathione-S-transferase.

The ratio of IgE to IgG4 levels has previously been associated with protection to re-infection by *S. haematobium* infection (11) and the association of a high IgE/IgG4 ratio with little or no infection in our present study is consistent with these findings. The inverse relationship between IgG4 and IgE has been used to suggest that IgG4 antibodies might block IgE effector functions by competitively binding to epitopes recognised by both isotypes and thereby inhibit IgE-dependent cell cytotoxicity mediated by monocytes, eosinophils or platelets (12, 39). The IgG4 subclass is up-regulated in association with anti-inflammatory factors(for example, IL-10 (40)) and its own anti-inflammatory characteristics are thought to help the immune system to dampen inappropriate inflammatory reactions (41). This would only be possible if the

two isotypes recognised the same antigens. In this study, IgG4 bound to the fewest antigenic spots while IgE recognised all those recognised by IgG4. IgE reacted with 7 of the 10 vaccine candidates, 5 of which were also recognised by IgG4. The recognition intensities (for each antigenic spot. representing both affinity and avidity) of these two antibodies were the most strongly correlated in the study suggesting a tightly regulated association between them. Earlier studies in filariasis using western blotting of one dimensional gels indicated that the level of cross reactivity between IgE and IgG4 differed with the level of pathology (42) which suggests that during the course of infection, there is dissociation in antigen recognition patterns between the two antibodies. How this dissociation occurs is unclear but may be related to the order of antibody class switching where IgG4 secreting B cells can switch to IgE but not the converse (43) and/or a threshold being reached in antigen levels (33).

The antigens recognised by IgE but not IgG4, and by IgG1 but not IgA, are of interest since they may be associated with the development of resistance to schistosomiasis; this is supported by the fact that most of these antigens are already known vaccine candidates (24). Studies are now underway to identify the proteins which were not serologically reactive, particularly those that are abundant in the soluble proteome which might play an important role in immune evasion and/or modulation of the host immune response (44). Similarly there is a need to identify the intensely recognised proteins which occurred at insufficient concentrations in the adult worm proteome to be identified by mass spectrometry, as these may indicate highly immunogenic vaccine candidates as yet undiscovered.

Overall, our study has indicated that although the majority of the antigens are recognised by all the four antibody isotypes tested, there may be restriction in antigen recognition for some antigens, for example prevalent isotypes such as IgG4 appear to react to relatively few of the major adult worm antigens. This may partly explain differences observed in population level isotype dynamics in human schistosomiasis. Furthermore, there are differences in the recognition of isoforms of some antigens such as the leading vaccine candidate, 28kDa glutathione-S-transferase and glyceraldehyde-3-phosphate dehydrogenase. These isoform-specific differences are an important consideration for vaccine development, where recombinant vaccines lack post-translational modifications and therefore epitopes crucial for induction of the relevant isotype-specific antibody responses. **Studies on antigen recognition patterns by different antibodies present in sera from individuals or partitioning the study population by age and infection status as**

we previously reported for total IgG will be valuable for identifying further vaccine candidates (33).

Acknowledgements

The investigation received financial support from the Medical Research Council, UK, The Wellcome Trust, UK (Grant no WT082028MA), and the Carnegie Trust for the Universities of Scotland. FM acknowledges support from the MRC, UK (Grant no G81/538). We are grateful for the co-operation of the Ministry of Health and Child Welfare in Zimbabwe, the Provincial Medical Director of Mashonaland East, the Environmental Health Workers, residents, teachers and school children in Mutoko and Rusike. We also thank Members of the National Institutes for Health Research (Zimbabwe) for technical support.

Figure legends

Figure 1: Description of the individual study participants partitioned by age group. (A) Infection intensity for each individual calculated as a mean of eggs /10ml of urine from at least 2 urine samples collected on consecutive days. (B) Antibody levels measured as optical densities for each individual (C) The relationship between the ratios of IgG1/IgA and IgE/IgG4 calculated from the values in (1B) above and schistosome infection intensity for each individual participant. These show a significant negative association between the ratio IgE/IgG4 and infection intensity and no significant association between the IgG1/IgA ratio and infection intensity.
Figure 2: Antigen recognition patterns of the serum pool compared by 2-dimmensional western blotting. (A) IgA vs. IgG1. (B) IgE vs. IgG4

Figure 3: Comparison of recognition intensity for each antigenic spot by the different isotypes showing a significant positive correlation between all 4 isotypes. Italicised numbers refer to the spot identity of antigenic spots preferentially recognised by one of the two isotypes and their protein identities are given in Table 1. (A) IgG1 vs. IgA. (B) IgG4 vs. IgE. (C) IgG4 vs. IgG1. (D) IgG4 vs. IgA. (E) IgE vs. IgG1. (F) IgE vs. IgA.

Tables

Table 1: The table gives identities of the antigenic spots in Figure 3, relating the spot number to its protein identity from the peptide searches and recognition intensity by each of the isotypes. The WHO-listed schistosome vaccine candidates are highlighted in bold. The intensity of recognition is coded from no colour (antigenic spot not detected), very light, 1st quartile, (excluding the undetected spots in former category, i.e. recognition intensity >0), light (2nd quartile), medium (3rd quartile) and darkest (4th quartile). The inter-quartile ranges were calculated separately for each isotype.

Table 1

Spot	Protein name	IgA	IgG1	IgE	IgG4
1	**Fatty acid-binding protein Sm14**				
2	No significant hit				
3	Myosin light chain				
4	Putative mucin-like protein				
5	Putative mucin-like protein				
6	**Triosephosphate isomerase**				
7	**28kDa glutathione-S transferase**				.
8	**28kDa glutathione-S transferase**				
9	**Phosphoglycerate kinase**				
10	**Myosin heavy chain**				
11	Proteasome subunit				
12	14-3-3 epsilon				
13	Myosin heavy chain				
14	Heat shock protein HSP70				
15	No significant hit			.	
16	ENSANGP00000014266				
17	**Phosphoglycerate kinase**	.			
18	Putative mucin-like protein				
19	Tropomyosin 1				
20	Tropomyosin 2				
21	**Glyceraldehyde-3-phosphate dehydrogenase**				
22	**Glyceraldehyde-3-phosphate dehydrogenase**				
23	Fructose 1,6 bisphosphate aldolase				
24	Actin				
25	Fructose 1,6 bisphosphate aldolase				
26	Enolase				
27	Enolase				
28	Fimbrin				
29	Heat shock protein HSP70				
30	Actin 1				
31	Actin				
32	Actin				
33	Actin				
34	Actin				
35	Immunophilin				
36	Immunophilin				
37	No significant hit				
38	Calreticulin				
39	Protein disulfide isomerase				
40	Enolase				
41	Enolase				
42	Enolase				
43	Enolase				
44	Putative mucin-like protein				
45	Enolase				
46	Enolase				
47	Enolase				
48	Putative cytosol aminopeptidase				
49	Putative cytosol aminopeptidase				
50	No significant hit				
51	No significant hit				
52	No significant hit				
53	ENSANGP00000019187				
54	Phosphoglucomutase				
55	No significant hit				
56	Phosphoglucomutase				
57	Heat shock protein HSP60				
58	Predicted Zn-dependent peptidases				
59	No significant hit				
60	No significant hit				
61	No significant hit				
62	No significant hit				
63	Heat shock protein HSP70				
64	Heat shock protein HSP70				
65	Heat shock protein HSP70				
66	**Actin-binding/filamin-like protein**				
67	**Actin-binding/filamin-like protein**				
68	**Actin-binding/filamin-like protein**				
69	**Actin-binding/filamin-like protein**				
70	**Paramyosin**				
71	No significant hit				

References

1. Utzinger J, Raso G, Brooker S *et al.* Schistosomiasis and neglected tropical diseases: towards integrated and sustainable control and a word of caution. *Parasitology* 2009; **136**: 1859-1874.
2. http://www.who.int/vaccine_research/diseases/soa_parasitic/en/index5.html Accessed 16th September 2010
3. Anderson RM and May RM. *Infectious diseases of humans dynamics and control*, Oxford Science, 1992.
4. Woolhouse MEJ, Taylor P, Matanhire D and Chandiwana SK. Acquired immunity and epidemiology of Schistosoma haematobium. *Nature* 1991; **351**: 757-759.
5. Mutapi F, Hagan P, Ndhlovu P and Woolhouse MEJ. Comparison of humoral responses to *Schistosoma haematobium* in areas with high and low levels of infection. *Parasite Immunol* 1997; **19**: 255-263.
6. Dunne DW, M. JF, L. C and A. MN. Passively transferable protection against *Schistosoma japonicum* induced in the mouse by multiple vaccination with attenuated larvae: the development of immunity, antibody isotype responses and antigen recognition. *Parasite Immunol* 1994; **16**: 655-668.
7. Maloney N, Hinchcliffe P and Webbe G. Passive transfere of resistance to mice with sera from rabbits, rats or mice vaccinated with ultraviolet-attenuated cercariae of schistosoma japonicum. *Parasitology* 1987; **94**: 497-508.
8. Sher A, Smithers SR and Mackenzie P. Passive transfer of acquired resistance to *Schistosoma mansoni* in laboratory mice. *Parasitology* 1975; **70 Part 3**: 347-357.
9. Mangold BL and Dean DA. Passive transfer with serum and IgG antibodies of irradiated cercaria-induced resistance against *Schistosoma mansoni* in mice. *J Immunol* 1986; **136**: 2644-2648.
10. Dunne DW, Butterworth AE, Fulford AJ, Ouma JH and Sturrock RF. Human IgE responses to *Schistosoma mansoni* and resistance to reinfection. *Mem Inst Oswaldo Cruz* 1992; **87**: 99-103.
11. Hagan P, Blumenthal UJ, Dunne D, Simpson AJG and Wilkins AH. Human IgE, IgG4 and resistance to reinfection with *Schistosoma haematobium.* *Nature* 1991; **349**: 234-245.
12. Demeure CE, Rihet P, Abel L, Ouattara M, Bourgois A and Dessein AJ. Resistance to Schistosoma mansoni in humans: influence of the IgE/IgG4 balance and IgG2 in immunity to reinfection after chemotherapy. *J Infect Dis* 1993; **168**: 1000-1008.
13. Grogan JL, Kremsner PG, van Dam GJ, Deelder AM and Yazdanbakhsh M. Anti-schistosome IgG4 and IgE at 2 years after chemotherapy: infected versus uninfected individuals. *J Infect Dis* 1997; **176**: 1344-1350.
14. Mutapi F, Ndhlovu PD, Hagan P *et al.* Chemotherapy accelerates the development of acquired immune responses to *Schistosoma haematobium* infection. *J Infect Dis* 1998; **178**: 289-293.
15. Hagan P and Sharaf O. Schistosomiasis vaccines. *Expert Opin Biol Ther* 2003; **3**: 1271-1278.
16. Capron A and Dessaint JP. Molecular basis of host-parasite relationship: towards the definition of protective antigens. *Immunol Rev* 1989; **112**: 27-48.

17. Correa-Oliveira R, Pearce EJ, Oliveira GC *et al.* The human immune response to defined immunogens of *Schistosoma mansoni*: elevated antibody levels to paramyosin in stool-negative individuals from two endemic areas in Brazil. *Trans R Soc Trop Med Hyg* 1989; **83**: 798-804.
18. Zhang Y, Taylor MG, McCrossan MV and Bickle QD. Molecular cloning and characterization of a novel *Schistosoma japonicum* "irradiated vaccine-specific" antigen, Sj14-3-3. *Mol Biochem Parasitol* 1999; **103**: 25-34.
19. Butterworth AE, Bebsted-Smith R and Capron A. Immunity in human schistosomiasis: prevention by blocking antibodies of the expression of immunity in young children. *Parasitology* 1987; **94**: 281-300.
20. Gryseels B, Stelma F, Talla I *et al.* Immuno-epidemiology of *Schistosoma mansoni* infections in a recently exposed community in Senegal. *Mem Inst Oswaldo Cruz* 1995; **90**: 271-276.
21. Ndhlovu P, Cadman H, Vennervald BJ, Christensen NO, Chidimu N and Chandiwana SK. Age-related antibody profiles in *Schistosoma haematobium* in a rural community in Zimbabwe. *Parasite Immunol* 1996; **18**: 181-191.
22. Webster M, LibrandaRamirez BDL, Aligui GD *et al.* 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* 1997; **114**: 383-393.
23. Fitzsimmons CM, Stewart TJ, Hoffmann KF, Grogan JL, Yazdanbakhsh M and Dunne DW. Human IgE response to the *Schistosoma haematobium* 22.6 kDa antigen. *Parasite Immunol* 2004; **26**: 371-376.
24. Al-Sherbiny M, Osman A, Barakat R, El Morshedy H, Bergquist R and Olds R. In vitro cellular and humoral responses to *Schistosoma mansoni* vaccine candidate antigens. *Acta Trop* 2003; **88**: 117-130.
25. Mutapi F, Mduluza T, Gomez-Escobar N *et al.* Immuno-epidemiology of human *Schistosoma haematobium* infection: preferential IgG3 antibody responsiveness to a recombinant antigen dependent on age and parasite burden. *BMC Infect Dis* 2006; **6**: 96.
26. Mutapi F, Winborn G, Midzi N, Taylor M, Mduluza T and Maizels RM. Cytokine responses to *Schistosoma haematobium* in a Zimbabwean population: contrasting profiles for IFN-gamma, IL-4, IL-5 and IL-10 with age. *BMC Infect Dis* 2007; **7**: 139.
27. Mutapi F, Burchmore R, Foucher A *et al.* Praziquantel treatment of people exposed to *Schistosoma haematobium* enhances serological recognition of defined parasite antigens. *J Infect Dis* 2005; **192**: 1108-1118.
28. Katz N, Chaves A and Pellegrino J. A simple device for quantitative stool thick smear technique in schistosomiasis mansoni. *Revi Instit Med Trop Sao Paulo* 1972; **14**: 397-400.
29. Mott KE. A reusable polyamide filter for diagnosis of *S. haematobium* infection by urine filtration. *Bull Soc Pathol Exot* 1983; **76**: 101-104.
30. Agnew AM, Murare HM and Doenhoff MJ. Immune attrition of adult schistosomes. *Parasite Immunol* 1993; **15**: 261-271.
31. Reilly LJ, Magkrioti C, Cavanagh DR, Mduluza T and Mutapi F. Effect of treating *Schistosoma haematobium* infection on Plasmodium falciparum-specific antibody responses. *BMC Infect Dis* 2008; **8**: 158.
32. Woolhouse MEJ and Hagan P. Seeking the ghost of worms past. *Nature Medicine* 1999; **5**: 1225-1227.

33. Mutapi F, Burchmore R, Mduluza T, Midzi N, Turner CM and Maizels RM. Age-related and infection intensity-related shifts in antibody recognition of defined protein antigens in a schistosome-exposed population. *J Infect Dis* 2008; **198**: 167-175.
34. Capron A, Dessaint JP, Capron M, Joseph M and Pestel J. Role of anaphylactic antibodies in immunity to schistosomes. *Am J Trop Med Hyg* 1980; **29**: 849-857.
35. Dunne DW, Butterworth AE, Fulford AJC *et al.* Immunity after treatment of human schistosomiasis: association between IgE antibodies to adult worm antigens and resistance to reinfection. *Eur J Imm* 1992; **22**: 1483-1494.
36. Sturrock RF, Kimani R, Cottrell BJ *et al.* Observations on possible immunity to reinfection among Kenyan school children after treatment for *Schistosoma mansoni. Trans R Soc Trop Med Hyg* 1983; **77**: 363-371.
37. Webster M, Fulford AJC, Braun G *et al.* Human Immunoglobulin E responses to a recombinant antigen from *Schistosoma mansoni* adult worms are associated with low intensities of reinfection after treatment. *Infect Immun* 1996; **64**: 4042-4046.
38. Yildiz B and Kural N. IgG1 deficiency and high IgA level with juvenile idiopathic arthritis. *Eur J Pediatr* 2007; **166**: 1179-1180.
39. Rihet P, Demeure CE, Dessein AJ and Bourgois A. Strong serun inhibition of specific IgE correlated to competing IgG4, revealed by a new methodology in subjects from a *S. mansoni* endemic area. *Eur J Imm* 1992; **22**: 2063-2070.
40. Satoguina JS, Weyand E, Larbi J and Hoerauf A. T regulatory-1 cells induce IgG4 production by B cells: role of IL-10. *J Immunol* 2005; **174**: 4718-4726.
41. Aalberse RC, Stapel SO, Schuurman J and Rispens T. Immunoglobulin G4: an odd antibody. *Clin Exp Allergy* 2009; **39**: 469-477.
42. Hussain R and Ottesen EA. IgE responses in human filariasis. III. Specificities of IgE and IgG antibodies compared by immunoblot analysis. *J Immunol* 1985; **135**: 1415-1420.
43. Stavnezer J and Amemiya CT. Evolution of isotype switching. *Semin Immunol* 2004; **16**: 257-275.
44. Hewitson JP, Grainger JR and Maizels RM. Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity. *Mol Biochem Parasitol* 2009; **167**: 1-11.