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Helminth parasite proteomics: from experimental models to human infections

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SUMMARY

Schistosomiasis is a major human helminth infection endemic in developing countries. Urogenital schistosomiasis, caused by *S. haematobium*, is the most prevalent human schistosome disease in sub-Saharan Africa. Currently control of schistosome infection is by treatment of infected people with the anthelmintic drug praziquantel, but there are calls for continued efforts to develop a vaccine against the parasites. In order for successful vaccine development, it is necessary to understand the biology and molecular characteristics of the parasite. Ultimately, there is need to understand the nature and dynamics of the relationship between the parasite and the natural host. Thus, my studies have focused on molecular characterization of different parasite stages and integrating this information with quantitative approaches to investigate the nature and development of protective immunity against schistosomes in humans. Proteomics has proved a powerful tool in these studies allowing the proteins expressed by the parasite to be characterized at a molecular and immunological level. In this review, the application of proteomic approaches to understanding the human-schistosome relationship as well as testing specific hypotheses on the nature and development of schistosome-specific immune responses is discussed. The contribution of these approaches to informing schistosome vaccine development is highlighted.

Key words: Schistosomiasis, proteomics, *Schistosoma haematobium*, immunity, antibody, human, vaccine.

INTRODUCTION

Schistosomiasis is a neglected tropical disease (NTD) affecting between 200–400 million people globally (Bethony *et al.* 2011). The recent first World Health Organisation (WHO) report on NTDs cites that 90% of the world's schistosomiasis cases now occur in sub-Saharan Africa (WHO, 2011). *Schistosoma haematobium* is the most prevalent human schistosome species in Africa and causes urogenital schistosomiasis whose clinical symptoms include haematuria, dysuria, hydronephrosis and calcification of the bladder. Urogenital schistosomiasis is also associated with bladder cancer (Parkin, 2008) and an increased risk of HIV infection (Kjetland *et al.* 2006). Control is primarily by treatment with the antihelminthic drug praziquantel which is cheap (approximately US\$ 0.50/dose), efficacious (cure rates >90%) and safe; it is used in mass drug administration programmes aimed primarily at school children. Despite all these attributes of praziquantel, the WHO report on NTDs notes that the goal of reaching 75% of school age children by the end of 2010 advocated for by the World Health Assembly (WHA) Resolution 54.19

and adopted by the WHA in 2001 was not realised with less than 10% treatment coverage having been achieved by 2008 (WHO, 2011). One of the problems associated with helminth control is the need for repeated treatment due to rapid re-infection, thus vaccination has been suggested as an additional tool for integrated helminth control.

Successful vaccine development requires an understanding of the immunological interaction (both protective and pathological) between the host and the parasite in addition to understanding the nature and development of protective acquired immune responses stimulated by the parasite. Methodologies and technologies for characterizing immune responses to parasites have become more sophisticated with the advent of both parasite genome sequence (Berriman *et al.* 2009; Zhou *et al.* 2009) data and high throughput platforms such as microarrays. Despite these technological and scientific advances, vaccine development for human helminth parasites has yet to produce a single licensed vaccine product compared to advances in animal helminth vaccines (Lightowlers *et al.* 2003). In this current climate of vast amounts of sequence data and rapid technological developments, the challenge is to identify and pose the 'right' biological questions and use those tools at our disposal to answer them. One of the main obstacles to this process is that many fundamental questions on the nature and dynamics of protective immune responses in human helminths remain

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unanswered. Questions such as (1) which parasite stage stimulates a protective immune response? (2) Against which parasite stage are protective responses directed? (3) How do naturally acquired protective responses differ from vaccine-induced protective immune responses? For example, natural anti-IgE responses against the hookworm antigen *Ancylostoma* secreted protein (ASP-2) are protective against hookworm infection but vaccine-induced responses trigger pathological responses whose clinical outcome is urticaria (Bethony *et al.* 2011). (4) Why do single antigen recombinant vaccines provide a large percentage of protection in some helminth species (e.g. the EG95 recombinant vaccine in *Echinococcus granulosus* is 100% protective in sheep) and only very weak protection in others (the 26 kDa GST recombinant *Schistosoma japonicum* recombinant vaccine elicited 24% protection in mice) (Liu *et al.* 1995). In addition to these questions, the applicability of vaccine-induced protective immunity generated in animal models to humans remains to be evaluated. Differences in the history of infection occurring in human and naturally infected animals which are not reproduced in experimental studies may affect the development and type of protective immunity in heterogeneous human populations (Wherry *et al.* 2003).

My own studies are concentrated on molecular characterization of schistosome parasites and integrating these studies with quantitative approaches to investigate the nature and development of protective immunity against schistosomes in humans. In this review, I focus on the application of proteomic approaches that we have used to characterize schistosome parasites as well as to test specific hypotheses on the development of naturally acquired and anthelmintic drug altered schistosome-specific immune responses in human populations.

CHARACTERIZATION OF SCHISTOSOME PARASITES THROUGH COMPARATIVE PROTEOMICS

There are similarities in immune responses stimulated by different helminth species in humans (Allen and Maizels, 2011) which have contributed to several shared epidemiological patterns in human populations exposed to different helminth species such as aggregation of infection, convex age-infection curves, peak shifts and pre-disposition to heavy infection (Anderson and May, 1992; Woolhouse, 1998). However, different helminth groups differ in their life histories and interaction with their hosts highlighting fundamental differences and phenotypic plasticity within host-helminth interactions (see review by Bourke *et al.* 2011). Thus, molecular comparisons between different helminth species should highlight differences between parasites that may be related to specific interactions with their

hosts. While genomic and transcriptomic differences are informative for evolutionary studies as well as giving indications on the level of genetic differences, they do not take into account post-transcriptional regulation of protein expression and cannot determine the degree of epitope cross-reactivity between parasite species (Lopez, 2007). Therefore, proteomic comparisons can give a better indication of phenotypic differences between different parasites. In our studies, we have compared the proteome of adult *Schistosoma haematobium* to that of the animal schistosome *S. bovis*, an important parasite of cattle, sheep and goats. These two schistosome species have different mammalian definitive hosts and occupy different niches within the vasculature of these hosts, and they utilise the same intermediate host (Rollinson *et al.* 1997). Furthermore, hybridisation has been reported between the two species demonstrating their close phylogenetic relationship (Huysse *et al.* 2009). Sequences of small and large nuclear subunit ribosomal RNA genes and partial mitochondrial cytochrome oxidase 1 sequences indicate a close similarity between *S. haematobium* and *S. bovis* (Bowles *et al.* 1993; Webster *et al.* 2006). Similarities between *S. haematobium* and *S. bovis* also translate to some level of antigenic similarity leading to a greater degree of heterologous immunity than that observed between *S. haematobium* and the other major human schistosomes, *S. mansoni* and *S. japonicum* (Grogan *et al.* 1997). Therefore, comparing these closely related schistosome species would give an indication of any differences that may be due to specific host-parasite interactions. The current lack of genome sequence data for *S. haematobium* and *S. bovis* means some proteins present in the proteome cannot be identified if they lack homologues in the sequenced *S. mansoni* (Berriman *et al.* 2009) or *S. japonicum* genomes (Zhou *et al.* 2009). In my laboratory, we conducted proteomic comparisons using the Difference Gel Electrophoresis (DiGE) (Alban *et al.* 2003). The approach is summarized in Fig. 1. Indeed, this comparison showed some interesting differences, mostly related to proteins involved in response to stress (e.g. changes in pH, attack by reactive oxygen metabolites and depletion of essential nutrients) (Kaufmann, 1990a,b), Heat Shock Protein (HSP) 70 despite 91% similarity between the two species as shown in Fig. 2A (adapted from Cook *et al.* 2011; Higon *et al.* 2011). The biological implications of the difference in HSP70 expression (at least 5-fold difference) has yet to be determined. HSP70 is expressed in cercariae and adult-stage schistosomes. Generally HSP70 expression is induced early during infection in parasitic organisms and plays a critical role during adaptation to the new environment (Maresca and Kobayashi, 1994). In most organisms as well as in parasites, several genes coding for HSP70 exist, but only some are stress-inducible. In

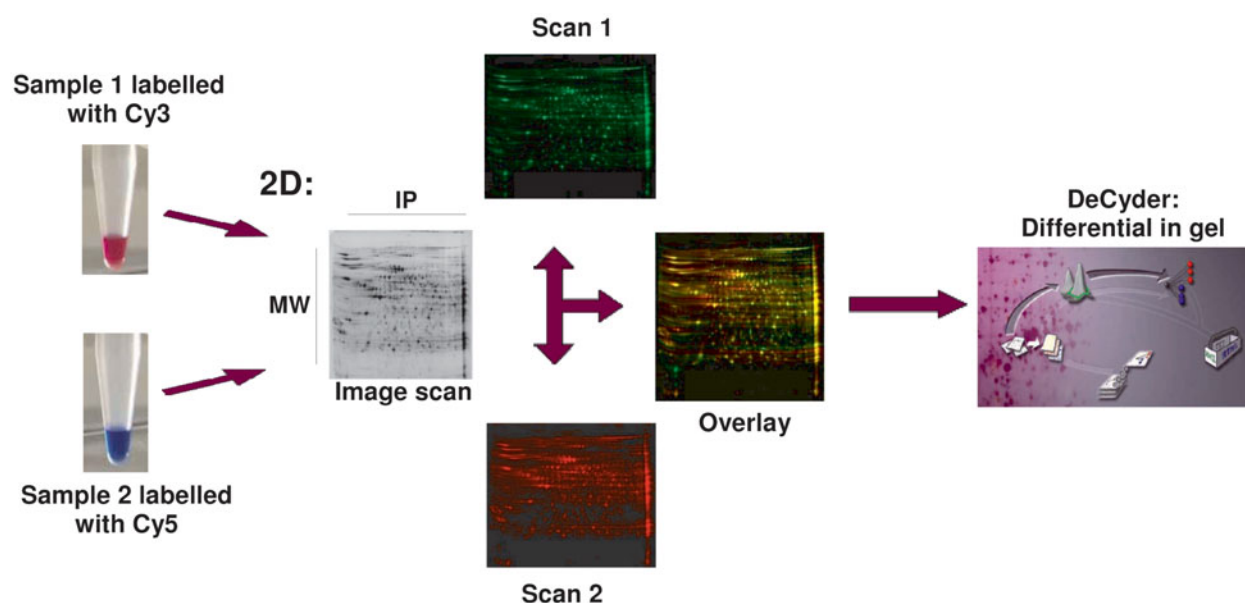


Fig. 1. A summary of the difference gel electrophoresis (DiGE) used to compare abundance of proteins between difference proteomes. The proteins from each proteome are labelled with fluorescent dyes (for example Cy3, Cy5, Cy2) prior to two-dimensional electrophoresis. After the gel electrophoresis, the gel is scanned with the excitation wavelength of each dye one after the other. Quantitative analysis is then conducted through differential in gel analysis (DIA) with image analysis software such as DeCyder from GE Healthcare.

schistosomes, expression of HSP70 in adults can be induced by increasing temperature and stress (Maresca and Kobayashi, 1994). Therefore, the differences in HSP70 between the two schistosome species may be related to differences in host-related stresses. This would be consistent with results from previous inter-species comparisons in *Fasciola* trematodes where *F. hepatica* and *F. gigantica* show different levels of HSP70 expression (Smith *et al.* 2008). Furthermore, host-related differences in HSP70 expression occur in *Echinostoma* species with parasites from less permissive hosts expressing different levels from parasites in hosts with which they are more compatible (Higon *et al.* 2008).

To further determine differences associated with different life histories, we compared the adult worm proteomes of the two schistosome species to that of another trematode, the experimental model parasite *Echinostoma caproni* which does not have a tissue phase in the mammalian host and resides in the gastrointestinal tract (Fig. 2B). Despite the fact that echinostomes belong to a different genus, they show protective immunological cross-reactivity with parasites of the genus *Schistosoma* (Sirag *et al.* 1980). Unsurprisingly, there are differences in the expression levels of both HSP70 and HSP60 whose expression was increased at least 5 fold in *S. haematobium* compared to that in *E. caproni*. Although this proteomic comparison provides an important insight into the molecular features underlying their distinct life histories, demonstrating differences in the *E. caproni* and *S. haematobium* proteome alone is not enough to fully understand phenotypic differences in the host-parasite

interactions. In this respect, we have commenced looking at differences in immunogenicity and cross-reactivity between the trematodes and have already shown varying degrees of cross-reactivity, with the greatest cross-reactivity unsurprisingly occurring between the two schistosome parasites (Cook *et al.* 2011). Further studies to characterize abundantly expressed but non-immunogenic proteins are currently underway. Such studies will be useful in informing vaccine development for, (1) cross-species protection (e.g. *S. haematobium* and *S. mansoni* exist as co-infections in some areas in Africa), and (2) determining suitable experimental models for vaccine evaluation prior to human clinical trials.

IDENTIFICATION OF IMMUNOGENIC SCHISTOSOME PROTEINS IN HUMAN HOSTS

Parasite vaccine candidate discovery studies have focused on identifying antigens that stimulate protective immune responses during natural infections in target hosts and attempting to reproduce these responses artificially. In order to identify candidate antigens, it is necessary to determine which of the parasite proteins are immunogenic. The approach used in our studies has been to separate the parasite proteins using 2-D electrophoresis, followed by Western blotting with sera from populations naturally exposed to schistosome infections, and thus screen for antigens reactive with these sera (Mutapi *et al.* 2005). Recognised antigens (spots on the Western blot gel) are then matched to a Coomassie-blue stained 2-D gel of the parasite proteome (preparative gel) and the specific spots excised from

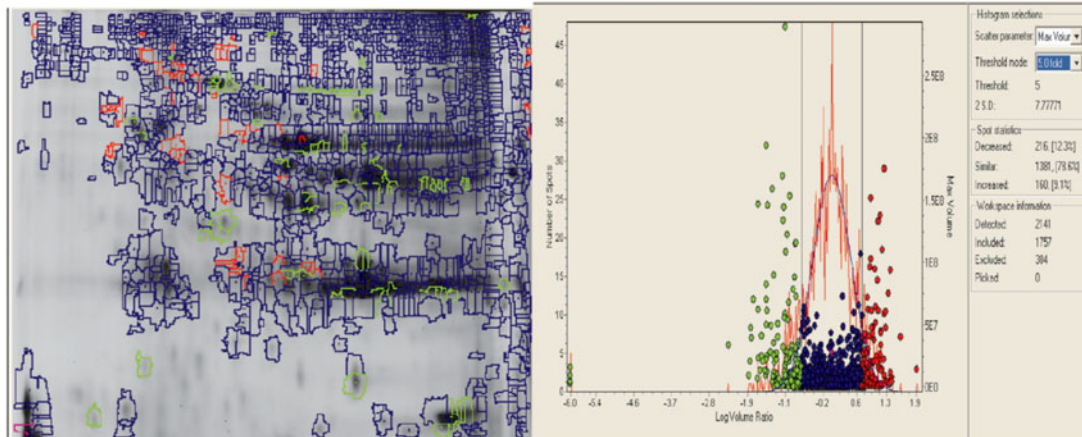
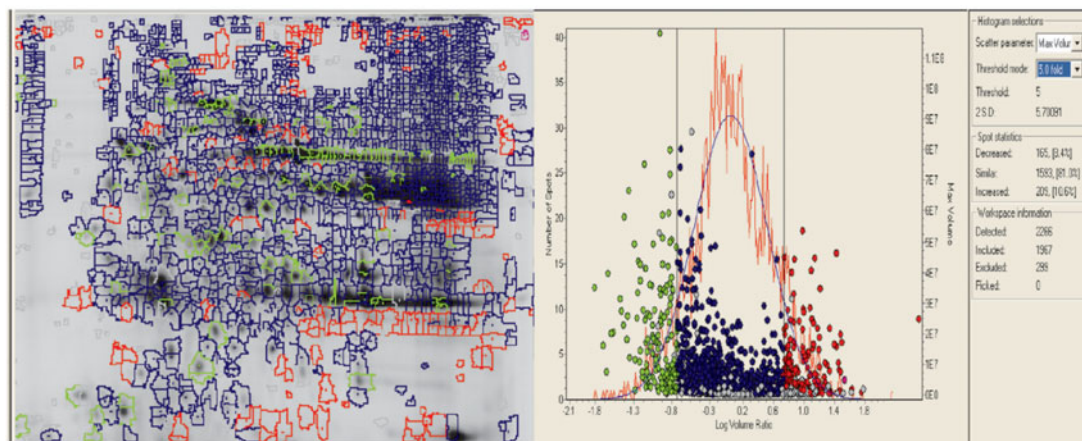
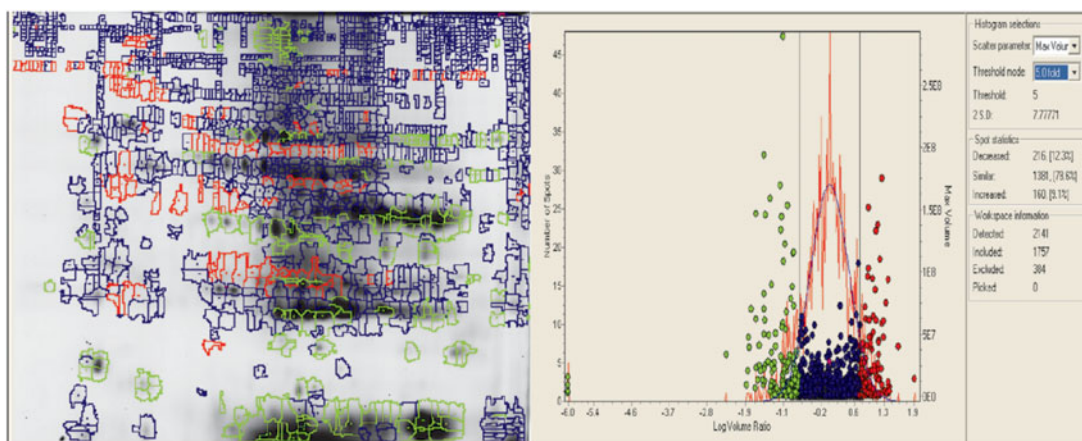
A *S. haematobium* vs. *S. bovis* analysis: 91% similarity between proteomes**B** *E. caproni* vs. *S. haematobium* analysis: 81 % similarity between proteomes**C** *E. caproni* vs. *S. bovis* analysis: 78.6 % similarity between proteomes

Fig. 2. Differential in gel analysis (DIA) images for the comparison between schistosomes and the echinostome parasites showing the different abundances of different proteins and the corresponding histogram comparing the abundance of each protein spot on the gel as well as the overall % similarity in the proteomes.

A) Comparing *S. haematobium* with *S. bovis*. Green spots = higher expression in *S. haematobium*; Red spots = higher expression in *S. bovis*; Blue spots = expression levels similar in both species.

B) Comparing *E. caproni* with *S. haematobium*. Green spots = higher expression in *S. haematobium*; Red spots = higher expression in *E. caproni*; Blue spots = expression levels similar in both species.

C) Comparing *E. caproni* with *S. bovis*. Green spots = higher expression in *E. caproni*; Red spots = higher expression in *S. bovis*; Blue spots = expression levels similar in both species. Fig. adapted from Higon *et al.* 2011.

the preparative gel, digested and subjected to tandem mass spectrometry to obtain identifications (Mutapi *et al.* 2005). This approach provides information both on the characteristics of immunogenic parasite antigens as well as the nature of the serological response directed against them during a natural infection in the human host (e.g. antibody isotype-specific reactivities (Mutapi *et al.*, 2011). A further advantage is that these variations can be related to host characteristics, including age and infection intensity, providing a link between molecular parasitology and epidemiology (Mutapi *et al.* 2008). The advantages of this approach over the traditional methods of screening expression libraries with serum samples from infected or vaccinated animals include the ability to detect immunogenic epitopes arising from post-translation modifications. Our studies determining the immunogenic proteins using the combined 2-D Western and proteomics approach have identified more novel schistosome antigens in a single experiment than the collective effort in the field in the preceding 10 years demonstrating the power of the approach, i.e. at least 71 antigens comprising different proteins as well as different isoforms of the same proteins (Mutapi *et al.* 2005) as shown in Table 1. This study showed that the heat shock proteins were immunogenic, consistent with previous reports from other studies and also from other parasite species (Kaufmann, 1990).

However, there is a limitation to the number of antigens that can be identified through the gel-based proteomic approaches. This is due to the differences in sensitivities of the stains on the preparative gels, solubility of parasite proteins and the Western blotting technique. This means that some highly immunogenic proteins seen on the Western blots cannot be visualised on the prep gel and therefore cannot be identified. This may also be occurring in the case of less abundantly expressed proteins. Advances in the protein array approaches in antigen discovery offer some promise in overcoming some of these limitations (Driguez *et al.* 2010) though integrating the two methods may give a more comprehensive array of parasite antigens and therefore, be more informative.

CHARACTERIZING THE ANTIBODY SUBCLASSES THAT RECOGNISE IMMUNOGENIC SCHISTOSOME PROTEINS IN HUMAN HOSTS

Different antibody subclasses have different effector functions and several human field studies have focused on characterizing the dynamics of different antibody subclass responses to helminth parasites (for example, Correa-Oliveira *et al.* 1988; Dunne *et al.* 1992; Grzych *et al.* 1993; Quinnell *et al.* 1995; Wherry *et al.* 2003). Therefore, some antibody subclasses, such as IgM, have been described as a marker of exposure to schistosome parasite antigens

(Wherry *et al.* 2003), IgG4 as a marker of infection status in filarial infections (Kwan-Lim *et al.* 1990), others such as schistosome and hookworm IgE as a marker of resistance to re-infection (Hagan *et al.* 1991; Hagan, 1992; Hagel *et al.* 2003). We earlier demonstrated that the production of some antibody subclasses is inversely correlated; specifically, high IgA producers did not produce high levels of IgG1 and vice versa (Wherry *et al.* 2003). Furthermore, only high IgG1 and low IgA producers had little or no infection. An inverse relationship in these two subclasses has also been reported in a case of idiopathic arthritis where patients deficient in IgG1 had high titres of IgA (Yildiz and Kural, 2007) suggesting that the expression of these two subclasses is differentially regulated. What was not clear from our immuno-epidemiological studies of the IgA and IgG1 profiles was whether these antibodies were recognizing the same antigens. Thus, the 2-D Western blotting and proteomics approach was used to determine the antigen recognition profile of the main antibody subclasses, IgE, IgG4, IgG1 and IgA implicated in the expression of resistance to schistosome re-infection. The aim of the study was to test the null hypothesis that the four subclasses recognise the same parasite antigens from adult schistosomes. This study showed that although the majority of the parasite antigens were recognised by all four antibody subclasses, there were some antigens whose reactivity was restricted to only one of the subclasses (Mutapi *et al.* 2011) as shown in Table 1. These differences occurred between different proteins as well as between isoforms of the same protein; for example, the two fructose 1-6-bis-phosphate aldolase isoforms were differentially recognized by IgA and IgG1. In general, IgG1 recognised the largest variety of antigens followed by IgE, IgA and IgG4, respectively. IgE recognized all antigens reactive with IgG4 as well as an additional four antigens, an isoform of 28-kDa GST, phosphoglycerate kinase, actin 1 and calreticulin. IgG1 additionally recognized fatty acid-binding protein, triose-phosphate isomerase and HSP70, which were not recognized by IgA. Knowing the subclasses that recognise antigens is now an important aspect of helminth vaccine development since people already exposed to the parasite in endemic areas will have had some immunological experience with the parasite or its antigens. This could potentially lead to adverse reactions to immunization, if the vaccine enhances certain types of already existing subclass responses. The human hookworm vaccine clinical trials evaluating the ASP-2 vaccine had to be discontinued after Phase I when it was shown that vaccinating people who already had an IgE response to the protein induced urticaria (Bethony *et al.* 2011). Thus, it seems a vaccine inducing/enhancing a parasite-specific IgE response is now less favoured compared to that inducing an IgG1 response, making studies

characterizing subclasses which recognise candidate vaccine antigens an essential component of the characterization of natural and vaccine-induced immune responses.

NATURE AND DEVELOPMENT OF PARASITE – SPECIFIC ACQUIRED IMMUNITY: EXPLAINING THE SLOW DEVELOPMENT OF NATURALLY ACQUIRED IMMUNE RESPONSES

Helminth infections are characterized by the slow development of protective acquired immunity (Anderson and May, 1992) and various explanations have been proposed for this (Yazdanbakhsh and Sacks, 2010). In my laboratory, we have been testing different hypotheses explaining this gradual development of protective acquired immunity including the immuno-suppressive effects of helminths (Maizels and Yazdanbakhsh, 2003) and the need for a threshold of parasite antigens (Woolhouse and Hagan, 1999) using a combination of quantitative 2-D Western blotting and proteomic approaches. The quantitative studies have shown that host immuno-suppression by parasites alone does not reproduce the epidemiological patterns observed in the field (Mitchell *et al.* 2008). The second proposition that we tested was the threshold hypothesis. We proposed that the host immune system is effective against parasites only after exposure to a threshold level of parasite antigens (Woolhouse and Hagan, 1999). During a natural schistosome infection, in which adult worms can survive for an average 3–7 years (Fulford *et al.* 1995), it may take several years for this threshold to be reached. To test the hypothesis that a host must experience a threshold amount of antigen exposure to mount a protective response, we compared antigen-recognition patterns (the number of antigens recognized and the intensity of antigen recognition assayed via 2-D Western blotting as described above) by Zimbabweans exposed to *S. haematobium* infection in three age-groups; young children (acquiring infection), older children (in whom infection levels are peaking) and in adults (where infection levels are declining rapidly). Individuals within these age-groups were further partitioned by infection level. The study clearly demonstrated that the repertoire of antigens recognized increased with age, peaking in the oldest participants harbouring little or no infection (Table 1), indicating that this increase in the repertoire of antigens recognized was associated with resistance to infection (Mutapi *et al.* 2008). Furthermore, the intensity of antigen recognition increased with age, peaking in the oldest participants with the heaviest infection intensity thus indicating a cumulative effect of parasite antigens on the immune responses mounted. Taken together, these observations demonstrate that the recognition of specific schistosome antigens, both in terms of the

diversity of antigens recognized and the intensity of antigen recognition, increased with cumulative exposure to parasite antigens consistent with the need for a threshold of antigen.

DETERMINING HOW NATURALLY ACQUIRED HUMAN ANTI-SCHISTOSOME ANTIBODY RESPONSES ARE AFFECTED BY ANTHELMINTIC TREATMENT

Control of schistosome infections is by treatment of infected people with the anthelmintic drug praziquantel. The drug damages the adult schistosome tegument exposing parasite antigens including 28-kDa glutathione *S*-transferase, the current leading vaccine candidate antigen for schistosomiasis (McManus and Loukas, 2008), Sm23, tubercle glycoprotein, alkaline phosphatase and actin on the worm surface to the host immune system (Andrews 1985; Fallon *et al.* 1994; Dupre *et al.* 1999) resulting in the killing of the parasites. Thus, if the threshold hypothesis holds, praziquantel treatment offers a straightforward way of testing it as it introduces a large amount of parasite antigens at once. This should accelerate the rate at which infected individuals reach threshold levels of adult schistosome antigens. Using the 2-D Western blotting and proteomics approach we compared antigen recognition profiles of the same population described above before and after curative praziquantel treatment. The results showed that praziquantel treatment induced quantitative and qualitative changes in schistosome antigen recognition patterns within 12 weeks of treatment (Mutapi *et al.* 2005). These changes were not observed in untreated people. Moreover, the antigens whose recognition was enhanced by treatment were also those antigens recognised naturally in untreated older people carrying little or no infection (so called endemic normals or putatively resistant people) (Mutapi *et al.* 2008). The fact that anthelmintic treatment can induce potentially protective immune responses presents a plausible additional vaccination approach and, as such, field studies are currently underway evaluating this method of inducing protective immunity to schistosomes (Black *et al.* 2010a,b). The idea of treatment-induced resistance to reinfection is neither a novel concept nor restricted to schistosome infections. In the veterinary field, a significant proportion of protozoan vaccines against some of the most economically significant parasites of livestock and companion animals are based on drug-abbreviated infections, using the so called infection and treatment (I&T) immunisation strategy (reviewed by Meeusen *et al.* 2007). Recently, a trial of the I&T protocol was attempted in human volunteers for control of *Plasmodium falciparum*, the protozoan causing the most severe form of malaria (Roestenberg *et al.* 2009) and was successful in inducing sterile immunity

against re-infection. Even if this approach is not eventually adapted as a vaccine strategy for schistosome control, it provides a vaccination model from which general lessons can be learned for the design of successful recombinant vaccines. Mechanistic studies in experimental models on the effect of praziquantel treatment on the development of protective immune responses against schistosome infections are already shedding light on the nature of immune responses protective against re-infection (Wilson *et al.* 2011).

CONCLUDING REMARKS

There has been significant progress made in understanding schistosome biology through proteomic approaches. The elegant work characterizing the schistosome infective stages as well as secreted and excreted products from the parasite has expanded our knowledge of the parasite's anatomical structure and physiological processes (Curwen *et al.* 2004; Mathieson and Wilson, 2010; Mathieson *et al.* 2011). Furthermore, the recent description of micro exon genes in schistosomes and their potential for generating antigen variation represents an exciting development in our current understanding of helminth antigen diversity and how the diversity is generated (DeMarco *et al.* 2010). Nonetheless, there are still more questions to address if we are to improve current understanding of the interaction between helminth parasites and their natural hosts. An understanding of the evolutionary, molecular and mechanistic processes involved in this interaction is essential for the development of additional intervention methods against the parasites. Due to variations arising from phenotypic differences and phenotypic plasticity in parasites (see (Machado-Silva *et al.* 2011)) and heterogeneities in human populations, studies characterizing the interaction of the whole organisms (schistosomes and host) should be more informative than reductionist approaches. This review has focused just on a few of the proteomic approaches that we are using to address fundamental questions in schistosome biology and immunology. Extension of these approaches to include assessment of protein activities, modifications and localization and studies of interactions of proteins in complexes (Tyers and Mann, 2003) will enable the description of systems-level cellular interactions between host and parasite proteins.

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