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Enzyme-linked immunosorbent assay with worm vomit and cercarial secretions of *Schistosoma mansoni* to detect infections in an endemic focus of Burkina Faso

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Abstract

Cercariae and adult *Schistosoma mansoni* were used to prepare, respectively, cercarial secretions (CS) and worm vomit (WoV). These were used as antigens in an enzyme-linked immunosorbent assay (ELISA) to test the IgG-reactivity of sera obtained in an *S. mansoni*-endemic area of Burkina Faso. Among the egg-excreting individuals ($n = 240$), 94.6% reacted positively with WoV, but only 62.9% with CS, thus suggesting a high diagnostic sensitivity of WoV, but not of CS. Among those individuals without detectable eggs in two Kato-Katz thick smears from different stool specimens ($n = 215$), the respective percentages of positive IgG reactivity were 78.1% and 63.3%. These positive reactions in the absence of detectable eggs are interpreted in terms of limited sensitivity of parasitological stool examinations. Optical density values in ELISA with CS, but not with WoV, correlated negatively with age, which may reflect decreasing exposure to cercariae in older individuals.

Introduction

Schistosomiasis remains one of the most important parasitic diseases of the tropics and subtropics. Its burden on human health is of concern as more than 120 million people are currently infected, with a risk of developing severe disease (Engels *et al.*, 2002). In the continued absence of an applicable vaccine, praziquantel-based chemotherapy will remain the strategy of choice for the control the disease (Bergquist *et al.*, 2002, 2005; Fenwick *et al.*, 2003; Hagan *et al.*, 2004; Utzinger & Keiser, 2004). Targeting chemotherapy to affected individuals and/or populations requires adequate diagnostic procedures, for which the superiority of serological methods over traditional egg counting by microscopy has long since been advocated (Mott & Dixon, 1982). The advantages and disadvantages of serologic testing versus diagnosis by microscopy were discussed by Doenhoff *et al.* (2003, 2004). Most antigens for serodiagnosis originate from schistosome eggs or adult worms. The schistosome gut is highly reactive with patient sera (Ruppel *et al.*, 1985a; Tarp *et al.*, 2000, Li *et al.*, 2004) and contains several identified specific and sensitive diagnostic antigens, including the cirulating cathodic and circulating anodic antigens (see Nash & Deelder, 1985; van Lieshout *et al.*, 2000) as well as proteolytic enzymes (Ruppel *et al.*, 1985a; Klinkert *et al.*, 1989; Caffrey *et al.*, 2004). If schistosomes are induced *in vitro* to vomit, they release their gut contents, which contains several cathepsins (Caffrey & Ruppel, 1997) and, thus, may be suspected to be rich in diagnostic antigens. On the other hand, cercarial antigens have also been advocated for diagnostic use

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(Ramzy *et al.*, 1997) and cercarial secretions (CS) in particular are rich in immunogenic molecules (Bahgat *et al.*, 2001).

Against this background, we evaluated and compared the reactivity with worm vomit (WoV) of *Schistosoma mansoni* and of CS using sera of patients infected with *S. mansoni* and residing in an endemic area of Burkina Faso.

Materials and methods

Study population and sera

Sera were obtained during a cross-sectional survey carried out in October 2002 from 455 subjects of both sexes, aged from 6 to 46 years, and living in the Kou valley, Burkina Faso. Further details of this survey, including informed consent by those enrolled, have been described (Sorgho *et al.*, 2005). Briefly, active infection by *S. mansoni* was detected by two Kato-Katz thick smears (Katz *et al.*, 1972). Overall, 52.7% of the study paricipants were infected, with a prevalence declining from between 50–60% in children to about 35% in individuals aged 35 years or older. The prevalence of infections with *S. haematobium* was less than 1%. Sera were supplemented with a trace of sodium azide, frozen and transported to Heidelberg, Germany, where all further tests were performed.

Parasite antigens

Worm vomit (WoV) was produced from a Puerto Rican strain of *Schistosoma mansoni* maintained in Heidelberg in NMRI-mice. Worms were obtained 6–7 weeks after infection by portal perfusion of mice with warm (room temperature) Dulbecco's Modified Eagle's medium (DME, Gibco-Europe, Karlsruhe, Germany) supplemented with 5% inactivated newborn calf serum (NCS, Gibco) plus heparin (2.5 units ml^{-1}). Groups of 20 worm pairs were placed overnight at 4°C in small Petri dishes containing 1 ml of distilled water. This treatment visibly induces regurgitation by the worms of their gut contents, although release of additional material from the worms is not excluded. The liquid was harvested, aliquoted and frozen at −80°C until use. The protein concentration was determined by the bicinchoninic acid (BCA) protein content test (Pierce Chemical Co., Rockford, Illinois, USA).

Cercarial secretions were prepared from cercariae freshly shed from *Biomphalaria glabrata* snails following published procedures (Bahgat *et al.*, 2001). Briefly, around 10,000 cercariae in distilled water were placed in a Petri dish previously painted with linoleic acid (0.9 g ml^{-1}; Sigma, St Louis, Missouri, USA). This treatment for 30 min at 37°C induces the release of acetabular gland contents from cercariae. Water containing CS was collected, sedimented for 1 h on ice and centrifuged at 3000 *g* for 2 min. The supernatant contained the CS and was lyophilized, reconstituted with distilled water in one tenth of the original volume, frozen and stored at −80°C until use. This preparation of CS contained 150–200 mg ml^{-1} of protein.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed in Maxisorp microtitre plates (Nunc, Roskidle, Denmark) using buffers and procedures essentially as previously described (Bahgat *et al.*, 2001). Coating with WoV (0.5 μg protein per well) or CS as (0.1 μg per well) as antigens was first done for 3 h at room temperature then overnight at 4°C. Blocking was with 0.01 M phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 plus 5% NCS (PBST-NCS) for 1 h at 37°C. Sera were diluted 1:100 in PBST-NCS and incubated at 37°C for 2 h. Proxidase-conjugated goat anti-human IgG (Dako, Hamburg, Germany) was diluted 1:5000 in PBST-NCS and left for 1 h at 37°C. The substrate was *o*-phenylenediamine (Sigma, St Louis, Missouri, USA), the reaction was stopped with 4 N H_2SO_4 and the optical density (OD) determined at 490 using a reference filter of 630 nm and distilled water as blank. IgG-reactivity was considered as positive if the OD exceeded a value of 2 standard deviations above the mean obtained with 22 sera of German healthy blood donors.

Data were analysed with the Statistical Package for Social Scientists (version 10.1; SPSS Chicago, Illinois, USA). The non-parametric Mann-Whitney U-test was used to compare the means of reactivity between groups. Correlations between variables (e.g. ELISA OD, egg counts, age, etc.) were assessed by Spearman's rank correlation.

Results

The IgG-reactivities of the complete study population in ELISA with WoV and CS are shown in table 1. The OD values obtained with WoV ranged between 0.061 and 1.633 and those with CS between 0.025 and 0.932. Positive reactions with WoV were much more frequent (86.8%) than with CS (63.1%).

Table 1. Reactivity of sera of the study population ($n = 455$) in ELISA with *Schistosoma mansoni* worm vomit and cercarial secretions.

		Positive reactions*		Negative reactions	
Antigen	Cut-off (OD*)	Number (%)	Mean OD ± SD	Number (%)	Mean OD ± SD
Worm vomit	0.23	395 (86.8)	0.68 ± 0.34	60 (13.2)	0.16 ± 0.04
Cercarial secretions	0.16	287 (63.1)	0.37 ± 0.18	167 (36.7)	0.10 ± 0.03

*Positive reactions were those with an optical density (OD) above the cut-off value (see Materials and methods).

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The IgG-reactivity with respect to *S. mansoni* egg excretion is shown in table 2. Almost all subjects, for whom eggs had been detected in their stool samples, showed positive reactions against WoV (94.6%). However, also among those individuals with two egg-negative stool samples, the majority reacted with WoV (78.1%), but the mean OD values were significantly lower for the egg-negatives ($P < 0.001$). No sex-dependent differences were observed between OD values of egg excretors and egg negatives. In contrast to WoV, similar percentages among egg excretors and egg negatives reacted positively with CS (62.9% versus 63.3%), and the mean OD values were neither different between both groups (table 2) nor between male and female participants (not illustrated).

The reactivity of sera with worm and cercarial antigens was compared for all sera (regardless of egg excretion) using the Spearman rank test and was found to correlate significantly (fig. 1). However, neither reactivity against WoV nor against CS correlated significantly with egg counts (not illustrated). A significant negative correlation between age and reactivity with CS was found not only for *S. mansoni* egg-excretors (fig. 2), but also for the whole study population ($r = -0.098$, $P < 0.001$; not illustrated). Reactivity with WoV revealed neither age dependency nor sex influence.

Discussion

This work compared two sets of *S. mansoni* antigens released from either cercariae or adult worms. With respect to adult worm antigens, WoV is likely to contain several gut enzymes (reviewed by Caffrey *et al.*, 2004) and excretion-secretion proteins (Cutts & Wilson, 1997). With respect to CS, the immune reactivity is based on acetabular gland secretions, excluding the 'cercarial elastase' (Bahgat *et al.*, 2001). However, we cannot rule out that CS may also contain antigens derived from the cercarial surface, which react strongly with sera from schistosomiasis patients (Harrop *et al.*, 2000; Bahgat *et al.*, 2001). Although the molecular identities of antigen preparations used here were not further analysed, the results allow several conclusions.

Firstly, the sensitivity of antigens in the WoV to detect specific IgG in sera of patients living in an *S. mansoni* endemic area is much higher than that of antigens in CS. The antigens in WoV include at least gut-released material, but probably also tegument-derived molecules. The gut is the first worm tissue against which antibodies become detectable by immunoflorescence after infections, which may occur only 40 days after exposure of humans (Ruppel *et al.*, 1985a) or 28 days after experimental infection of mice (Ruppel *et al.*, 1985b). On the other hand, antibodies against the gut persist also in chronic human infections and thus can be considered as a continuous marker of infection (Li *et al.*, 2004). This agrees with the observed high sensitivity (over 95%) of gut-associated fluorescence observed previously with the same study populations (Sorgho *et al.*, 2005) or another population in Oman, where *S. mansoni* has recently emerged (Idris *et al.*, 2003). Cercarial antigens, in contrast, appeared to be less sensitive (around 83% positive IgG-responses among sera of patients in an *S. haematobium* endemic area; Ramzy *et al.*, 1997), although cross-reactivity of cercarial secretions between schistosome species is pronounced (Bahgat *et al.*, 2001). Since sera of mice exposed to lethally irradiated cercariae did not react in Western blots with adult worm antigens (Ruppel *et al.*, 1985b), cercarial antigens apparently contribute to a lesser degree than adult worm antigens to the antibody response observed in patent infections. The direct comparison with both sets of antigens was done here using a much higher number of sera than used previously for cercarial antigens (Ramzy *et al.*, 1997). The relatively low sensitivity of CS does not favour cercarial antigens as diagnostic tools, as has already been stressed (Hamilton *et al.*, 1998).

Secondly, the immune response against CS showed an inverse age-dependency and this finding on cercarial antigens is reported here, to our knowledge, for the first time. Although the result is suggestive of a higher exposure of the younger age group to cercariae, field observations in this context still need to be done for the present study cohort. In an apparent contrast, Ramzy *et al.* (1997) reported for an *S. haematobium*-endemic area (23% prevalence) that the percentage of sera reacting with *S. mansoni* cercarial elastase increased with age (between 2 and 20 years) and the mean IgG units increased up to 14 years (their fig. 1). Although the identity of the antigen in that report has later been questioned (Bahgat *et al.*, 2001; see below), there is no doubt that the antigen preparations used by Ramzy and co-workers and by our group overlap antigenically, at least in part, by sharing common epitopes. However, if the calculations of the age-

Table 2. Reactivity of sera from *Schistosoma mansoni* egg excretors and egg-negatives in ELISA with *S. mansoni* worm vomit (WoV) and cercarial secretions (CS).

	Reaction of egg excretors ($n = 240$)		Reaction of non-excretors ($n = 215$)	
Antigen in ELISA	Positive (%) (mean OD ± SD)	Negative (%) (mean OD ± SD)	Positive (%) (mean OD ± SD)	Negative (%) (mean OD ± SD)
WoV-ELISA	227 (94.6) (0.79 ± 0.34*)	13 (5.4) (0.17 ± 0.05)	168 (78.1) (0.54 ± 0.28*)	47 (21.9) (0.16 ± 0.04)
CS-ELISA	151 (62.9) (0.36 ± 0.18)	89 (37.1) (0.11 ± 0.03)	136 (63.3) (0.39 ± 0.18)	79 (36.7) (0.11 ± 0.03)

*Values are significantly different ($P < 0.05$).

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dependency are limited to the strata between 6 years (the lowest age in our study) to 14 years (the age where IgG reactivity peaks in the other study), both the negative correlation (which is particularly influenced by the older ages, see our fig. 2) and the positive correlation (which is influenced by the low reactivities clustering in the very young age, see fig. 1 in Ramzy *et al.*, 1997) become absent. Future studies which take particularly younger and older age groups into account, may detect similar patterns of reactivity with cercarial antigens in both *S. mansoni*- and *S. haematobium*-endemic areas.

Thirdly, a substantial number of sera from individuals with two egg-negative Kato-Katz thick smears gave positive results with the antigens tested. As discussed earlier with respect to adult worm and egg antigens (Ruppel *et al.*, 1990; Idris *et al.*, 2003; Sorgho *et al.*, 2005), apparently false positive reactions reflect the limited sensitivity of microscopical egg detection (de Vlas & Gryseels, 1992; Engels *et al.*, 1996; Kongs *et al.*, 2001; Utzinger *et al.*, 2001) combined with a high sensitivity of antibody detection (for a comprehensive review see Doenhoff *et al.*, 2004). This report provides the first evidence that this discussion must be extended also to cercarial antigens.

Fourthly, with respect to the potential in serodiagnosis of schistosomiasis, the present study confirms the superior sensitivity of adult worm antigens over cercarial antigens (Mott & Dixon, 1982). Whether the anti-cercarial antibody response measured here reflects exposure to schistosome larvae, as had been suggested (Ramzy *et al.*, 1997), remains open. The antigen (the 'cercarial elastase') held responsible in the latter report for immune reactivity was not found to induce a detectable antibody response in natural infections of both humans and mice (Bahgat *et al.*, 2001). The presently used CS contain the 'elastase', but also other components (Bahgat *et al.*, 2001). Additional research would be needed to identify antigens which might detect very early infections or which might indicate exposure to cercariae in the absence of adult worm development. To this aim, a cDNA library from cercariae or sporocysts could be screened with sera from young children reacting with CS or with sera from mice infected with lethally irradiated cercariae. To identify individuals having been exposed to infection, but not harbouring adult worms, although living in endemic areas, would be of particular relevance to study acquired immune resistance and possibly to monitor control programmes.

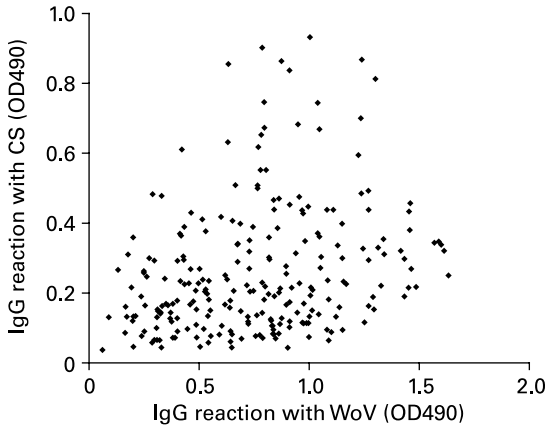

Fig. 1. Correlation between results in ELISA IgG reactivity obtained with *Schistosoma mansoni* cercarial secretions (CS) or worm vomit (WoV) as antigens. The correlation is statistically signficant ($r = 0.262$, $P < 0.001$).

Fig. 2. Correlation between IgG reactivity with *Schistosoma mansoni* cercarial secretions (CS) and host age (years) among all egg excretors of the study group ($n = 240$). The correlation is statistically significant ($r = -0.357$, $P < 0.001$).

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