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The antibody responses to adult-worm antigens of *Schistosoma haematobium*, among infected and resistant individuals from an endemic community in southern Ghana

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Antibody responses to antigens from adult *Schistosoma haematobium* were investigated in an endemic community in Ghana, using microplate-ELISA. The results of a survey of egg output in urine and of a questionnaire-based investigation of water-contact activities were used to select 'endemic normal' (EN) and patently infected (PI) individuals as subjects. The plasma levels of antibodies reacting with the adult-worm antigens were determined and compared and the correlations between these levels and the age, water-contact index and egg output of each subject were evaluated. Compared with the EN subjects, the PI generally had higher levels of anti-worm IgG and IgE but lower levels of anti-worm IgA. When the data for the EN and PI groups were combined, the levels of anti-worm IgG and IgE were found to be positively correlated with egg output and with each other. Whichever the antibody class considered, levels of anti-worm antibodies were never negatively correlated with egg output. These results indicate that anti-worm IgE and IgG could be used as markers to reflect current infection intensity, and that anti-worm antibodies may not act as protective antibodies in the natural course of urinary schistosomiasis.

Schistosomiasis is one of the most prevalent parasitic diseases of humans, affecting 200 million people world-wide (Chitsulo *et al.*, 2000). Despite long-term efforts to limit the disease — based on snail control, health education, safe-water supplies and mass chemotherapeutic treatment — many hyper-endemic areas remain, especially in sub-Saharan Africa (Chitsulo *et al.*, 2000). Although chemotherapy can be very effective, high levels of transmission often lead to rapid re-infection.

An effective vaccine, if it could be developed, may prevent infection and re-infection, especially if backed up by the older methods of control. There are two main ways in which such a vaccine could be developed. One is to identify the molecules that are the targets of protective immune responses in experimentally infected animals; *Schistosoma mansoni* glutathione-S-transferase (Balloul *et al.*, 1987), the paramyosins of *S. mansoni* and *S. japonicum* (James *et al.*,

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1985; Kojima *et al.*, 1987; Pearce *et al.*, 1988; Nara *et al.*, 1994) and *S. mansoni* calpain (Jankovic *et al.*, 1996) are examples of vaccine candidates identified in this way. The other main method of producing an effective vaccine is to identify the schistosome antigens that are specifically recognized by sera or lymphocytes from resistant individuals in an endemic area. Post-treatment follow-up can identify those who appear resistant to re-infection and then the immune responses of these individuals can be investigated. Only subjects resistant to re-infection with *S. mansoni* seem to have antibodies to a 37-kDa glyceraldehyde-3P-dehydrogenase (Goudot-Crozel *et al.*, 1989) or another, 22.6-kDa, antigen of the parasite (Webster *et al.*, 1996; Dunne *et al.*, 1997). Any investigation in which the immune responses of subjects of different infection statuses are compared may provide data useful for vaccine development. In a study of urinary schistosomiasis, for example, Hagan *et al.* (1991) found that the risk of re-infection was negatively correlated with levels of worm-antigen-specific IgE and positively correlated with levels of worm-antigen-specific IgG_4. It appears that IgG_2, IgG_4 and IgM may act as blocking antibodies that prevent protective immune responses (Khalife *et al.*, 1986; Butterworth *et al.*, 1987; Demeure *et al.*, 1993), although Caldas *et al.* (2000) reported that levels of IgM were higher in their resistant subjects than in the susceptible. IgG_1, IgG_3, IgA and IgE all seem to be protective isotypes in human schistosomiasis (Butterworth *et al.*, 1985; Khalife *et al.*, 1989; Hagan *et al.*, 1991; Rihet *et al.*, 1991; Dunne *et al.*, 1992; Demeure *et al.*, 1993; Grzych *et al.*, 1993). In terms of cell-mediated immunity, lymphoproliferative responses and production of interleukin-5 (IL-5) were found to be negative correlated with the level of re-infection (Roberts *et al.*, 1993).

As well as re-infection studies, there have been a few comparative investigations of the immune responses in patently infected (PI) and 'endemic normal' (EN) subjects in areas where *S. mansoni* is endemic. The EN subjects were found to have more prominent lymphocyte proliferation and higher levels of interferon-γ production than the PI, and they had levels of IgE reacting with the antigens on the schistosomulum tegument that were higher (not lower, as in the PI) than those of the IgG_4 reacting with the same antigens (Viana *et al.*, 1994, 1995). Individuals with low levels of *S. mansoni* infection showed higher lymphoproliferative responses than those with high levels of infection (Ribeiro de Jesus *et al.*, 1993). For urinary schistosomiasis in particular, the immune responses occurring specifically in EN have not been well characterized. In the present study, PI and EN subjects were identified in a community in Ghana where *S. haematobium* is endemic. Microplate ELISA were then used to explore the subjects' humoral antibody responses to adult-worm antigens. The relationships between the levels of anti-worm IgA, IgE, IgG and IgM observed and infection status are discussed.

SUBJECTS AND METHODS

Subjects

The subjects were residents of Okyereko, a coastal village in the Gomoa district of Ghana's Central region, where *S. haematobium* is endemic. The results of a preliminary baseline survey revealed that the prevalence of urinary schistosomiasis in this community was approximately 30% (data not shown).

The aims and methods of the study were explained to the village leaders and other villagers in their native languages. All the subjects of the study were aged $\geqslant$10 years and all gave their informed consent. A baseline survey of helminth infection status (including urinary and intestinal schistosomiasis) was carried out using filtration with Nucleopore membranes (Nucleopore, Pleasanton, CA) to check urine samples and the Kato–Katz method (Katz *et al.*,

1972) to check stool samples. Water-contact activities were investigated by questionnaire, as previously described (Viana *et al.*, 1995; Caldas *et al.*, 2000). Briefly, a water-contact index (WCI) was calculated, for each subject, as $\Sigma(R \times F)$, where R was a score for the reason for the water contact and F a score for the frequency of each type of activity. R was scored 5 (for bathing, swimming, or playing in a water body), 4 (for laundering, watering agricultural fields, or sand extraction from streams), 3 (for collecting water for the household or dishwashing) or 2 (for fishing or wading across the streams). F was scored 28 (for at least one contact/day), 4 (for at least one contact/week), 2 (for at least two contacts/month) or 1 (for fewer than two contacts/month). The WCI were calculated for the village's three major water-contact sites where infection seemed possible: the Ayensu river, a dam site and an irrigation field. The intermediate host snail of *S. haematobium* (*Bulinus truncatus rohlfsi*) was found in the water behind the dam and the water for the irrigation field was derived from the dam. Although no host snails were found in the river, there were infected individuals in the village who had water contact only in the river.

Individuals with WCI of at least 300 were selected and considered potential PI (if schistosome eggs had been found in the urine samples they had supplied for the baseline survey) or potential EN (if their baseline urine samples had been found egg-negative). Several further urine samples from each of these potential subjects were checked for eggs and only those consistently found negative or positive, for at least three samples collected on different days, were enrolled as EN and PI, respectively. All the urine samples were checked by filtration and those from potential EN were re-checked both by the microscopical examination of the sediments produced by centrifuging the remains of the samples and by using dipsticks designed to detect *S. haematobium* antigen in urine samples (Bosompem *et al.*, 1996, 1997). The intensity of the *S. haematobium* infection in each of the PI subjects was evaluated, as the mean egg output/10 ml urine, for two or three urine samples collected on different days. Normal control (NC) individuals, who had never lived in an *S. haematobium*-endemic area, were recruited from the personnel in the Noguchi Memorial Institute for Medical Research. Like the EN, the NC were screened, both by urine filtration and using dipsticks, to confirm that were not infected.

Preparation of Adult-worm Antigen

Eggs of *S. haematobium* were isolated from urine samples of some of the infected individuals, by centrifugation (at $200 \times g$ for 3 min at room temperature), and hatched in aged tap water. The miracidia were used to infected *B. truncatus rholfsi* snails, by exposing each snail overnight to four miracidia in a well of a 24-well culture plate. More than 5 weeks later, the snails were exposed to light and the emerging cercariae were collected. Outbred ddY or ICR mice were then infected with the cercariae percutaneously. Three to 4 months later, the infected mice were perfused with physiological saline containing 0.45% trisodium citrate, to recover the adult worms. The collected worms were washed with the perfusion solution, frozen in liquid nitrogen and subsequently dispersed in phosphate-buffered saline (PBS; pH 7.4) by ultrasonic treatment. The homogenate was centrifuged at $950 \times g$ for 10 min and the resultant supernatant solution was collected and used as the worm-antigen preparation in the ELISA.

Microplate ELISA

The antigen preparation was diluted in 50 mM bicarbonate buffer (pH 9.6) to a final concentration of 5 μg/ml. This dilution, at 50 μl/well, was used to coat 96-well microplates overnight at 4°C. After the plates were rinsed three times with washing buffer (PBS containing 0.05% Tween 20), a 50-μl sample

of diluted plasma from one of the subjects — diluted 1:40 (if IgG was being detected) or 1:10 (for IgE, IgA and IgM) with dilution buffer (washing buffer containing 0.5% bovine serum albumin) — was added to each of two wells and incubated at 37°C for 1 h. The plates were then washed three times with the washing buffer before 50 µl of a 1:1000 dilution of peroxidase-conjugated anti-human-IgG, -IgE, -IgA or -IgM (Sigma) in dilution buffer were added to each well. After incubation at 37°C for 30 min, the plates were washed five times with the washing buffer before the substrate — the diammonium salt of 2,2′ azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS; Sigma) — was added. Optical densities (OD) were measured at 415 nm in a Model 550 microplate reader (Bio-Rad, Hercules, CA), and recorded using the Microplate Manager III for Windows software package (Bio-Rad).

Statistical Analysis

The sex distributions of the various groups of subjects were compared using Fisher's exact probability test, whereas *F*-tests were used to analyse the differences in variance of each parameter recorded. Based on the results of the *F*-tests, the most suitable unpaired *t*-test for each analysis (Student's or Welch's) was used to make inter-group comparisons of age, WCI and OD (as indicators of antibody levels). The level of correlation between each pair of parameters was evaluated as a Pearson's correlation coefficient. All of the statistical analyses were performed using commercial software: Excel 97 (Microsoft) and StatView 4.5 (SAS Institute, Cary, NC).

RESULTS

Age, Egg Output, WCI and their Correlation

The characteristics of the EN and PI subjects are summarized in Table 1. The EN subjects were generally older than the PI ($P<0.001$). This was consistent with the fact that, when the data from the EN and PI groups were combined, intensity of infection, represented by mean egg output, was found to be negatively correlated with age ($P<0.05$; Table 2). The male:female ratio was higher in the PI group than in the EN group but the difference was not statistically significant ($P>0.05$; Table 1). For the PI subjects, and also for the combination of PI and EN, egg output was found to be negatively correlated with WCI ($P<0.05$; Table 2).

Anti-worm Antibody Levels

In terms of the antibodies reacting with the preparation of adult-worm antigens, the EN had similar levels of IgM [Fig. (a)], higher levels of IgA [$P<0.05$; Fig. (b)] and lower levels of IgE [$P<0.01$; Fig. (c)] and IgG [$P<0.05$; Fig. (d)] than the PI subjects. When the data for the PI and EN subjects were combined, males were found to have similar WCI and levels of worm-specific

TABLE 1. *Characteristics of the 'endemic normal' (EN) and patently infected (PI) subjects*

			P-value from:	
	EN	PI	Unpaired *t*-test	Fisher's exact test
Mean age and (S.D.) (years)	42 (15)	20 (12)	<0.001	–
No. of males/no. of females	5/6	11/5	–	>0.05
Mean egg output and (S.D.) (eggs/10 ml urine)	0 (0)	327 (495)	–	–
Mean water-contact index and (S.D.)	527 (167)	434 (80)	>0.05	–

TABLE 2. *Correlations between egg output, age, water-contact index (WCI) and levels of worm-antigen-specific antibodies for the 'endemic normal' (EN) and patently infected (PI) subjects*

		Pearson's correlation coefficient					
Subjects	Parameter	Egg output	Age	WCI	IgM	IgA	IgE
EN + PI	Egg output	–	–	–	–	–	–
	Age	−0.451*	–	–	–	–	–
	WCI	−0.386*	0.395*	–	–	–	–
	IgM	−0.112	0.216	0.049	–	–	–
	IgA	0.019	0.353	0.157	0.422*	–	–
	IgE	0.501†	−0.259	−0.299	−0.031	0.114	–
	IgG	0.652‡	−0.269	−0.294	0.118	0.091	0.650‡
EN only	Egg output	–	–	–	–	–	–
	Age	–	–	–	–	–	–
	WCI	–	0.188	–	–	–	–
	IgM	–	0.041	−0.013	–	–	–
	IgA	–	−0.003	0.132	0.497	–	–
	IgE	–	−0.151	−0.103	−0.108	0.272	–
	IgG	–	−0.281	0.120	0.017	0.649*	0.763†
PI only	Egg output	–	–	–	–	–	–
	Age	−0.386	–	–	–	–	–
	WCI	−0.558*	0.320	–	–	–	–
	IgM	−0.053	0.201	−0.079	–	–	–
	IgA	0.483.	0.113	−0.425	0.101	–	–
	IgE	0.408	0.114	−0.283	0.193	0.697†	–
	IgG	0.620†	0.057	−0.438	0.421	0.359	0.557*

*$P<0.05$.
†$P<0.01$.
‡$P<0.001$.

IgM, IgA and IgE to the females but significantly higher levels of worm-specific IgG ($P<0.05$; data not shown). To determine whether the PI subjects had higher levels of worm-specific IgG than the EN simply because they were more likely to be male, the IgG levels of the male EN, female EN, male PI and female PI were compared. For each sex, the mean level of worm-specific IgG was higher in the PI group than in the EN, although the differences were not statistically significant ($P>0.05$ for each; data not shown).

Correlation Between Egg Output, Age, WCI and Antibody Levels

Table 2 summarizes the strengths of the correlations observed between the levels of worm-specific antibody (of each immunoglobulin class) and egg output, age or WCI.

When the PI and EN subjects were considered as a single group, egg outputs were found to be positively correlated with the levels of specific IgE ($P<0.01$) and IgG ($P<0.001$). In the PI group, egg output appeared to be positively correlated with the levels of specific IgG, IgA and IgE but only the correlation with IgG was statistically significant ($P<0.01$).

The levels of specific IgG and those of specific IgE were positively correlated, for the EN only ($P<0.01$), for the PI only ($P<0.05$) and for EN and PI combined ($P<0.001$). The positive correlations observed between the levels of specific IgM and those of specific IgA were only statistically significant when the data for the EN and PI were pooled ($P<0.05$), not for the EN only or PI only. The levels of specific IgA and IgE were positively correlated in the PI group ($P<0.01$)

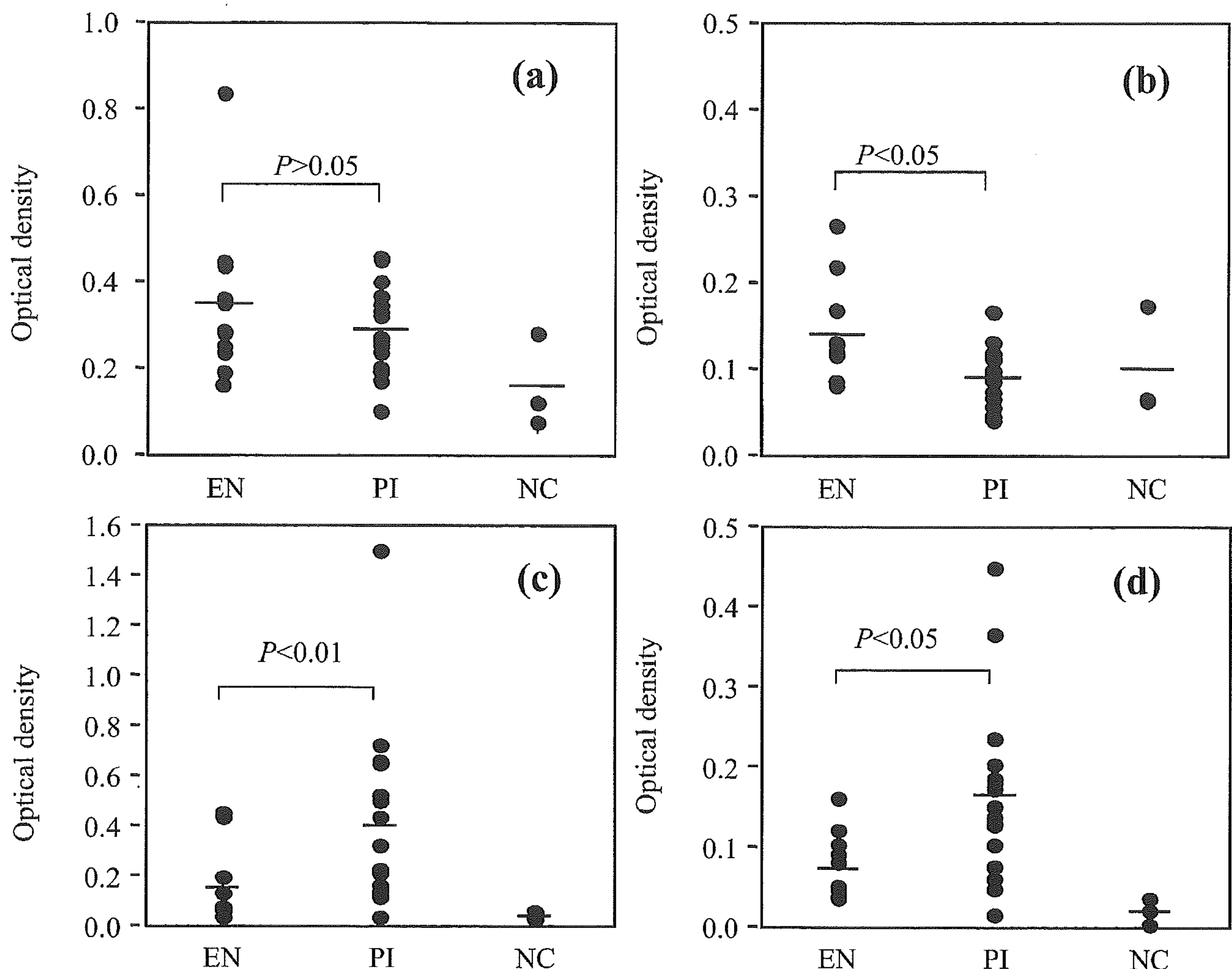

FIG. Actual (●) and mean (—) plasma levels of worm-antigen-specific IgM (a), IgA (b), IgE (c) and IgG (d) measured, as ELISA optical densities, in the 'endemic normal' (EN), patently infected (PI) and 'normal control' (NC) subjects. Statistical differences were assessed using unpaired *t*-tests.

but not in the combined group, and the levels of specific IgA and IgG were positively correlated in the EN group ($P<0.05$) but again not in the combined group.

DISCUSSION

For the present study, EN and PI individuals were identified in a community where *S. haematobium* is endemic. The EN group, composed of individuals who appeared uninfected despite their high WCI, is assumed to represent those who were resistant to current exposure to infective cercariae. This does not mean that the EN subjects had never been infected or that they were innately resistant; most, if not all, had probably been infected and had acquired protective immunity. Such acquired immunity may explain why, even in the absence of treatment, the prevalence of *S. haematobium* infection is generally found to be much higher in young, school-age children than adults (Ross *et al.*, 2000). It may also explain why the EN subjects of the present study were generally much older than the PI (Table 1) and why egg output tends to decrease with increasing age (Table 2). Since the EN and PI subjects had similar risks of cercarial infection (at least as indicated by their WCI), it seems likely that at least some of the EN had slowly acquired protective immunity. Abortion of the infection before worm maturation, single-sex infections, and infections that are so light that they fall

below the detection limits of the method used to evaluate prevalence may also, however, give rise to individuals who appear to be EN (Corrêa-Oliveira *et al.*, 2000).

The male:female ratio in the PI group was higher than that in the EN group (Table 1). In terms of mean age, WCI and antibody levels, the EN males were not significantly different to the EN females and the PI males were not significantly different to the PI females. The one exception was in the levels of specific IgG, which were significantly higher in the PI males than in the PI females. It seemed possible that the observation of a higher mean level of specific IgG in the PI group than in the EN group therefore simply represented gender bias, since, compared with an EN subject, a PI subject was more likely to be male (Table 1). However, levels of specific IgG were higher in the PI group than in the EN group not only for the males but also for the females (although the differences were not statistically significant).

Curiously, egg output was found to be negatively correlated with WCI (Table 2). This seems to contradict the fact that human–water contact is essential for schistosome infection. This surprising result may reflect the limitation of the present study to individuals with high WCI (i.e. with WCI of $\geqslant 300$ — probably enough for infection) and/or the failure of the questionnaire-based method used to estimate the level of exposure to cercariae accurately.

Viana *et al.* (1994, 1995) described the differences in the immune responses of EN and PI subjects for schistosomiasis mansoni. They found that the level of schistosomulum-tegument-specific IgE was higher (EN) or lower (PI) than the level of specific IgG_4, depending on the subject's infection status (Viana *et al.*, 1995). Compared with the PI, their EN group exhibited higher IgM responses against the schistosome antigens. The present data, for *S. haematobium*, do not always indicate the same trends: the levels of specific IgM were similar for the PI and EN subjects and those of specific IgE were higher in the PI group than in the EN (Fig.). These results imply that IgE and IgM that react with the adult-worm antigens do not act as the main protective antibodies in *S. haematobium* infection. The present results seem to be inconsistent with those of Hagan *et al.* (1991), who suggested that IgE may act protectively against re-infection with *S. haematobium* after chemotherapy. It is very possible, however, that worm antigens that induce protective IgE can be released only by the drug-related destruction of adult worms or eggs. Praziquantel treatment of urinary schistosomiasis, for example, is known to trigger a significant increase in the levels of anti-egg antibodies (Mutapi *et al.*, 1998*a*, *b*). It is also possible that the preparation of soluble worm antigens used for the present ELISA lacked the tegumental antigens that may serve as the targets of protective IgE.

The mean level of specific IgA in the PI group was low, comparable with that in the NC, and significantly lower than that in the EN group [Fig. (b)]. These results indicate that, although worm-specific IgA is not produced much in the natural course of infection, protective IgA might be produced in EN individuals. In fact, it has been reported that IgA could act as a protective antibody in *S. mansoni* infection (Grzych *et al.*, 1993). In western blots, however, IgA in plasma from EN did not recognize any antigen bands that were not recognized by IgA in plasma from PI (data not shown). Taken together, this observation and the lack of correlation between IgA and egg output (Table 2) indicate that anti-worm IgA does not play a protective role against *S. haematobium* infection.

Levels of specific IgG and IgE were positively correlated with egg output (Table 2) and, in western blots, IgG and IgE in plasma from PI recognized more antigens than IgG and IgE in plasma from EN (data not shown). Relatively high levels of anti-worm IgG and IgE may directly reflect 'active' current infection. Another possibility is that the high level of specific IgG seen in the PI group may reflect the presence of blocking

antibodies. As IgG subclasses were not investigated in the present study, it is impossible to say whether blocking IgG subclasses, such as IgG_4, recognized some of the worm antigens. Further investigation is needed to clarify this point.

Although levels of specific IgG and IgE were positively correlated (Table 2), western blots indicated that the IgE from the PI group did not recognize the same antigen bands as the IgG from the same group (data not shown). These results indicate that the positive correlation observed between IgG and IgE levels was not the result of the strong antigenicity of certain antigens, but probably a reflection of a common regulating mechanism for these antibody isotypes. Human IL-4, for example, is known to enhance the production of both IgE and IgG_4 antibodies (Ishizaka *et al.*, 1990).

The immune responses of EN generally differ from those of individuals who appear resistant to re-infection after chemotherapy (Corrêa-Oliveira *et al.*, 2000). In schistosomiasis mansoni, those considered EN and those with very light infections not only have peripheral-blood mononuclear cells (PBMC) that show an intense proliferative response but also have high levels of interferon-γ production and high IgE:IgG_4 ratios (Ribeiro de Jesus *et al.*, 1993; Viana *et al.*, 1994, 1995). In schistosomiasis japonica in China, resistant individuals have PBMC that produce high levels of IL-10 (McManus *et al.*, 1999). In the present study, only the level of specific IgA was significantly higher in the EN group than in the PI group (Fig.), although the PI group had relatively high levels of IgE and IgG (Fig.) — the isotypes generally considered to be protective in humans (Khalife *et al.*, 1989; Hagan *et al.*, 1991; Rihet *et al.*, 1991; Dunne *et al.*, 1992; Demeure *et al.*, 1993). In western blots, there appeared to be no antigens recognized by plasma from the EN group that were not recognized by plasma from the PI group (data not shown). The apparent protective immunity of the EN group was not therefore based on humoral antibody responses to the antigens contained in adult worms. Antibodies specific to the migrating larvae or cellular immune responses may be involved in the protective immunity of the EN group. Such a possibility needs to be further investigated if the mechanisms of protective immunity against urinary schistosomiasis are to be elucidated.

In conclusion, anti-worm IgE and IgG can be used as markers to reflect current infection intensity, and anti-worm antibodies do not act as protective antibodies in the natural course of urinary schistosomiasis.

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SENSITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY WITH URINE SAMPLES : A TOOL FOR SURVEILLANCE OF SCHISTOSOMIASIS JAPONICA

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Abstract. An enzyme-linked immunosorbent assay (ELISA) to detect antibodies to *Schistosoma japonicum* soluble egg antigens (SEA) in un-concentrated urine was developed. The urine ELISA was applied to samples collected in a schistosomiasis-endemic village in China. The levels of anti-SEA antibodies detected in urine correlated well with those obtained with paired serum samples ($r = 0.694$, $p<0.0001$). Among 129 serum ELISA positives, 112 (86.8%) were positive by urine ELISA, while all 40 serum ELISA negatives from a non-endemic area were negative. The levels of anti-SEA in urine samples were stable up to 8 weeks of storage at 37°C, with sodium azide as a preservative. Therefore, ELISA with urine samples can be used for the surveillance of schistosomiasis.

INTRODUCTION

Schistosomiasis is one of the most important infectious diseases in the world. It has been estimated that 200 million people harbor the parasites, while 20 thousand die from the disease annually. To control schistosomiasis japonica, simple diagnostic methods suitable for mass surveys are essential. Serodiagnostic methods, such as the enzyme-linked immunosorbent assay (ELISA) have been used to detect antibodies specific to the parasite antigens in serum samples, since eggs are not always detected in stool samples (Yogore *et al*, 1983; Lewert *et al*, 1984; Yu *et al*, 1998).

Compared with serum samples, the collection of which has a risk of accidental infection with blood-borne diseases, such as human immunodeficiency virus (HIV) and hepatitis viruses, urine samples can be collected safely and easily. Collection of urine samples does not require trained staff, syringes or a centrifuge. Furthermore, compliance is easy to obtain. ELISAs with urine samples used to diagnose lymphatic filariasis and visceral leishmaniasis have shown high sensitivity and specificity (Itoh *et al*, 2001; Islam *et al*, 2002).

In this study, we investigated anti-*Schistosoma japonicum* specific IgG in urine samples and showed that un-concentrated urine contained enough antibodies to diagnose schistosomiasis japonica.

MATERIALS AND METHODS

Serum and urine samples

Paired serum and urine samples were collected from 373 habitants in Rinjiang Village, an endemic area of schistosomiasis japonica, located close to Dongting Lake, 45 km northwest of Yueyang City, Funan Province, People's Republic of China, in 1995 and 1996. Forty in habitants of Yueyang City were used as negative controls.

Serum samples were kept at -20°C until used. NaN_3 (at a final concentration of 0.1%) was added to urine samples just after collection, and kept at 4°C until used.

Enzyme-linked immunosorbent assay (ELISA)

Preparation of *S. japonicum* soluble egg an-

tigens (SEA) was carried out according to a method described by Tanaka *et al* (1983). Anti-SEA antibodies in serum and urine samples were measured by an ELISA method. A 96-well microtiter plate was coated with 5 μg/ml of SEA overnight. After blocking the plate with 0.05M Tris-HCl buffer, pH 7.6, containing 1% casein for 2 hours at room temperature, 100 μl of 2,000 times diluted serum or un-concentrated urine samples were added to the wells. The plate was incubated at 37°C for one hour for serum and two hours for urine samples. After washing the plate three times with a washing buffer, 0.067M phosphate-buffered saline, pH7.4, containing 0.05% Tween 20, 100 μl of anti-human IgG conjugated with peroxidase (4,000 times diluted, Bio Source International Inc, USA) were added to each well and the plate was incubated at 37°C for an hour. Then the plate was washed three times with the washing buffer and 100 μl/well of ABTS (Kirkegaard and Perry Laboratories, USA) were added as a substrate. The optical density of each well was measured at 415 nm with a reference at 492 nm after one hour incubation at room temperature.

Antibody levels were expressed as units based on a standard curve. To construct the curve, pooled sera from five schistosomiasis japonica patients were threefold serially diluted with the casein buffer (1:1,000 to 1:729,000 for serum ELISA and 1:3,000 to 1:2,187,000 for urine ELISA), and a set of the serially diluted sera was prepared for each microtiter plate. As antibody units, a value of 21,870U was arbitrarily assigned to the 1:1,000 dilution and a value of 10U to the 2,187,000 dilution. Antibody units of serum samples higher than 21,870U and those of urine samples higher than 7,290U were regarded as 21,870U and 7,290U, respectively. Cut-off values for urine and serum ELISA were defined as the average unit of control samples + 3 standard deviations. A geometric mean of (antibody unit +1) was used for the average calculation. The cut-off unit for urine and serum ELISA were 124 and 949 units, respectively.

Effect of storage of urine samples on antibody levels

In order to study how long urine samples could be kept without deterioration at ambient temperatures, urine was kept in an incubator at 37°C for up to 8 weeks and the change in antibody units was examined at 5 and 8 weeks. Twelve samples (4 with high units, 4 with lower units and 4 negatives) were used.

RESULTS

Antibodies to SEA in urine samples

As shown in Fig 1, IgG to SEA could be detected in urine samples and the levels correlated well with those obtained with serum samples (r=0.694, p<0.0001). Out of 129 serum ELISA positives, 112 (86.8%) were positive with urine ELISA. On the other hand, all the 40 urine samples from the non-endemic area were negative.

Positive rates of serum and urine ELISA by age groups are shown in Fig 2. Both positive rates of serum and urine ELISA increased with age and the rates of urine ELISA in all age groups were the same or more than those of serum ELISA. All four urine ELISA positives in the age group ≤10, were also serum ELISA positive.

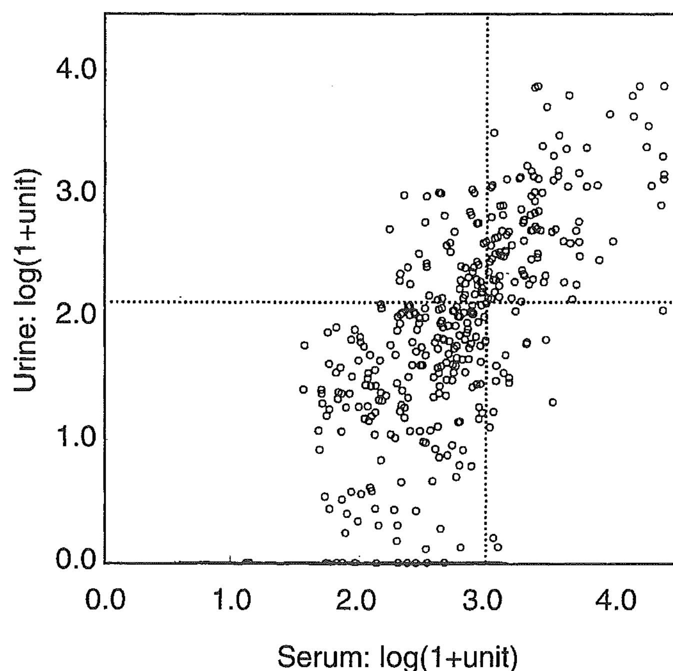

Fig 1–Correlation of anti-SEA IgG detected in urine and serum samples. Anti-SEA IgG levels in urine (without dilution) and serum (1,000 times diluted) samples were measured by ELISA. Antibody levels were indicated as log (1 + unit). Dotted lines indicate cutoff points of both urine and serum ELISAs.

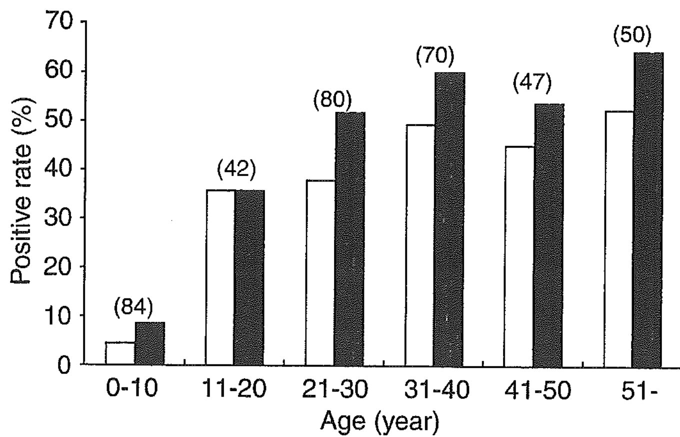

Fig 2–Changes in positive rates of urine and serum samples by age group. Open and dotted bars indicate urine and serum samples, respectively. Sample numbers are in parentheses.

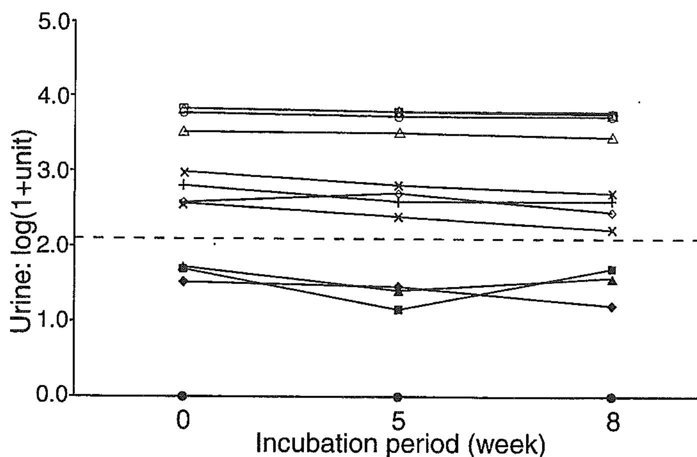

Fig 3–Effect on anti-SEA IgG levels of keeping urine samples at 37°C. Four samples were used for each of the high, low and negative groups.

Effect of storage of urine samples on their antibody levels

Anti-SEA levels in urine samples stored at 37°C for 5 and 8 weeks are shown in Fig 3. The storage condition affected little on anti-SEA levels of samples with not only high but also low antibody levels. None of the positive samples became negative and vice versa.

DISCUSSION

ELISA with SEA as antigens has been used for the serodiagnosis of schistosomiasis (Hillyer *et al*, 1979; Ishii and Owhashi, 1982; Tanaka *et al*, 1983). The ELISA method is more sensitive and accurate than stool examination in obtaining prevalence rate (Yogore *et al*, 1983) and incidence (Lewert *et al*, 1984) of schistosomiasis japonica. A drawback of antibody detection is that it cannot distinguish previous and current infections. However, the ELISA method is still a useful and sensitive diagnostic tool for surveillance, especially in finding new endemic foci. It can also be useful for evaluating a control program; successful control will reduce antibody levels.

Recently, urine was successfully substituted for serum as samples to diagnose lymphatic filariasis and visceral leishmaniasis with ELISA (Itoh *et al*, 2001; Islam *et al*, 2002). Compared with serum samples, urine can be easily and safely collected, especially from children. This can facilitate compliance of people in field activities. This study revealed that urine samples from schistosomiasis patients contained anti-SEA IgG as well, and the levels significantly correlated with those of serum samples. Anti-SEA IgG was detected in 86.8% of urine samples from serum-positives and all the urine samples from a schistosomiasis non-endemic area were negative with the urine ELISA. Leak of IgG into urine caused by nephritis ,which may occur in chronic patients (Tada *et al*, 1975) is unlikely, since positive rates of urine and serum ELISA were almost the same in all age groups and protein levels of all the urine samples were judged normal with a reagent strip (data not shown). As a successful control program will stop transmission of the parasite and make young age groups anti-SEA free, anti-SEA levels of the group provide useful information to evaluate control programs. Storage of urine samples for 8 weeks at 37°C had little effect on their anti-SEA levels, making the urine ELISA more practical, especially in remote areas.

The effects of the Three Gorges Dam, currently under construction on the Yangtze River in China, on the transmission of *S. japonicum* have been suggested, and it is submitted that systematic surveillance and preventive strategies against the disease are necessary (Xu *et al*, 2000). This sensitive and safe urine ELISA will be one of the essential tools for the surveillance of schistosomiasis. Application of this urine ELISA to school-based examination will elicit information on the prevalence of the disease and aid in the evaluation of control programs. Combination of the urine ELISA with other simple urine examinations, *eg*