The Effect of Treatment on the Age-antibody Relationship in Children Infected with *Schistosoma mansoni* and *Schistosoma haematobium*

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The effect of praziquantel treatment on the age-antibody relationship was studied in 174 children aged between 6 and 17 years from a schistosome endemic area in Zimbabwe. The children were co-infected with Schistosoma mansoni *and* S. haematobium *with infection prevalences of 74% and 53% respectively. Antibody levels for the isotypes IgA, IgE, IgM, IgG1, IgG2, IgG3 and IgG4, directed against soluble egg antigen were measured using an indirect ELISA assay. Treatment resulted in a significant increase in levels of IgG2 and IgG3 while levels of IgA decreased significantly. In untreated children there were significant decreases in levels of IgG4. Treatment also resulted in significant alteration in the age-antibody profiles for the isotypes IgE, IgM, IgG1 and IgG2 in treated children but not in untreated children. The results are discussed in the context of factors believed to give rise to the age-antibody relationship; i.e. age-related exposure patterns, age-related development of acquired immunity, age-related hormonal changes and age-related changes in innate susceptibility to infection.*

Key words: *Schistosoma mansoni* - *Schistosoma haematobium* - antibody - age-infection experience - treatment

Numerous field studies have demonstrated several epidemiological and immuno-epidemiological patterns now accepted as characteristic for schistosome infections (Woolhouse & Hagan 1999). One such pattern is the distribution of immune responses across different ages in a host population. For both *Schistosoma mansoni* and *S. haematobium* responses directed against egg antigen, levels of IgE have been shown to increase with age, those of IgA, IgM and IgG2 have been shown to decrease with age, while levels of IgG1 and IgG3 have been shown to either increase or decrease (Demeure et al. 1983, Butterworth et al. 1988, Hagan et al. 1991, Dunne et al. 1992a,b, Grogan et al. 1996, Ndhlovu et al. 1996, Vandam et al. 1996, Mutapi et al. 1997, Webster et al. 1997). The explanations for these patterns have been controversial. They include the development of acquired resistance which is related to the development of parasite-specific immune responses as a function of the duration and frequency of exposure to parasite antigens (Anderson 1987). Immune responses are believed to develop slowly and to be short lived (Woolhouse & Hagan 1999), so that as the hosts age, their cumulative experience of parasite antigens increases, resulting in an increase in acquired resistance. This explanation is supported by both experimental (Crombie & Anderson 1985) and field studies (Woolhouse et al. 1991, Woolhouse 1998). The second explanation is age-related hormonal influence of the immune system. It has been suggested that hormones which change with age such as growth hormones affect the immuno-competence of the host (Gryseels 1994). There has yet not been a comprehensive field or experimental study to validate this explanation. Another explanation is related to the development of innate resistance to infection. This includes changes in skin thickness and changes in the distribution of fat, both of which are believed to reduce the success of cercariae in penetrating the skin (Gryseels 1994). Again there have not been any field studies to validate this. Finally age-related changes in exposure patterns have also been suggested to influence the age-antibody pattern. Field studies on exposure patterns have shown that while differing exposure patterns do affect the levels of antibodies produced they do not fully explain the patterns observed (Stelma 1997).

The aim of our study is to investigate the robustness of the age-antibody relationships in children infected with both *S. mansoni* and *S. haematobium.* To do this, we make use of a feature of the treatment-re-infection study design. Numerous field studies have demonstrated that treatment of schistosome infections in people from areas endemic for the infections results in significant alteration of their parasite-specific humoral and cellular responses (Mutapi 2001). In this study this knowledge is employed to study an immuno-epidemiological pattern that is consistent with the distribution of schistosome infection.

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MATERIALS AND METHODS

Study site and sample collection - The study was carried out as part of a larger study of school children from Chiredzi, Southeastern Zimbabwe, where *S. mansoni* and *S. haematobium* are endemic. Only inhabitants of the study areas who had never been treated for any helminth infection were selected for the study. Following explanation of the study aims to the community, school children and their teachers, 320 children (6 to17 years old) were surveyed. Parasitology samples were collected from each child as follows. At least two urine and two stool samples were collected on three consecutive days. Three replicate slides were prepared for each stool sample and processed following the Kato Katz procedure to detect any *S. mansoni* eggs and other intestinal helminths while the urine filtration method was used to detect any *S. haematobium* eggs in the urine samples (Katz et al. 1972, Mott 1983). The formal ether faecal concentration method was used on a random sub-sample of 53 children to confirm the Kato results. At the same time a blood sample was collected for serological assays. Parasitology and serology samples were collected in the same manner 6 and 18 weeks after treatment. Of the 320 children surveyed, 174 children fulfilled the criteria for inclusion in the cohort namely; each child had to have given at least two stool samples for intestinal helminth detection and at least two urine samples for *S. haematobium* detection, on any three consecutive days both before and after treatment. Each child had to be negative for all other intestinal helminths and had to have provided a blood sample for serological assays both before and after treatment. One hundred and thirty-two children (82 male, 91 female) received the recommended dose of praziquantel, 40 mg/kg body weight. Forty-two children (22 male, 20 female) who would not accept treatment on religious grounds or were absent from school on treatment days, but wished to remain part of the study cohort effectively became untreated controls. All treated children were confirmed egg negative for both *S. mansoni* and *S. haematobium* six weeks after treatment. Children who missed treatment and were prepared to be treated received treatment at the final field visit.

Serology - Freeze dried *S. mansoni* soluble egg antigen, SEA, obtained from Dr M Doenhoff (University of Bangor, Wales) was reconstituted with phosphate buffered saline (PBS) at pH 7.4. Antibody levels were determined by a standard indirect enzyme linked immuno-sorbent assay (ELISA). ELISA plates (Immulon 4, Dynatec, USA) were coated overnight at 4^{o}C with 100 µl of antigen at 10 µg/ml/well in carbonate bicarbonate buffer (pH 9.6) for all isotypes except IgM and IgA which were coated with antigen at 2.5 µg/ml. All plates except IgE plates were blocked with 200 µl/well skimmed milk for 1 hr and washed 3 times in PBS which was used for all washes; 100 µl of serum was added to each well at 1:10 dilution for IgE, IgG1, IgG2, IgG4; 1:20 for IgG3, 1:40 for IgA and 1:100 dilution for IgM. Plates were incubated at 37^{o}C for 1 h for all other isotypes and 3 h for IgE and then washed 3 times; 100 µl/well of monoclonal antibody were added at 1:500 dilutions for IgG1, IgG2, IgG3 and 1:1000 dilutions for IgA, IgE, IgM and IgG4, using horseradish peroxide conjugated IgA (A-7032 Sigma, St Louis), IgE (P-295, Dako, Denmark), IgM (A-9607, Sigma, St-Louis) and IgG1, IgG2, IgG3 and IgG4 (MCA514, MCA515, MCA516, MCA517 respectively, Serotec, UK). IgE plates were incubated at 37^{o}C for 2 h and plates for all other isotypes were incubated at room temperature for 1 h. All plates were washed six times, and 100 µl/well of substrate *o*-phenyldiamine in phosphate citrate buffer (pH 5) added. The reaction was allowed to take place at 37^{o}C for 30 min for all isotypes, stopped with 25 µl of H_2SO_4, and absorbance read at 492 nm. All sera were assayed in duplicate and three standards (also in duplicate) were included on each plate. These were: a positive control consisting of a pool of sera from 20 individuals presenting with the highest egg counts across the age range; a negative control consisting of a pool of sera from 20 schistosome-negative South Americans; and a blank control containing no sera. The background absorbance of reagents in the absence of serum was subtracted from all readings. Immunological assays were also conducted against whole worm homogenate antigens. The results from these are not presented here as responses to SEA gave clearer results.

Statistical analyses - Statistical analyses were conducted using the statistical package SPSS (SPSS, Chicago) to determine if treatment significantly affected either the levels of immune responses or the age infection-antibody relationships. To determine if there was a significant difference between pre-and post-treatment antibody levels, a paired t-test was conducted on square root transformed antibody data from treated children. The effect of treatment on the change in levels of antibodies produced by all children was determined using a repeated general linear model (GLM) analysis of variance (ANOVA) which as appropriate included sex, age-group and treatment as categorical variables and pre-treatment infection intensity [$\log_{10}(x+1)$ transformed] as a continuous variable. The dependent variables were the pre- and post-treatment antibody levels (square root transformed). The age groups were 1 (6-8 years old, n = 29); 2 (9-10 years old, n = 53); 3 (11-12 years old, n = 43) and 4 (13-17 years old n = 48) and the treatment categories were untreated controls and praziquantel treated. For infection intensity, the transformed *S. mansoni* and *S. haematobium* infection intensities were entered into the statistical model together with their interaction term. Sequential sums of squares were used to calculate F ratios allowing for the effects of all confounding variables before testing for the effects of treatment (Mutapi & Roddam 2002).

The effects of treatment on the age-antibody relations were analysed using a GLM ANOVA, with both age and antibody levels (pre- and post-treatment) as continuous variables. The dependent variable for this analysis was age and the independent variables were antibody level (square root transformed) and time (pre-treatment, post-treatment). Age was not categorised for this procedure because the model satisfied the assumptions for the parametric test. A significant interaction between time and antibody levels would indicate that the relationship between age and antibody level had altered between the two time points.

RESULTS

Before treatment, 49% of the children were carrying a mixed *S. mansoni* and *S. haematobium* infection. Overall, the prevalence of *S. mansoni* was 74% and mean infection was 65 epg (range 0-538). The prevalence of *S. haematobium* was 53% with a mean infection intensity of 31 eggs/10 ml of urine (range 0-400). The mean infection intensity for both *S. mansoni* and *S. haematobium* for each age group is shown in Fig 1.

Table I shows that there were significant changes in the levels of the isotypes IgA, IgE, IgM and IgG3 in treated children, while, levels of IgG2, IgG1 and IgG4 in the same children did not change significantly. However, Table II shows that only changes in levels of IgA and IgG3 were due to praziquantel treatment. Furthermore, while levels of IgG4 remained at pre-treatment levels six weeks following treatment, there were significant changes in the levels of these isotypes in untreated children six weeks later. Fig. 2 shows that levels of IgG4 increased significantly in untreated children. Levels of IgG2 decreases in untreated children while they increased in treated children. Levels of IgG3 increased significantly in treated children but remained unchanged in untreated children.

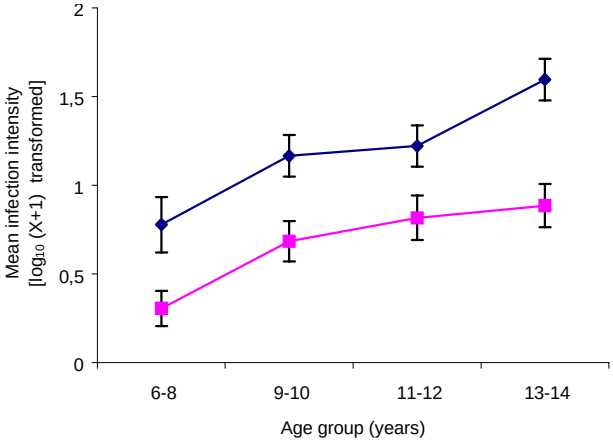

Fig. 1: mean infection intensity [$\log_{10}$ (x+1) transformed] for each age group. Diamonds represent *Schistosoma mansoni* and squares represent *S. haematobium*. Bars represent standard error of the mean.

TABLE I

T-values and degrees of freedom from the paired T-test determining if there are changes in mean antibody levels in treated children 6 weeks after treatment

	T-value	Degrees of freedom
IgA	6.72***	114
IgE	2.45*	117
IgM	-3.239**	116
IgG1	-0.56	64
IgG2	-0.56	64
IgG3	-3.43***	117
IgG4	1.29	120

Asterix represent significance levels as follows: * p < 0.05; ** p <= 0.01; *** p <= 0.001.

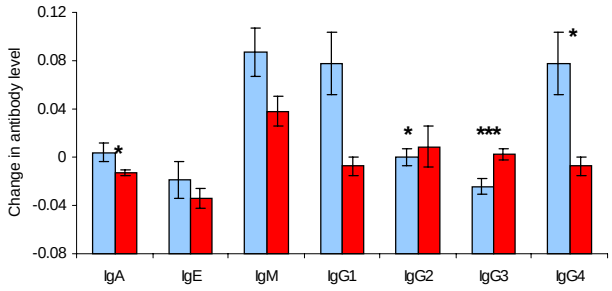

Fig. 2: change in antibody levels 6 weeks after treatment. Unshaded bars represent untreated children and shaded bars represent treated children. Asterix represent significance levels as follows: * p < 0.05; *** p <= 0.001.

Comparative analyses on the effect of praziquantel treatment on the age-antibody relationship showed a significant alteration of the relationship following treatment for the isotypes IgE, IgM, IgG1 and IgG2 in treated children. Such changes did not occur for any isotypes in untreated children as shown by the f- and p-values in Table III. Fig. 3 compares the relationship in untreated and treated children before and six weeks post treatment. The general trend is an increase in antibody level before treatment for both treated and untreated children at the start of the study and a decrease with age in treated children while the relationship in untreated children remains the same.

TABLE II

F-values from the repeated ANOVA determining factors affecting the magnitude of change in mean antibody levels in all children 6 weeks after treatment

	Sex	Age	*Schistosoma mansoni (SM)*	*Schistosoma haematobium (SH)*	*SM*SH*	Treatment
IgA	0.00	4.82**	5.3*	11.5***	2.6	5.04*
IgE	0.46	13***	9.21**	16.4***	6.97***	2.11
IgM	2.64	3.67*	0.33	0.89	4.1*	2.75
IgG1	0.27	3.59*	0.98	0.07	0.002	1.59
IgG2	0.166	1.57	2.88	0.80	0.12	4.33*
IgG3	0.27	0.64	8.12**	4.43*	0.05	23.8***
IgG4	0.000	3.78*	0.000	12.74***	0.08	4.07*

Asterix represent significance levels as follows: * p < 0.05; ** p <= 0.01; *** p <= 0.001.

TABLE III

F-values from the ANOVA determining if there is a change in the age-antibody profile (pre- vs 6 weeks post-treatment) in untreated and treated children

	Untreated F-value (degree of freedom)	Treated F-value (degree of freedom)
IgA	0.21 (1,64)	0.74 (1,239)
IgE	0.22 (1,64)	4.53 (1,243)*
IgM	2.26 (1,64)	10.5 (1,247)***
IgG1	0.35 (1,64)	7.04 (1,191)**
IgG2	0.33 (1,64)	18.3 (1,131)***
IgG3	0.04 (1,64)	0.04 (1,187)
IgG4	0.26 (1,64)	0.12 (1,245)

Asterix represent significance levels as follows: * $p < 0.05$; ** $p <= 0.01$; *** $p <= 0.001$.

DISCUSSION

Treatment of schistosomiasis with praziquantel results in changes in parasite specific humoral and cellular immune responses (e.g. Colley et al. 1986, Feldmeier et al. 1988, Grogan et al. 1996, 1998, Mutapi et al. 1998a,b, Ndhlovu et al. 1998) . This change is believed to be due to an increase in the amount of antigen exposed to the immune system by treatment (Mutapi 2001) and a removal of immunosuppressive effects of adult worm (Ottesen et al. 1978, Feldmeier et al. 1988, Grogan et al. 1998). Some of the changes occurring following treatment have been associated with resistance to infection/re-infection with schistosomiasis (Correa-Oliveira et al. 2000). This current study shows that treatment of children co-infected with *S. mansoni* and *S. haematobium* results in a significant change in the level of antibody responses directed against *S. mansoni* egg antigens. There was a significant decrease in levels of IgA, and a significant increase in levels of IgG2 and IgG3 in treated children when compared to untreated children. In addition while levels of IgG4 remain unchanged in treated children levels of the same isotype increases significantly in untreated children. Levels of IgM, IgG1 and IgE did not change significantly. Dunne et al. (1992a,b) have previously reported a lack of change in IgE levels directed against *S. mansoni* egg antigen following treatment (Dunne et al. 1992a,b). IgA levels have been shown to either remain unchanged in the case of *S. mansoni* infections (Gryzchl et al. 1993) or decrease for *S. haematobium* infections following treatment (Mutapi et al. 1998a/b). IgG1 and IgM responses have been shown to increase in *S. haematobium* infections (Mutapi et al. 1998a, Naus et al. 1998). IgG2 and IgG4 responses have been shown to both increase and decrease for *S. haematobium* infections (Grogan et al. 1996, Mutapi et al. 1998a, Naus et al. 1998). The causes of such heterogeneity in the treatment-induced changes in humoral responses include both host and parasite related factors (Mutapi 2001). There were changes in some isotypes, e.g. IgE, which were not related to treatment. This would be related to continued exposure to parasite antigens as a result of continued exposure to infective water or natural isotype switching.

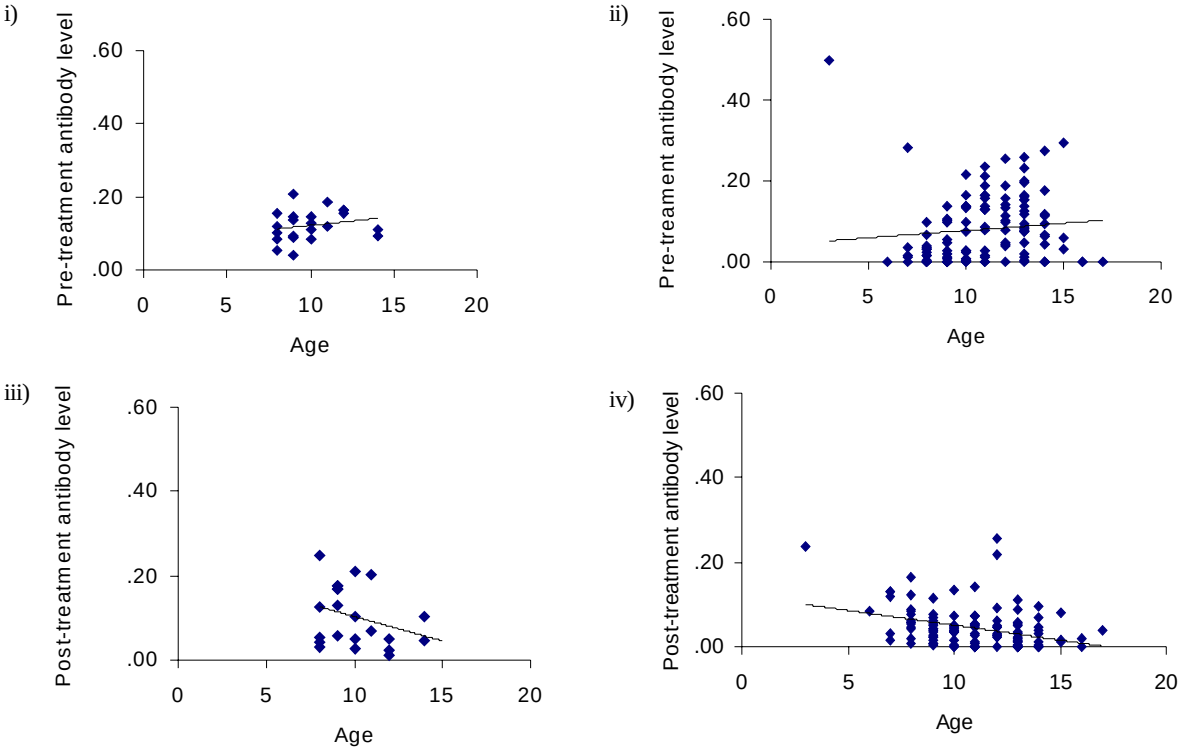

Fig. 3a: age-antibody relationship - IgE, where i) represents pre-treatment untreated control children; ii) represents pre-treatment praziquantel treated children; iii) represents post-treatment untreated control children and; iv) represents post-treatment praziquantel treated children.

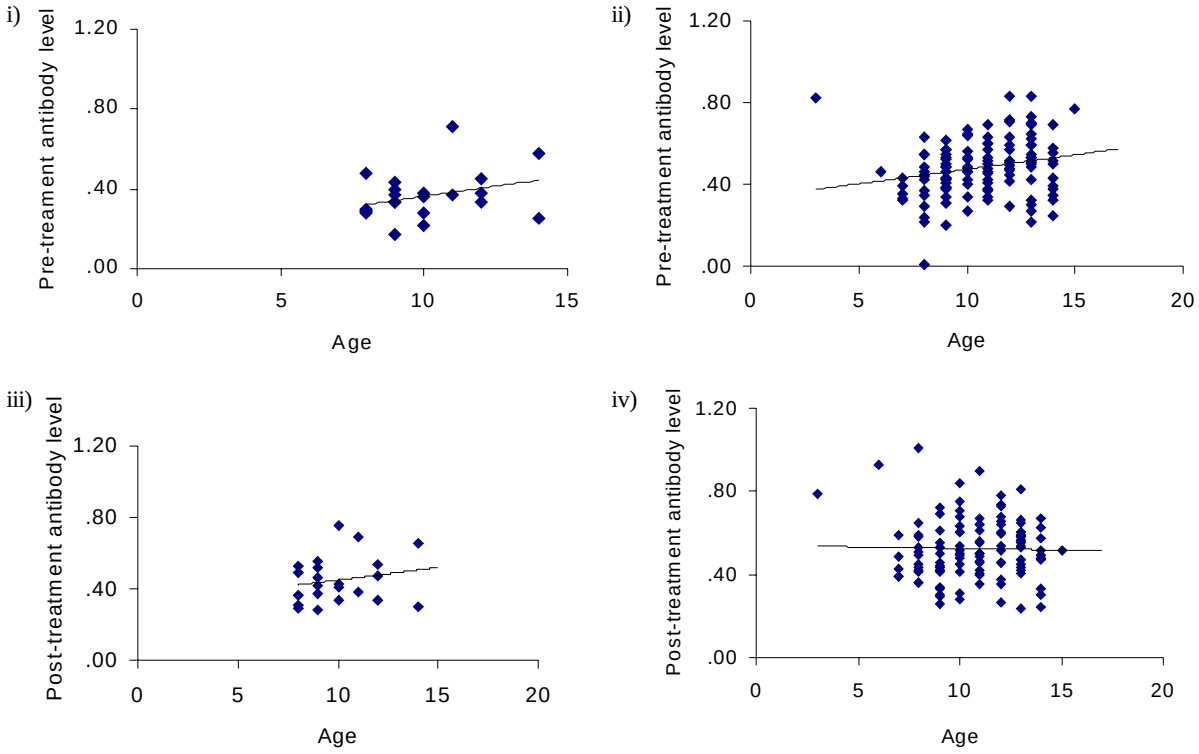

Fig. 3b: age-antibody relationship - IgM, where i) represents pre-treatment untreated control children; ii) represents pre-treatment praziquantel treated children; iii) represents post-treatment untreated control children and; iv) represents post-treatment praziquantel treated children.

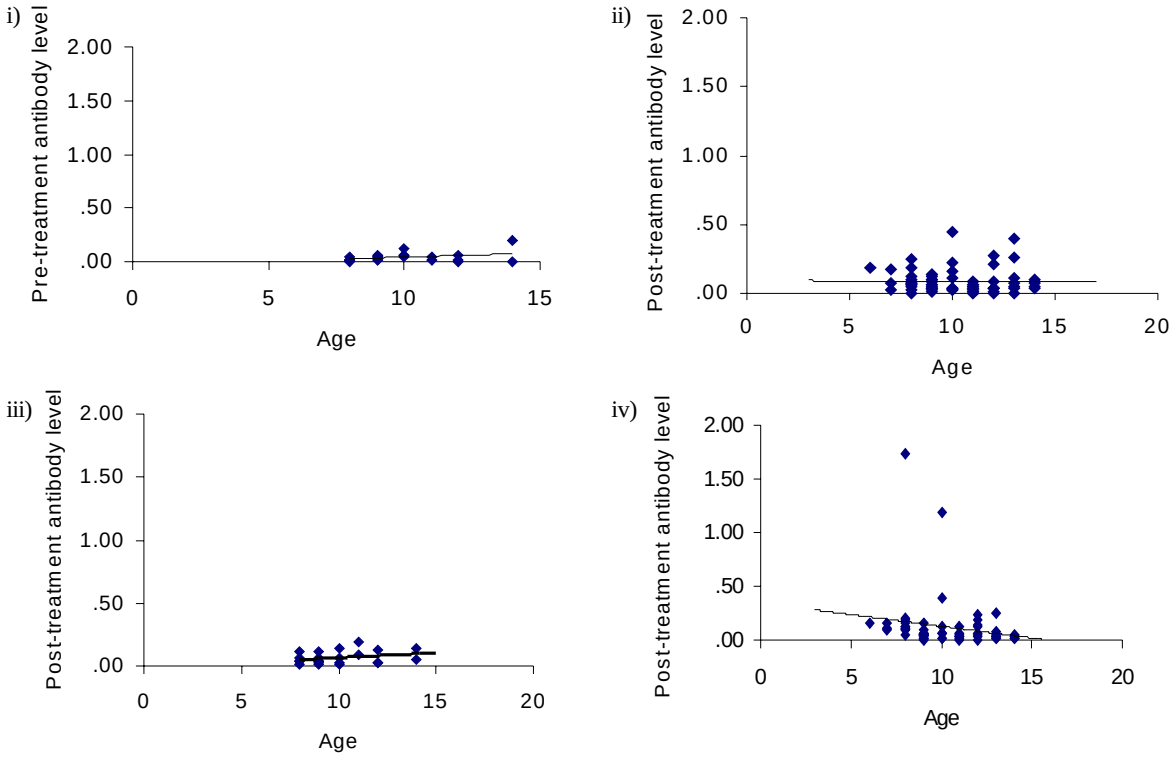

Fig. 3c: age-antibody relationship - IgG1, where i) represents pre-treatment untreated control children; ii) represents pre-treatment praziquantel treated children; iii) represents post-treatment untreated control children and; iv) represents post-treatment praziquantel treated children.

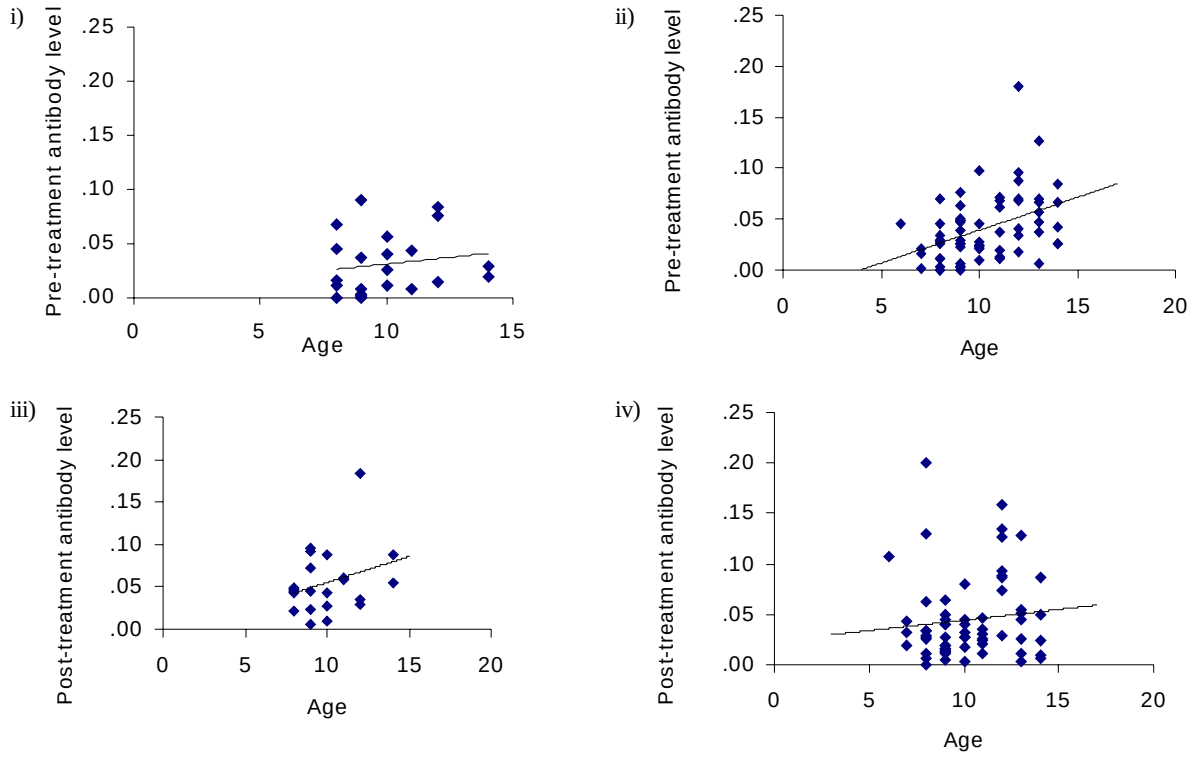

Fig. 3d: age-antibody relationship - IgG2, where i) represents pre-treatment untreated control children; ii) represents pre-treatment praziquantel treated children; iii) represents post-treatment untreated control children and; iv) represents post-treatment praziquantel treated children.

Both parasite species tended to affect the responses directed against the *S. mansoni* antigens separately, i.e. the only significant interactions were for IgE and IgM. The fact that an *S. haematobium* infection affects immune responses to *S. mansoni* antigens is largely due to shared/cross-reactive antigens between *S.mansoni* and *S. haematobium* for example the carbohydrate epitopes which induce the IgM response (Butterworth et al. 1988).

The study showed that treatment altered the relationship between the level of antibody produced and age for the isotypes IgE, IgM, IgG1 and IgG2. In all isotypes pre-treatment antibody levels increased with age. This is partly expected in this age-group where exposure to infective water also increases with age, so that the increase in antibody levels is attributed partly to increased exposure to parasite antigens (Woolhouse et al. 1991). However, other age-related processes such as hormonal influence on immune responses and innate susceptibility to infection/re-infection have also been implicated in giving rise to this observation (Gryseels 1994). Following treatment the positive relationship between isotype levels and age either decreased as occurred in IgM and IgG2 (Fig. 3) or became negative as occurred for IgE and IgG1. However these changes were not observed in untreated children as shown in Table III. This implies that the factors influencing this change in the relationship are not robust to treatment. The first factor, exposure to parasite antigens, is affected by treatment. As previously mentioned treatment results in an increase in the level of antigen available to the immune system. For example work in the mouse model has shown that praziquantel treatment induces the unmasking of the native glutathione-S-transferase enzyme, the leading antigen vaccine candidate for schistosomiasis enzyme, at the surface of the worms making it more accessible to the immune system (Dupre et al. 1999). The second factor, hormonal influence has been recently investigated. Sex hormones have been shown to affect immune response to glutathione-S-transferase (Remoue et al. 2000), however, to date, no studies have been shown of the effects of growth hormones (which would be related to age) on schistosome-specific responses. In addition, there have been no studies on the effects of treatment on any hormones. It is, however, unlikely that treatment alters the hormones for a prolonged enough period to allow the hormones to influence the immune responses and that the effects can still be observed six weeks after treatment. Indeed in a similar study of children infected with only *S. mansoni*, the effects of treatment on the age-antibody relationship were still apparent 18 weeks post treatment (submitted). Similarly the effects of treatment on innate susceptibility to infection/re-infection have not been studied and would not be expected to result in such a change in the age-antibody. Taken together this suggests that by altering the levels of antigens available to the immune system, treatment alters the age-antibody relationship in these children.

Interestingly these changes occurred in the isotypes which did not change significantly following treatment.

This means that while the overall mean antibody levels did not change, there were significant changes in individual antibody production. One possible explanation for this is that for the isotypes resulting in significant treatment-related changes following treatment, pre-treatment antibody levels were too low for any clear relationships with age to be observed. For example, only 58% of the children were producing IgG3; most of the children who produced this isotype produced very little before treatment.

In conclusion, this study confirms findings from previous studies that treatment alters immune responses. Second the study demonstrated that treatment alters the age-antibody relationship in children infected with *S. mansoni* and *S. haematobium* suggesting that this relationship is due, in part to the type and amount of antigens that the host's immune system has been exposed to.

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