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Immunity after Treatment of Human Schistosomiasis: Association between Cellular Responses and Resistance to Reinfection

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Previous studies have demonstrated the development of an age-dependent resistance to reinfection after chemotherapeutic cure of the helminthic parasite *Schistosoma mansoni*. Here we report on a longitudinal investigation of cell-mediated responses in infected individuals before and after treatment which was designed to outline those parameters important in mediating a protective response. A well-defined study group of 89 individuals with an age range of 9 to 35 years was selected from an area of high *S. mansoni* transmission in the Machakos district of Kenya. Peripheral blood mononuclear cell proliferation and cytokine production (interleukin-2 [IL-2], gamma interferon IL-5, IL-4, and tumor necrosis factor) in response to different crude life cycle-stage antigens of *S. mansoni* were assessed longitudinally in vitro before, 3 months after, and 1 year after treatment. Detailed statistical analyses of the results from this study have indicated a clear negative association between the proliferative responses to adult- and schistosomulum-stage antigens and subsequent reinfection intensity in older individuals (14 to 35 years) which was not present in the younger individuals (9 to 13 years). This association was significant even after the effects of age, sex, and exposure had been accounted for in multiple regression analyses. Cytokines were detected predominantly in response to adult worm and egg antigen extracts. An inverse association between the two cytokines gamma interferon and IL-5 was detected in response to all antigens at the three time points investigated, indicating cross-regulation in the production of these two mediators. Differences in antigen-specific cytokine levels between the two age groups were detected, with significantly higher IL-5 levels detected in the older (more resistant) age group. An inverse correlation between this cytokine and reinfection was detected but could not be dissociated from the effects of age and exposure in multiple regression analysis.

Over the last decade, evidence has accumulated that, following chemotherapeutic cure of *Schistosoma mansoni* or *S. haematobium* infection, older individuals display a resistance to reinfection in comparison to younger children (5, 6, 9, 55). This resistance has not been attributed to differences in exposure alone and has led to the suggestion of the development of an acquired immunity (48). Certain components, both humoral and cellular, of the immune response to human infection can be measured, but until recently it has proved difficult to associate resistance in the older age groups with any of these. However, in vitro studies have suggested a number of potential effector killing mechanisms involving both cells and antibody (3, 24, 29), directed primarily at the schistosomulum stage of the parasite. In addition, experimental models of infection and vaccination have outlined the importance of $CD4^+$ T cells in the induction of host responses to *S. mansoni* infection (16, 32, 43, 54). $CD4^+$ helper subsets in murine models (38) are defined by their cytokine profiles, Th1 producing gamma interferon (IFN-γ), interleukin-2 (IL-2), and lymphotoxin and Th2 producing IL-4, IL-5, and IL-10. There is evidence for the induction of similar Th1- and Th2-like $CD4^+$ cells in certain human diseases, with an additional subset, Th0, demonstrating an intermediate cytokine repertoire (reviewed in reference 47). Both Th1- and Th2-like cytokine responses have been identified in association with murine models of *S. mansoni* infection (reviewed in reference 20) in which Th1 mediators have been linked to protective responses in vaccination studies, whereas Th2 responses, induced following infection and the onset of egg laying, are thought to contribute to the development of pathology. However, the relative importance of these functionally distinct T-cell subsets and their contribution to egg-associated pathology or host protective immunity have yet to be established in other models of infection and in humans. Indeed, the hallmarks of human helminthic infection, B-cell activation, immunoglobulin E (IgE) production, and eosinophilia, are all Th2-mediated attributes, but an association of any of these parameters with protective immunity has proved difficult to demonstrate.

However, recent seroepidemiological data from studies of communities where human *S. haematobium* and *S. mansoni* infection is endemic have provided evidence for a relationship between raised IgE levels, specifically to adult worm (17, 27) or schistosomulum (44) antigens, and resistance to reinfection. As IgE production is regulated by cytokines derived from distinct T-helper subsets, such that IL-4 and IL-5 enhance and IFN-γ downregulates IgE production (34, 40, 41), further investigation of the spectra of cytokines generated in response to human *S. mansoni* infection seemed appropriate. In this context, a study has been designed to examine a number of immunological components of the response to reinfection in a well-defined cohort of highly exposed individuals in an area of endemicity in Kenya. For this purpose, rather than arbitrarily selecting a range of defined antigens and examining the clonal responses thus stimulated, a less reductionist and more holistic approach was deliberately adopted at this stage in an attempt to

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TABLE 1. Infection intensities in the two age groups, 9 to 13 and 14 to 35 years

Time of study	Sex of patients	n	9–13 yr (mean eggs per g)	n	14–35 yr (mean eggs per g)
Pretreatment	Females	25	2,159 (312)[a]	26	1,016 (190)[a]
	Males	18	1,128 (203)	14	1,387 (249)
	Total	43	1,727 (213)	40	1,146 (152)
Reinfection	Females	26	351[b] (45.7)[c]	25	104[b] (29.5)[c]
	Males	18	298 (203)	11	35 (10.6)
	Total	44	329 (32)	36	83.2 (21.3)

[a] Value in parentheses is standard error of the mean for pretreatment means.
[b] Cumulative reinfection score mean (7- to 21-month egg output).
[c] Value in parentheses is standard error of the mean for reinfection score means.

maximize the information obtained. The group of individuals was examined prior to treatment, and thereafter over a subsequent period of reinfection, for the capacity of their peripheral blood mononuclear cells (PBMCs) to respond in proliferative assays to crude antigen preparations from different life cycle stages of *S. mansoni*. In addition, the repertoire of cytokines IL-2, IFN-γ, IL-5, tumor necrosis factor (TNF), and IL-4 produced by these cells following antigenic challenge in vitro was assessed. This report presents an analysis of some of these cell-mediated immunological parameters and their association with age-dependent resistance among these individuals.

MATERIALS AND METHODS

Study group. A study group of 89 individuals, study I, from the community of Kitengei near Kambu in Machakos District, Kenya (25), was chosen on the basis of a preliminary stool examination in April 1989. Subjects aged between 9 and 40 years were selected from a group of households close to the Kambu river (high potential exposure) for high *S. mansoni* egg output. The oldest individual in the study group was 35 years old, and the group consisted of 56 females and 33 males. Forty-four individuals fell into the younger age group of 9 to 13 years, and 45 were in the older age group of 14 to 35 years.

Stool examination. The stool samples on which selection of individuals was based were not used in the subsequent analysis. Instead, in November 1989, three stool samples, from which duplicate Kato smears were prepared (49), provided pretreatment levels of infection (intensity), as shown in Table 1. In March 1990, all individuals within the group were clinically examined and received a treatment dose of 40 mg per kg (body weight) of praziquantel (Biltricide; Bayer AG, Leverkusen, Germany) per ml.

At 2 weeks and 4, 10, 14, and 21 months after treatment, follow-up stool examinations were carried out, five stools per individual and duplicate Kato smears per stool. For the purpose of analysis, an overall cumulative reinfection score (17) was calculated for each individual, on the basis of the 7- to 21-month mean egg output, with the score for each time point being weighted to avoid bias due to missing data. Results were analyzed by using the square root transformation of this score to normalize the data.

Water contact. Following selection, increased monitoring of the individuals' water contact commenced and was maintained throughout the study period. A water contact score for each individual was calculated from the frequency and duration of observed exposure, corrected for the type of activity, for use in the multiple regression analysis. A detailed report on the extensive water contact data gathered will be published elsewhere (25a).

Mononuclear cells and plasma. Blood samples (25 to 30 ml of venous blood extracted into heparin, 10 U/ml) were requested before treatment (bleed A) and at 3 (bleed B) and 12 (bleed C) months after treatment. Total cell counts were performed at bleeds B and C, and smears for differential counts were made at bleed C. Mononuclear cells were isolated from blood samples by density centrifugation on Histopaque 1077 (Sigma, Poole, United Kingdom), and the plasma was aliquoted and stored at −20°C. These PBMCs were washed three times in RPMI 1640 (ICN Flow Biomedicals, High Wycombe, United Kingdom), counted, and finally resuspended in Iscove's modification of Dulbecco's medium (IMDM) (ICN Flow) supplemented with 5% (final concentration), human AB serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 20 mM L-glutamine for lymphoproliferation assays and for the production of culture supernatants.

Antigens and mitogens. The concentration of antigen was optimized in preliminary experiments: soluble adult worm extract (SWA) and soluble egg extract (SEA) (17), each at a 10-μg/ml final concentration. Soluble schistosomulum preparation (SOM) was used at a 5-μg/ml final concentration, and concanavalin A (Sigma C5275) was used at 1 μg/ml. All antigens were diluted from the same batch, aliquoted, and stored at −20°C for use throughout the study period.

Cellular responses. (i) Lymphoproliferation. Aliquots of 100 μl of a PBMC suspension (1.5×10^6 per ml) from each individual were incubated in triplicate with 100 μl of antigen (stock concentration of antigen, 20 μg/ml; stock concentration of mitogen, 2 μg/ml) for 5 days at 37°C in 5% CO_2-air. Cell stimulation was monitored by an 18-h incorporation of tritiated [^{3}H]thymidine (Amersham, Little Chalfont, United Kingdom) overnight between days 5 and 6. Cells were harvested, and [^{3}H]thymidine incorporation was monitored in a Beckman scintillation counter. Results were analyzed as both mean counts per minute of triplicate wells and [(mean cpm of cells + antigen) − (mean cpm of cells + medium)] = δ cpm.

(ii) Cell supernatants. Replicate cultures of 1.5×10^6 cells were incubated with antigens (final concentration, 10 μg/ml) in a total volume of 1 ml. At 2 and 4 days, cell-free culture supernatants were collected following centrifugation, aliquoted, and stored at −20°C.

Cytokine analysis. (i) Functional assays. Using the two cytokine-dependent cell lines CTLL-2 (IL-2 dependent; American Type Collection Culture TIB 214) and B13 (IL-5 dependent [46]), the IL-2 and IL-5 activities present in the cell culture supernatants at both 48 h and 4 days were determined. These two cell lines were maintained exclusively on the recombinant human or murine cytokines (rhIL-2 and rmIL-5; Genzyme Corp.) and periodically checked for specificity. Recombinant cytokines (rhIL-2, rmIL-5, or rhIL-5; Genzyme) were used within each assay to determine a standard curve of activity. Briefly, 100 μl of indicator cells at 10^6 per ml (CTLL2) or 5×10^6 per ml (B13) was incubated with 100 μl of test sample or standard in 96-well, flat-bottom tissue culture plates at 37°C in 5% CO_2–air. Cytokine-dependent cell growth was monitored at 2 and 3 days, respectively, for CTLL-2 and B13 by incorpo-

ration of MTT (37) over 6 h. Cells were subsequently lysed by addition of 100 μl of 10% sodium dodecyl sulfate, and solubilized color release was measured at 540 nm in a Titertek enzyme-linked immunosorbent assay (ELISA) reader. The cytokine levels present in culture supernatants were determined by interpolation from the standard curve produced on each occasion. The sensitivity of the assay for the standards was reproducible down to values of 40 pg/ml for IL-2 and 5 ng/ml for IL-5.

The fibroblastic cell line L929 (CCL1, American Type Culture Collection) was used to detect TNF in supernatants in a rapid cytotoxicity assay (36). Aliquots of 100 μl of sample or standard were incubated with 2.5×10^5 L929 adherent cells in wells of a 96-well flat-bottom microtiter plate (final volume, 200 μl) in the presence of actinomycin D (2 μg/ml; Sigma A 4639). After 18 h, surviving cells were stained with 0.5% crystal violet in methanol. The plates were allowed to dry, and the stain was solubilized by the addition of 100 μl of 10% acetic acid solution. Color release was read spectrophometrically at 540 nm. Recombinant TNF-β (Genzyme) was titrated in a standard curve for each plate (the assay is nonspecific, however, for TNF-α or TNF-β). The sensitivity of this assay was ~70 pg/ml.

(ii) Immunological assays. A capture ELISA was used to measure IFN-γ in antigen-specific culture supernatants. Briefly, mouse anti-IFN-γ monoclonal antibody (Interferon Sciences, New Brunswick, N.J.) was used to coat Maxisorb (Nunc) plates in carbonate-bicarbonate coating buffer (pH 9.6) overnight at 4°C. Plates were then washed 10 times with phosphate-buffered saline (PBS)-Tween (PBS-Tween was used for all subsequent washes between incubation steps) and blocked with coating buffer containing 0.5% bovine serum albumin for 1 h at 37°C. Thereafter, samples or standards were added to appropriate wells and incubated for 2 h at 37°C. Recombinant human IFN-γ (1050494, Boehringer-Mannheim) diluted in RPMI 1640 supplemented with 5% human AB serum and 5% fetal calf serum, was titrated to form a standard curve. After washing, incubations of 1 h each were with polyclonal rabbit anti-human IFN-γ (1:800) (prepared in Cambridge University) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (1/5,000) (Dako Ltd., High Wycombe, United Kingdom). Finally, the substrate ABTS (catalog 50-62-00, Kirkegaard and Perry) revealed the presence of IFN-γ in culture supernatants. The color reaction was read at 405 nm in a Titertek ELISA reader. The sensitivity of this assay was reproducible at 5 pg/ml.

A commercial kit ELISA (Genzyme kit IT-4) which detects IL-4 in the 45- to 3,000-pg/ml range was used for the measurement of IL-4. Replicate wells and repeated assays were carried out for the majority of cytokine assays.

Statistical analysis. Relationships between the various immune responses and between each response and a number of epidemiological parameters (age in 1989, sex, pretreatment, and reinfection intensities) were initially examined by Spearman's rank correlation coefficients (all correlations given in the subsequent text are rank correlations). Relationships of particular interest were plotted and further analyzed by analysis of variance and multiple regression.

RESULTS

Intensity of infection before and after treatment. Individuals who presented with no detectable egg output in the definitive pretreatment November 1989 stool survey, in spite of giving positive output in the initial selection screen in April 1989, and individuals who did not receive treatment in March 1990 for reasons of "no show" or pregnancy have been excluded from the analysis (five individuals). In addition, complete data sets for each individual were not always achieved due to a limited number of peripheral blood cells being isolated from the blood sample taken. Each data set presented therefore details the number of individuals (n) used for the analysis.

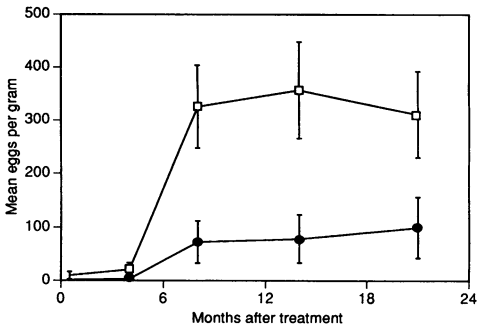

FIG. 1. Reinfection intensity (arithmetic mean eggs per gram) in months after treatment in the two age groups, 9 to 13 (□) and 14 to 35 (●) years. Bars represent 95% confidence intervals.

Following the main transmission period between July 1990 and February 1991 (4 to 11 months after treatment), the majority of individuals in study I became reinfected with *S. mansoni*. As in previous studies (17), the younger children (9 to 13 years) acquired much higher reinfection levels of intensity than the older individuals (14 to 35 years) (Fig. 1 and Table 1). This difference could not be accounted for solely by differences in the degree of exposure (data not shown; a detailed analysis of water contact and exposure to reinfection in this study area will be published elsewhere [25a]). In the younger age group, a significant positive correlation between the pretreatment and reinfection intensity of infection (correlation coefficient, $r = 0.328$; $n = 45$; $P < 0.02$) was observed. This was not the case for the older age group ($r = -0.040$; $n = 44$; not significant [NS]).

Proliferative responses. (i) Proliferative responses to different life cycle stages of *S. mansoni* antigens with time. In general, the highest proliferation responses were elicited by SWA preparations, with lower levels of response to the SOM and SEA preparations. These responses, however, were positively correlated within individuals at the two time points bleeds A and B. The pretreatment (bleed A) coefficients for the rank correlations between proliferative responses to SWA and SOM were $r = 0.637$, $n = 84$, and $P < 0.001$; those between SWA and SEA were $r = 0.476$, $n = 84$, and $P < 0.001$, and those between SOM and SEA were $r = 0.798$, $n = 84$, and $P < 0.001$. Three months after treatment (bleed B), the coefficients were as follows: between proliferation to SWA and SOM, $r = 0.855$, $n = 77$, and $P < 0.001$; between SWA and SEA, $r = 0.326$, $n = 77$, and $P < 0.01$; and between SOM and SEA, $r = 0.413$, $n = 77$, and $P < 0.001$.

Interestingly, at the final time point investigated, bleed C (1 year after treatment), the responses elicited with schistosomulum antigen were inversely related to those of both adult worm and egg such that correlations between different life cycle stages were as follows: between SWA and SOM, $r = -0.553$, $n = 74$, and $P < 0.001$; between SWA and SEA, $r = 0.738$, $n = 74$, and $P < 0.001$; and between SOM and SEA, $r = -0.484$, $n = 74$, and $P < 0.001$.

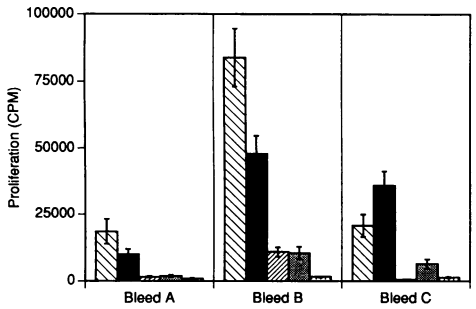

FIG. 2. Study group's mean (arithmetic) PBMC proliferation in response to the different life cycle-stage antigens of *S. mansoni* (SWA [▩, SOM [▨], and SEA (▧]), the mitogen concanavalin A (▧), and a medium control (□) at three time points during the study period; before treatment (bleed A), 3 months after treatment (bleed B), and 1 year after treatment (bleed C). Bars represent 95% confidence limits.

Bleed B (3 months after treatment) proliferative responses (Fig. 2) were markedly higher than those before treatment, an effect noted previously by other authors (2, 14). Treatment may have had a short-lived nonspecific effect on the immune responsiveness of individuals as reflected in the transiently increased response to the mitogen concanavalin A. This was in marked contrast, however, to the sustained antigen-specific response observed 3 months to 1 year after treatment directed at SWA.

(ii) Proliferative responses in relation to age and intensity of infection. When the correlation coefficients for the relationship between each individual's age and proliferative response to the different antigens, before treatment or 3 months and 1 year after treatment, were examined, no significant values were found (data not shown). This suggests that age does not directly affect the potential to respond to *S. mansoni* antigens. Figure 3 illustrates the mean proliferative response in the two age groups 9 to 13 years and 14 to 35 years at the three time points examined. Although the mean reinfection intensity in these two age groups differs markedly, this is not reflected in the mean antigen-specific proliferative responses.

Since no significant relationships between proliferation and age were detected, the data were reexamined focusing on proliferative responses and resistance to reinfection. The correlation coefficients of proliferative responses (δ cpm) to *S. mansoni* antigens at the different time points, and pretreatment or reinfection intensities, are given in Table 2. Negative correlations were detected between proliferative

TABLE 2. Spearman's rank correlation coefficients between PBMC proliferation to *S. mansoni* antigens and intensity of infection

Proliferation to:	Pretreatment intensity at bleed A (n = 84)[a]	Reinfection intensity	
		Bleed B (n = 77)[b]	Bleed C (n = 74)[c]
Concanavalin A	−0.098[d]	−0.017	−0.111
SWA	0.076	**−0.232**	0.126
SOM	0.156	−0.221	−0.006
SEA	0.088	0.025	0.098

[a] Bleed A = before treatment.
[b] Bleed B = 3 months after treatment.
[c] Bleed C = 1 year after treatment.
[d] Spearman's rank coefficient; values significant at $P < 0.05$ are in boldface.

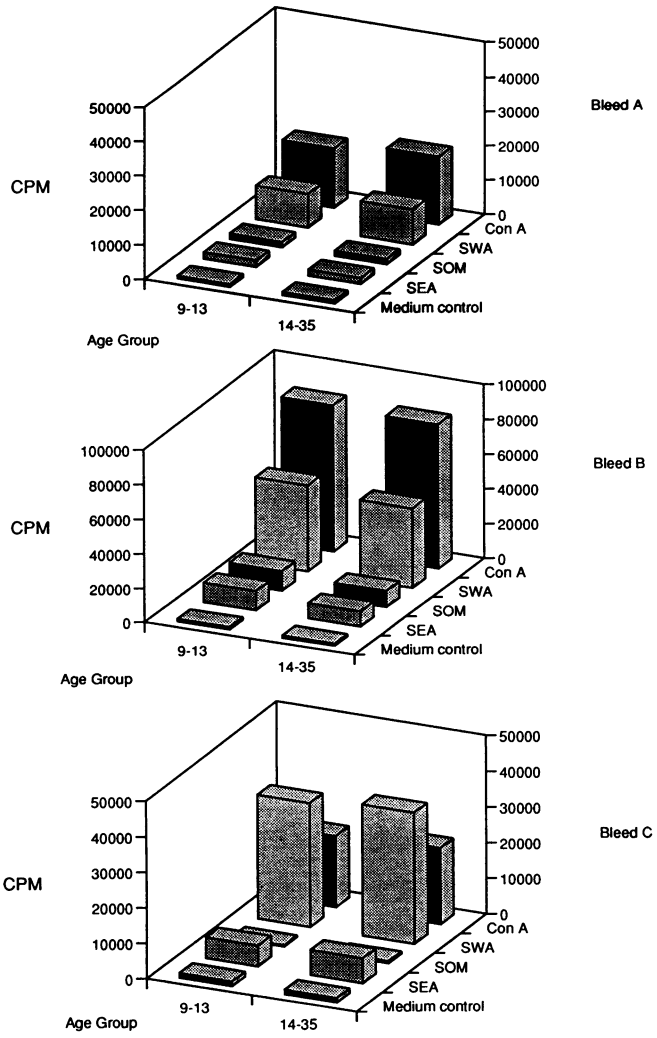

FIG. 3. Mean (arithmetic) proliferation (counts per minute) in the two different age groups, 9 to 13 and 14 to 35 years, in response to *S. mansoni* antigens, concanavalin A (Con A), and a medium control before treatment (bleed A), 3 months after treatment (bleed B), and 1 year after treatment (bleed C).

responses to both SWA and SOM with reinfection intensity at bleed B (3 months after treatment). The correlation coefficient was only significant, however, with SWA. This suggests that heightened proliferative responses to the adult and schistosomulum stages of the parasite after treatment but before transmission begins to occur are associated with low subsequent reinfection intensity. Interestingly, this correlation is lost when the 1 year after treatment (bleed C) time point is examined.

To investigate further the extent to which the relationship between proliferation to *S. mansoni* antigens and reinfection may be affected by age, the results were reexamined after having first stratified them into the two age groups, 9 to 13 and 14 to 35 years. As a result, a highly significant negative correlation between reinfection intensity and proliferation to SWA 3 months after treatment (bleed B) was found in the older age group (n = 35; r = −0.758; $P < 0.001$) which was absent in the younger age group (n = 42; r = −0.115; P = NS). This was also reflected in the association between reinfection intensity and responses to SOM, bleed B (9 to 13

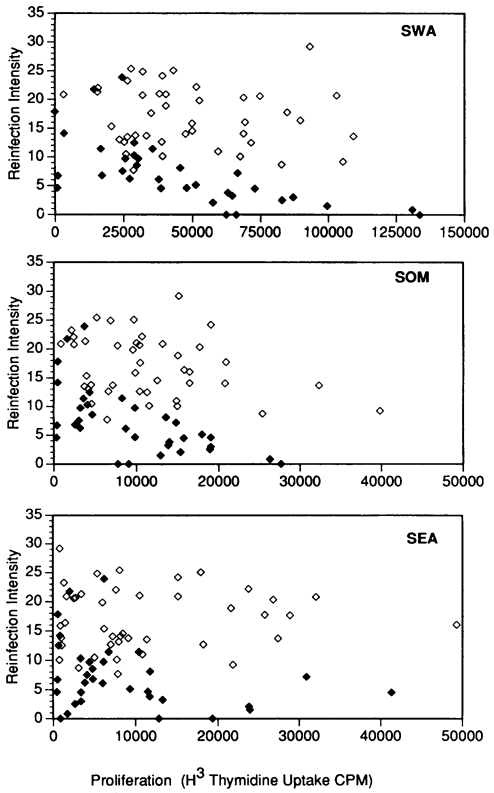

FIG. 4. Correlation between the proliferative response (δ cpm) to different *S. mansoni* antigens (SWA, SOM, and SEA) and reinfection intensity (expressed as the square root of the individual's reinfection score) in the two age groups, 9 to 13 (◇) and 14 to 35 (◆) years. Correlation in the 9 to 13 year olds (n = 42) gave rank coefficients for SWA (r = −0.115), SOM (r = −0.205), and SEA (r = −0.100); all values were nonsignificant. In the older age group (n = 35), rank coefficients were as follows: SWA (r = −0.758; P < 0.001); SOM (r = −0.644; P < 0.001); SEA (r = −0.270; P = NS).

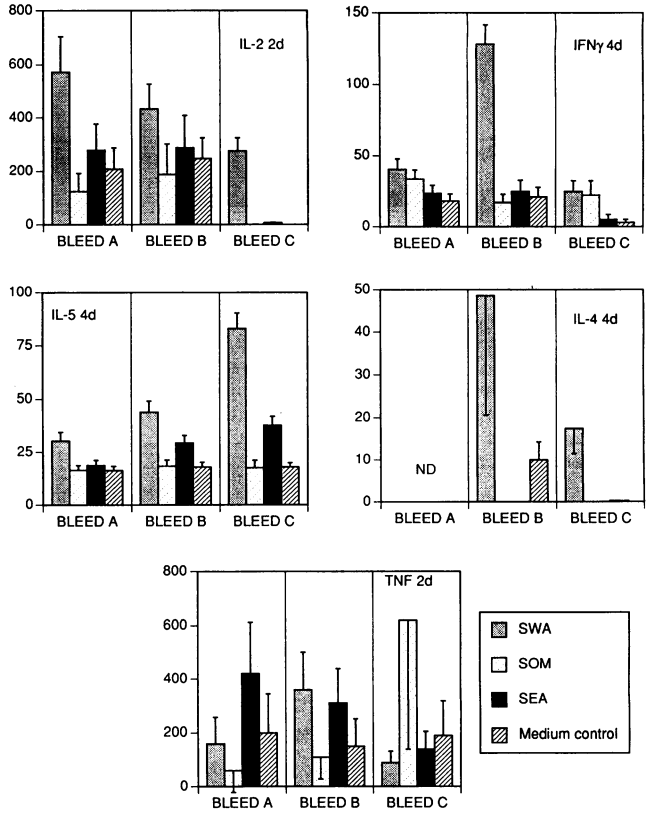

FIG. 5. Study group's mean (geometric) cytokine levels produced in PBMC culture supernatants in response to the different *S. mansoni* antigens SWA, SOM, and SEA and a medium control at the three different time points during the study. Vertical bars represent the standard error. Cytokine levels are in picograms per milliliter except those for IL-5, which are expressed in nanograms per milliliter.

years, n = 42, r = −0.205, and P = NS; 14 to 35 years, n = 35, r = −0.644, and P < 0.001), but was not present in response to SEA (9 to 13 years, n = 42, r = 0.100, and P = NS; 14 to 35 years, n = 35, r = −0.270, and P = NS) (Fig. 4).

Multiple regression analyses of the proliferation to SWA and SOM antigens in the older age group showed a significant effect on reinfection intensity after allowing for the effects of age, sex, and exposure, in a linear regression model, such that for proliferation to SWA, F value = 30.3 (P < 0.001), and for SOM, F = 23.14 (P < 0.001), both with 1/27 df. This effect was absent in the younger age group; for SWA, F = 0.03 (P = NS); for SOM, F = 1.46 (P = NS), both with 1/37 df. These results would suggest that among the older individuals there was a strong association between the ability of their cells to respond to the adult worm antigens and the development of an age-related resistance, which could be dissociated from the effects of age itself.

Cytokines. (i) Cytokines detected in response to *S. mansoni* antigens. When possible, each individual's supernatants from bleeds A, B, and C were stored and assayed together. Cytokine levels detected in repeat assays showed good correlations in general, although the absolute values declined in those supernatants which, by necessity, underwent repeat freeze-thaw cycles. The results were compiled as a large data base from which certain information has been selected for comment and analysis here.

Peak levels of IL-2 and TNF tended to occur in 2-day culture supernatants, in comparison to IFN-γ and IL-5, which peaked at 4 days. IL-4 was only measured in 4-day supernatants from SWA-stimulated cultures because of reagent constraints. Background levels of cytokine production (i.e., in the absence of exogenous antigen) were present in some PBMC culture supernatants, in particular, TNF, and have been incorporated into the data base. In specific instances, maximal antigen-specific levels of cytokines detected were in the order of 40 ng of IL-2, 220 ng of IL-5, 3.7 ng of IFN-γ, 5 ng of TNF, and 0.20 ng of IL-4 per ml.

The mean levels of cytokines produced in antigen-stimulated cultures at each of the three study time points, from all individuals assayed, are shown in Fig. 5. The highest levels of most cytokines were detected in PBMC cultures in response to SWA at each of the three time points examined. An interesting exception were the higher (although variable) levels of TNF produced in response to SEA antigen. Taking the study group as a whole, the mean levels of TNF production were not elevated significantly above background levels (cells plus medium) in response to any of the life cycle antigens in PBMC culture supernatants obtained at each of the three bleed time points (bleeds A, B, and C). In contrast, however, differences with time are seen for the

TABLE 3. Relationship between proliferation to *S. mansoni* adult worm or egg antigens and cytokine detection in PBMC culture supernatants

Cytokine (age of culture supernatant)	Bleed A[a]				Bleed B[b]				Bleed C[c]			
	n	SWA	*n*	SEA	*n*	SWA	*n*	SEA	*n*	SWA	*n*	SEA
IL-2 (2 days)	43	0.103[d]	36	0.019	40	0.125	29	0.084	52	**0.585**	43	**0.526**
IFN (4 days)	74	0.077	67	0.087	54	0.122	45	−0.100	69	0.092	61	0.166
IL-5 (4 days)	72	**0.360**	67	**0.243**	61	−0.008	58	0.139	68	**0.720**	57	**0.392**
TNF (2 days)	64	**0.280**	60	−0.197	64	0.080	51	0.229	48	−0.040	36	−0.016

[a] Bleed A = before treatment.
[b] Bleed B = 3 months after treatment.
[c] Bleed C = 1 year after treatment.
[d] Spearman's rank correlation coefficient; boldfaced values are significant at $P < 0.05$.

study group's mean IL-2, IFN-γ, and IL-5 release in response to the different antigens at different time points throughout the course of treatment and reinfection. Significant levels of IL-2 were observed in response to adult worm antigen, but not the other life cycle-stage antigens, before and 1 year after treatment but not at the 3-month after treatment time point (when reinfection had not commenced). In contrast, peak levels of IFN-γ were produced in response to SWA antigen at the 3-month time point which fell by the 1-year-posttreatment time point; for all other antigens, there was no IFN-γ production significantly above background levels. Significant levels of IL-5 were produced in response to both SWA and SEA at all three time points in the study period, and indeed the levels of IL-5 produced following treatment were higher than those before treatment. Unfortunately, the levels of IL-4 detected in response to SWA antigen were extremely variable and did not differ significantly from the mean background levels.

When associations between proliferation to antigen and cytokine release were examined (Table 3), there was a strong correlation before treatment and 1 year after treatment between proliferation and the IL-5 levels produced in response to SWA and SEA antigens. At the bleed B time point, however (that is, after treatment but before transmission of infection recommences), there was no correlation between in vitro proliferation and cytokine release.

In many individuals, both IFN-γ and IL-5 could be detected in culture supernatants from antigen-stimulated PBMCs. Negative correlation coefficients were found between the levels of IFN-γ and IL-5 detected in both 2- and 4-day culture supernatants in response to each of the antigens throughout the study period. By way of example, Table 4 shows the correlation coefficients for cytokine levels in response to SWA and SEA antigens 3 months after treatment. These data might indicate a reciprocal production of these two cytokines in response to antigen.

(ii) Cytokines, age, and reinfection. An extract from the correlation matrix for relationships among cytokine release, age, and reinfection is shown in Table 5 (adult and egg antigens at the three bleed time points). Very few of the correlations approached significance at the $P < 0.05$ level; however, a significant association between age and the level

TABLE 4. Correlation between the different cytokines detected in SWA and SEA antigen-stimulated PBMC culture supernatants

Antigen	Cytokine	Spearman's rank coefficient correlation (no. of individuals)[a]					
		IL-2, 2 days	IL-2, 4 days	IFN-γ, 2 days	IFN-γ, 4 days	IL-5, 2 days	IL-5, 4 days
SWA	IL-2, 4 days	**0.420** (*n* = 37)					
	IFN-γ, 2 days	0.057 (*n* = 37)	−0.055 (*n* = 36)				
	IFN-γ, 4 days	0.213 (*n* = 37)	0.098 (*n* = 36)	0.218 (*n* = 37)			
	IL-5, 2 days	−0.067 (*n* = 37)	**−0.399** (*n* = 36)	−0.253 (*n* = 37)	−0.068 (*n* = 37)		
	IL-5, 4 days	−0.297 (*n* = 37)	−0.195 (*n* = 36)	**−0.555** (*n* = 37)	−0.189 (*n* = 37)	**0.479** (*n* = 37)	
	IL-4, 4 days	0.062 (*n* = 37)	−0.017 (*n* = 36)	−0.174 (*n* = 37)	0.021 (*n* = 37)	0.113 (*n* = 37)	0.072 (*n* = 37)
SEA	IL-2, 4 days	**0.653** (*n* = 23)					
	IFN-γ, 2 days	−0.006 (*n* = 25)	0.083 (*n* = 23)				
	IFN-γ, 4 days	−0.189 (*n* = 25)	0.131 (*n* = 23)	0.240 (*n* = 43)			
	IL-5, 2 days	0.031 (*n* = 25)	0.092 (*n* = 23)	−0.190 (*n* = 35)	0.067 (*n* = 35)		
	IL-5, 4 days	−0.030 (*n* = 25)	−0.048 (*n* = 23)	**−0.445** (*n* = 34)	**−0.381** (*n* = 34)	**0.525** (*n* = 34)	

[a] Values in boldface are significant at $P < 0.05$.

TABLE 5. Correlations of antigen-stimulated cytokine production with age and reinfection

Bleed	Response to SWA	n	Age correlation	Reinfection score	Response to SEA	n	Age correlation	Reinfection score
A	IL-2, 2 days	43	0.292[a]	0.067	IL-2, 2 days	36	0.256	−0.125
	IFN-γ, 4 days	74	0.203	−0.041	IFN-γ, 4 days	67	0.115	0.007
	IL-5, 4 days	72	−0.017	0.032	IL-5, 4 days	67	0.024	−0.110
	TNF, 2 days	64	0.149	−0.271	TNF, 2 days	60	−0.004	−0.227
B	IL-2, 2 days	40	−0.155	−0.22	IL-2, 2 days	29	−0.189	0.044
	IFN-γ, 4 days	54	−0.226	0.241	IFN-γ, 4 days	45	0.001	−0.060
	IL-5, 4 days	61	0.157	−0.028	IL-5, 4 days	58	**0.378**	**−0.320**
	IL-4, 4 days	54	−0.092	0.239	IL-4, 4 days	ND[b]	ND	ND
	TNF, 2 days	64	−0.259	0.156	TNF, 2 days	51	−0.199	0.075
C	IL-2, 2 days	36	0.017	−0.097	IL-2, 2 days	43	0.248	−0.197
	IFN-γ, 4 days	69	0.059	−0.087	IFN-γ, 4 days	61	0.067	0.279
	IL-5, 4 days	68	−0.029	−0.026	IL-5, 4 days	57	0.170	0.098
	IL-4, 4 days	54	−0.092	0.239	IL-4, 4 days	ND	ND	ND
	TNF, 2 days	48	0.278	−0.102	TNF, 2 days	36	0.009	0.005

[a] Spearman's rank correlation coefficient; values significant at $P < 0.05$ are in boldface.
[b] ND, not done.

of IL-5 released in response to SEA ($r = 0.378$; $n = 43$; $P < 0.02$) 3 months after treatment was noted. In addition, when the data were examined further for associations between cytokine release and reinfection, only one correlate with reinfection, the amount of IL-5 detected in 4-day culture supernatants from cells stimulated with SEA, showed significance ($r = -0.320$; $n = 43$; $P < 0.05$). The data suggested, therefore, that higher levels of IL-5 were released by individuals of the older age group in response to SEA antigens; these were also those individuals who tended to demonstrate lower reinfection intensity. A repeat assay set, however, did not confirm this significant association, and additional caution should be taken with interpretation of these results since both IL-5 release and reinfection intensity are strongly associated with age (Fig. 6). Multiple regression analysis in this instance was unsuccessful in dissociating the three parameters, as the age effects were too strong to allow the detection of a residual effect of IL-5 on reinfection with the data available. It is hoped that current analysis of data from additional cytokine immunoassays will extend these results.

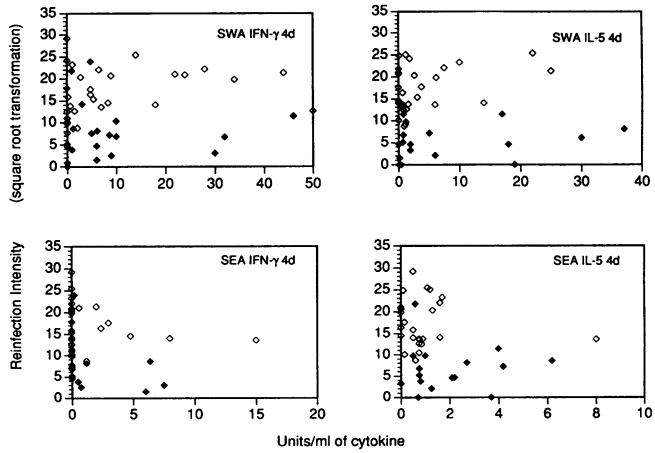

FIG. 6. Correlation between PBMC cytokine production (IL-5 and IFN-γ) in response to adult worm (SWA) and egg (SEA) antigens of *S. mansoni* and reinfection intensity 3 months after treatment in the two age groups, 9 to 13 (◇) and 14 to 35 (◆) years.

Interestingly, there was a significant correlation between the total number of circulating eosinophils observed at bleed C, i.e., 1 year after treatment and at a time when reinfection was occurring, and the levels of IL-5 detected in culture supernatants from PBMC cultured with egg antigen ($r = 0.412$; $n = 48$; $P < 0.01$). There was no significant association, however, between the detected eosinophil number and reinfection intensity ($r = 0.074$; $n = 74$).

DISCUSSION

In this latest investigation of human schistosomiasis, we again observed a clear age-dependent resistance to reinfection. This agreed with previous studies in this area of Kenya and provided a basis for the investigation of immunological parameters contributing to the resistance observed in older individuals.

As with previous studies (2, 14), proliferative responses peaked (3 months) after treatment, gradually declining over the following year, with the adult worm preparation eliciting the highest, and the egg preparation the lowest, antigen-stimulated cellular responses in vitro. Colley and his associates (14, 42) noted some differences in levels of cellular response to schistosome antigens by groups of non-reinfected and reinfected individuals, and recently de Jesus et al. (15) observed an association between antigen-specific responses and resistance to reinfection. The current study, however, included a sufficient number of individuals to allow segregation into relatively large groupings of younger and older individuals, allowing separate age group analysis. As transmission in this area is very high, it gave rise to differences in reinfection intensity even among the older (more resistant) individuals, so it was possible to detect clear negative correlations between reinfection intensity and proliferation in the older age group even after the effects of age, sex, and exposure had been taken into account in multiple regression analyses.

Our data indicated, moreover, that the intense reinfection that occurred in the younger age group (9 to 13 years) was not the consequence of an inability of their PBMCs to respond to *S. mansoni* antigens. Indeed, cells from individuals of all ages demonstrated substantial proliferative responses in vitro to the adult, schistosomulum, and egg

antigens. However, the continued susceptibility observed in the younger age group would suggest that the cellular responses induced were ineffective at mediating protection or were inhibited from doing so. In the latter context, the "blocking" of potential host protective responses is a hypothesis which has been proposed previously by our group for *S. mansoni* infection (9). Levels of IgM and IgG2 (4, 8) antibody isotypes elicited in response to *S. mansoni* egg-associated carbohydrate epitopes have been shown to correlate positively with susceptibility to reinfection in younger individuals, and it has been suggested that these antibodies may prevent recognition or binding of cross-reactive epitopes on the maturing schistosomulum (10). In a separate series of studies, Rihet et al. (44, 45) have detected an inhibition of protective IgE responses by antigen-specific competitive IgG4 in individuals less resistant to reinfection with *S. mansoni*, and in their seroepidemiological study (27), Hagan et al. have detected an association between high levels of IgG4 and increased likelihood of reinfection in *S. haematobium*. These observations have led to the suggestion that specific antigen-elicited isotypic responses contribute to the differential regulation of ADCC effector mechanisms and, thus, the expression of protective immunity.

Alternatively (or in addition), an inability to induce appropriate protective effector mechanisms in the younger age group might have arisen through the stimulation of differential responder (helper) cells. Whether this selective induction would have occurred as a result of antigen recognition or presentation events cannot be postulated at this stage. However, it is interesting to note that in the older age group resistance correlated to proliferative responses to adult and schistosomulum stages of the parasite but not to the egg stage. These results reflect those of the earlier IgE study (17), in which it was specifically anti-adult worm IgE levels which showed correlation with resistance. The *S. mansoni* preparations used for the current study were crude extracts, and it was considered that the three life cycle stages expressed partially distinct antigenic profiles, of which only certain components may have elicited appropriate protective responses. A more detailed investigation of responses to fractionated adult worm antigen is anticipated to ascertain the principal immunogenic components, including responses to a 22-kDa antigen initially identified on Western blots (immunoblots) of adult worm antigen, the recognition of which by individuals' IgE was associated with resistance to reinfection (17).

In this study, the use of a combination of crude antigen and unfractionated cell populations limits further deductions about the particular cell type producing each cytokine, and it leaves open whether raised levels of particular cytokines are the result of increased production from individual cells or the result of the expansion of a particular cell subset. However, the overall balance of cytokines determined in stimulated culture supernatants should reflect the general response to the antigen in question and indicate the type of effector response which might potentially be induced.

Differences in the levels of cytokines produced in response to *S. mansoni* antigens were detected at two levels. First, on an individual basis, there was an inverse relationship of the amounts of IL-2 and/or IFN-γ with IL-5 detected in antigen-stimulated cultures, compatible with the proposal that cytokines exert a cross-regulatory influence on mediator production (39). In murine models, the Th1 cytokine IFN-γ has been shown to downregulate expansion of Th2 cells (26), and conversely, Th2 cells have been observed to inhibit the synthesis of Th1 cytokines, predominantly via the mediator IL-10 (22, 39) and its effect on accessory cell function (22). Indeed, during *S. mansoni* infections mice mount an overwhelming Th2-like response after the onset of egg laying (53) to the extent that responsiveness to other antigens has been altered (33).

Surprisingly, in our study, we were unable to find any significant correlation between the levels of the Th2 mediators IL-5 and IL-4 produced in response to adult worm antigen. However, this may reflect the limited number of culture supernatants and/or the time point examined so far, and studies to examine all remaining culture supernatants for this cytokine are proceeding. Other authors investigating human infection with helminths have found significant correlation both in the levels of IL-5 and IL-4 secreted in 24-h mitogen-stimulated PBMC culture supernatants and in the frequency of cells producing these two cytokines in vitro (35).

In relation to the other cytokines examined, one interesting point to note is in the context of recently published results on TNF-α. In studies of schistosomiasis in SCID mice, Amiri and coauthors (1) have observed an important role for TNF-α in the induction of a granulomatous response to deposited eggs and, more intriguingly, TNF-α enhancement of adult worm fecundity and egg laying. Our results would suggest that following treatment there was an apparent decline in the in vitro TNF levels elicited, specifically in response to egg antigens. Extensive variation in the TNF levels induced, however, combined with high background levels of production deters the formulation of specific conclusions.

Second, our results indicated an association between the levels of IL-5 secreted in response to antigen with resistance to reinfection, such that the older, more resistant individuals produced higher levels of this Th2-type cytokine than the younger, more susceptible individuals; however, a dissociation could not be made for the effects of IL-5 as distinct from the effects of age itself on reinfection. If these results are confirmed, they would apparently contrast with findings in the murine model in which Th2 responses have been primarily associated with egg production and pathology (48) and host protective responses have been linked to the presence of a strong Th1 response (30, 48). Indeed, until recently, it was considered that in the majority of parasitic diseases Th1-type responses were prerequisite for protective immunity. There is, however, increasing evidence from different models of helminthic disease that Th2-type responses do play a role in host protection (21). BALB/c mice infected with the gut nematode *Heligmosomoides polygrus* display a resistance to oral challenge infection following antihelminthic drug treatment. This resistance was blocked by anti-IL-4 and anti-IL-4 receptor monoclonal antibodies; furthermore, recombinant IFN-γ exacerbated a primary infection with *H. polygrus* or with another nematode, *Nippostrongylus brasiliensis* (52). In addition, other authors (19) have shown a correlation between the ability to mount a Th2-type response to *Trichuris muris* (whip worm) infection and resistance to chronic infection in different mouse strains. Moreover, preliminary collaborative experiments with R. A. Grencis and K. Else have suggested that prior infection with *S. mansoni* can confer resistance to *T. muris* in genetically susceptible (normally Th1 responder) mice and that this resistance is accompanied by the induction of Th2-type responses (14a).

A variety of effector mechanisms may contribute to the observed resistance to reinfection following treatment, predominantly involving both cells and antibody in ADCC

reactions directed at the schistosomulum (3, 11, 12, 31). Although direct evidence for any particular mechanism is lacking, as the induction of different effector pathways is regulated by different cytokine-secreting cells, it was interesting to find an association between levels of the Th2-type cytokine IL-5 and resistance. This cytokine has been linked to the generation of eosinophilia (13), and in this study an association was detected between IL-5 levels produced in vitro to *S. mansoni* egg antigens 1 year after treatment and the number of circulating eosinophils detected at this time. There was no correlation, however, between the numbers of eosinophils at this time point and reinfection intensity. Previous studies have varied in the ability to show any association between circulating eosinophil levels and resistance to reinfection (9, 28, 50) even though these cells have been observed to mediate good killing of schistosomula in vitro (7, 51). One explanation for this is that measurement of circulating eosinophils per se does not reflect either the activation state of the cells or their tissue accumulation, both of which may be more relevant to effective resistance.

Th2 mediators in general have predominantly been associated with B-cell help and, particularly in the context of helminthic disease, the levels of IgE production. The measurement of the different antibody isotypes present in plasma from individuals of this study, at all three time points, is in progress.

In conclusion, we have found a number of cellular responses which correlate with the age-dependent resistance of older individuals to reinfection with the helminthic parasite *S. mansoni* which can be dissociated from the effects of age and exposure. These results are important not only in that they contribute to an understanding of the complex responses involved in the development of immunity in humans, but also, in light of current investigation of potentially protective recombinant antigens, in that they contribute in the context of their potential predictive value in delineating the rationale for a vaccine against schistosomiasis.

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