

Amelia M. Ribeiro de Jesus<sup>▲</sup>,  
 Roque P. Almeida<sup>▲</sup>,  
 Olivia Bacellar<sup>▲</sup>,  
 Maria Ilma Araujo<sup>▲</sup>,  
 Christian Demeure<sup>●</sup>,  
 Jose Carlos Bina<sup>▲</sup>,  
 Alain J. Dessein and  
 Edgar M. Carvalho<sup>▲</sup>

Division of Immunology,  
 Department of Medicine University  
 of Bahia<sup>▲</sup>, Salvador, Centre  
 D'Immunologie de Marseille –  
 Luminy<sup>●</sup>, Marseille

## Correlation between cell-mediated immunity and degree of infection in subjects living in an endemic area of schistosomiasis\*

Cell-mediated immunity to *Schistosoma mansoni* antigens, unrelated antigens and mitogens was evaluated in 50 subjects with the same degree of exposure to infection living in an endemic area of schistosomiasis. The degree of infection, assessed by the number of eggs/g of stool, was variable in this population (0–5604), suggesting differences in susceptibility to infection. Absence of lymphoproliferative response was observed in 56% of this group, despite having a response to purified protein derivative of tuberculin (PPD) and tetanus toxoid (TT) antigens and to pokeweed mitogen. The 50 subjects were divided into two groups, according to their degree of infection. The lymphoproliferative responses to schistosomula and adult worm antigens in the group with a low degree of infection (<400 eggs/g of stool) were higher than the ones documented in patients with a high degree of infection (>400 eggs/g of stool), and these differences were statistically significant ( $p < 0.001$ ). An inverse correlation between the lymphocyte proliferation in response to *S. mansoni* antigens and the degree of infection was also observed ( $p = 0.02$ ), indicating that subjects with a lower degree of infection have a higher lymphoproliferative response to schistosomula and adult worm antigens. No differences in the lymphocyte reactivity to other antigens (PPD and TT) were detected in these groups. An impairment of interferon- $\gamma$  *in vitro* production was observed when the lymphocytes from these subjects were stimulated with *S. mansoni* adult worm antigen, although they produced gamma interferon in response to phytohemagglutinin.

### 1 Introduction

*Schistosomiasis mansoni* is a major health problem for many tropical countries where hyperendemic areas are frequently documented [1, 2]. This parasitic disease has been controlled in selected endemic areas by programs combining chemotherapy and molluscicides [3–5]. However, these measures cannot be used on a large scale due to their high cost, the requirement for repeated treatments and trained personnel, and the risk of selecting for resistant parasite and snail populations [6–10].

An alternative to the conventional forms of control are measures which increase host resistance. Immune mechanisms effective against *Schistosoma mansoni* have been documented in experimental animals and in *in vitro* studies with human cells [11–16]. In addition, epidemiological studies have shown that susceptibility to infection is

variable in subjects from endemic areas, and evidence of an acquired immunity and consequently partial resistance to re-infection has been demonstrated [17–19]. Antibody-mediated killing of *S. mansoni* has been well documented in experimental systems [11, 20–26] and also in sera of patients with schistosomiasis [13, 14, 17–19]. Although cellular immune mechanisms have been well studied in experimental systems [24, 26–32] and infected subjects [33, 34] very little is known about the relationship between the specific cellular immune response to *S. mansoni* antigens and the degree of infection. In the present study, the cell-mediated immune response to schistosomula and adult worm antigens was evaluated in subjects, with comparable degree of water contact, living in an endemic area. The aim of this study was to evaluate if there is a correlation between immune response and partial protection against infection. The results show that, in this population, the lymphoproliferative responses to schistosomula and adult worm antigens are inversely correlated with the degree of infection.

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**Correspondence:** Edgar M. Carvalho, Hospital Universitário Prof. Edgard Santos, Rua João das Botas s/n, Laboratório de Imunologia, 5º andar, Salvador, Bahia, Brazil. CEP 40140.

**Abbreviations:** TT: Tetanus toxoid SI: Stimulation index

**Key words:** Cell-mediated immunity / Degree of infection / Schistosomiasis

### 2 Materials and methods

#### 2.1 Endemic area

Caatinga do Moura is a village located on the banks of a river in Bahia, a northeastern state of Brazil. It is one of the most important endemic areas of schistosomiasis in Brazil [19, 35] with a prevalence of up to 95% in the human population before the beginning of control programs in 1963 [35]. In spite of the use of molluscicide and mass chemotherapy, *S. mansoni* infection is widespread in this area.

## 2.2 Study subjects

In this study, subjects were selected from 49 families living within 200 meters of the river, and were followed from 1982 to 1989. The methodology used for selecting the study population, as well as the measures for controlling the disease carried out at the beginning of the study, have been previously published [19]. Briefly, the subjects were given an initial clinical and parasitological examination and were treated orally with Oxamniquine (15–25 mg/kg). They had different degrees of water contact and were therefore classified into three groups: a group of low water contact (less than 1 h per week), intermediate water contact (1 to 3 h once or twice a week) and high water contact (1 to 3 h per day). In the present study, only people from the group of high water contact were selected. In this way, both the opportunity to be infected and the exposure to parasite antigens, factors that could influence the immune response against *S. mansoni*, were standardized.

Fifty subjects (14 males and 36 females) were selected from patients in the high water contact group. Informed consent was obtained from the studied subjects or their parents or guardians. For the present study, clinical and parasitological evaluations were performed twice: once in 1986, before administration of chemotherapy with Oxamniquine and again in 1989, at the time of the first immunological evaluation. Their ages ranged from 5 to 44 years ( $15 \pm 9.6$ ): 15 (0–10 years old), 26 (11–20), 5 (21–30) and 4 (> 31).

They had different clinical forms of schistosomiasis but were otherwise apparently healthy. The group received a second treatment in 1989 together with the rest of the population, because some of them had high levels of infection.

A control group was formed with students and hospital employees who were free of parasitic infection or were infected with other parasites such as *Ascaris lumbricoides*, *Entamoeba histolytica* and *Tricocephalus trichiurus*.

## 2.3 Parasitological methods

Parasitological examinations were performed periodically using Kato-Katz's method [36, 37]. Three to five stool samples were collected from each subject on different days. The results represent the arithmetic mean of the number of eggs obtained at two time points, 1986 and 1989. Kato-Katz is the quantitative method of choice to measure degree of infection and has been used extensively in epidemiological studies due to its simplicity and reproducibility when three to five parasitological examinations are performed on different days [38–42]. The infectivity of the water in this endemic area was documented using mice [19].

## 2.4 Antigens

*S. mansoni* soluble extracts of adult worm and schistosomula were prepared as previously described [19]. Briefly, adult worms of the Puerto Rico strain of *S. mansoni* were sonicated and after centrifugation ( $100,000 \times g$ ) for 45 min, the supernatants were collected. The protein concentration

was measured by Lowry's method [43] and then stored at  $-20^\circ\text{C}$ . Cercariae were obtained from a colony maintained at the Centre d'Immunologie de Marseille – Luminy, Marseille, France. The schistosomula were prepared by Ramalho Pinto's method as modified by Lazdins *et al.* [44], and then diluted in saline and sonicated following the technique described for the adult worm antigen.

## 2.5 Immunological procedures

The cellular immune response was evaluated from December 1988 to July 1989 and this evaluation was repeated beginning 60 days after another drug treatment in September 1989.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient centrifugation using Ficoll-Hypaque (Pharmacia AB, Uppsala, Sweden).

After separation, the PBMC were collected from the interface and washed twice in sterile saline (0.9% NaCl) [45, 46]. For determination of T cell surface markers, PBMC were depleted of adherent cells and the lymphocytes were adjusted to a concentration of  $10^6$  cells/ml. Aliquots of 200  $\mu\text{l}$  were then incubated for 30 min at  $4^\circ\text{C}$  with monoclonal antibodies against T cell surface markers (Ortho Diagnostic System Inc., Raitan, NY), followed by addition of goat anti-mouse fluoresceinated IgG (Sigma, St. Louis, MO). The results were expressed as the percentage of positive cells [45, 47].

Lymphocyte proliferative tests were performed as previously described [45, 46]. Briefly, PBMC were adjusted to a concentration of  $10^6$  cells/ml in RPMI supplemented with 15% AB<sup>+</sup> heat-inactivated serum and aliquots of  $2 \times 10^5$  cells were then cultivated in triplicate in tissue microculture plates (Limbro Chemical Company, New Haven, CT). The cells were stimulated with PWM 1:100 (Gibco), PPD 10  $\mu\text{g/ml}$  (Connaught Laboratories, Ontario, Canada), TT 1 LF/ml (Wyeth-Ayerst Lab, Marietta, PA) and *S. mansoni* adult worm and schistosomula soluble extracts in concentrations of 0.01, 0.1, 1 and 10  $\mu\text{g/ml}$  of culture. The PPD and TT were chosen as control antigens because BCG and TT vaccinations are mandatory in Brazil. After 5 days of incubation ( $37^\circ\text{C}$ , in 5%  $\text{CO}_2$ ) 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine (6.7 Ci/mM, New England Nuclear, Boston, MA) was added to the cultures for 4.5 h. The cultures were then harvested and processed for scintillation counting. Lymphocyte blastogenic data is given as the mean of cpm of triplicate cultures  $\pm$  SE or as the mean of the stimulation index (cpm of the stimulated culture divided by the cpm of the unstimulated culture)  $\pm$  SD.

Determination of (IFN- $\gamma$  *in vitro* production was performed in supernatants of cultures. Briefly, PBMC ( $3 \times 10^6$ ) from eight subjects were stimulated with PHA (final dilution 1:10) and adult worm antigen, and five of them were also tested with schistosomula antigen, in concentrations ranging from 2 to 20  $\mu\text{g/ml}$ . The supernatants were collected after 24 to 48 h and the levels of IFN- $\gamma$  were determined by ELISA using commercial reagents (Genzyme Corp., Cambridge, MA). A standard curve was used to express the results in pg/ml [45].

## 2.6 Data analysis

The results were analyzed using the Wilcoxon Rank sum test and the Kendall Rank correlation test. These statistical methods are indicated in the figure legends.

## 3 Results

### 3.1 Degree of infection

Despite having the same degree of water contact, the 50 subjects of the present study had quite different degrees of infection. In 1986, the number of eggs per gram of stool ranged from 0 to 5604. Three years later, the subjects had a lower degree of infection, though the range (0–1804 eggs/g of stool) was still quite variable. A direct correlation was observed between the number of eggs per subject in 1986 and the number of eggs per subject in 1989 ( $p = 0.004$ ) (Fig. 1). Therefore, each subject tended to retain a similar level of infection relative to the levels of the other subjects within the group.

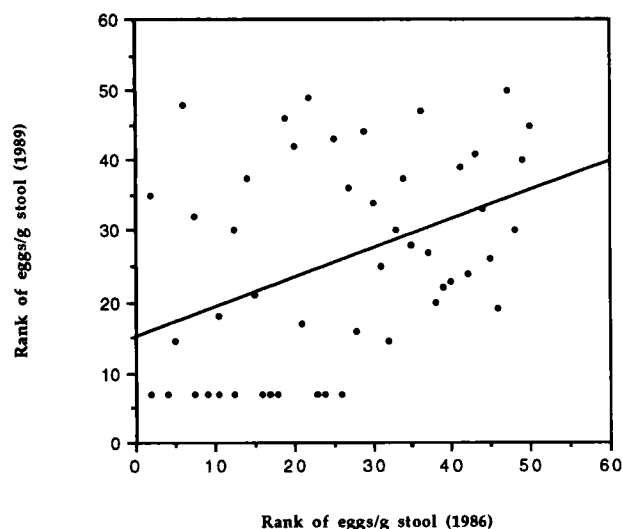


Figure 1. Direct correlation between the number of eggs/g of stool at two time points, in 1986 and in the period of immunological evaluation (1989). Kendall Rank correlation:  $p = 0.004$ .

### 3.2 Lymphoproliferative response

In preliminary experiments, both antigens (schistosomula and adult worm) had a similar stimulatory effect on lymphocytes from subjects infected by *S. mansoni*. Hence, schistosomula antigen was chosen to evaluate the best concentration able to produce a specific lymphocyte proliferative response. A dose-response curve with this antigen (0.01, 0.1, 1 and 10  $\mu\text{g/ml}$ ) was performed in 14 subjects from the study group with a high response to these antigens and 7 uninfected controls. A nonspecific response was observed with a concentration of 10  $\mu\text{g/ml}$  and therefore 0.01, 0.1 and 1  $\mu\text{g/ml}$  were chosen for use in further experiments. The results shown here represent the highest response obtained for each patient, usually achieved with a concentration of 1  $\mu\text{g/ml}$  of antigen.

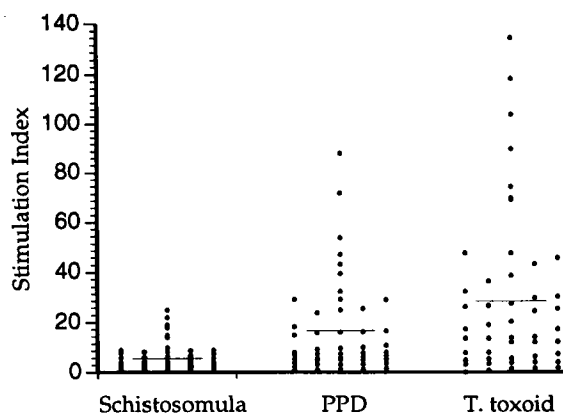


Figure 2. Lymphocyte reactivity, expressed as stimulation index (SI), to schistosomula, PPD and tetanus toxoid in all 50 studied subjects from an endemic area of schistosomiasis.

The lymphocyte reactivity to schistosomula antigen, PPD and TT of the 50 schistosomiasis subjects analyzed, expressed as stimulation index (SI), is shown in Fig. 2. The [ $^3\text{H}$ ]thymidine uptake of cultures from the 50 subjects, stimulated by schistosomula antigen, ranged from 772 to 41 319 cpm and the mean of the stimulation indices (SI) was  $6 \pm 5.6$ . A similar response (not shown) to adult worm antigen was observed, ranging from 541 to 110 299 cpm. There was a positive correlation between the lymphoproliferative responses to these two antigens ( $p = 0.001$ ). The 50 subjects response to PPD ranged from 2233 to 101 426 cpm (mean of SI =  $15 \pm 15.4$ ) and to TT from 140 to 65 371 cpm (mean of SI =  $28 \pm 31.9$ ). A poor lymphoproliferative response to schistosomula antigen was observed in 28 of the 50 patients (56%). Poor responses to schistosomula and adult worm antigens were considered when the SI was  $< 5$ . This value corresponds to the mean + 2 SD of the SI of similar cultures from healthy subjects. In contrast, a significant lymphoproliferative response to PPD and TT was observed in these 28 patients,  $12 018 \pm 2056$  and  $27 728 \pm 4956$  cpm, respectively and these responses were not statistically different from similarly stimulated cultures from the 22 subjects who did respond (SI  $\geq 5$ ) to *S. mansoni* antigens ( $p > 0.1$ ). All except 2 subjects had a lymphoproliferative response to PPD and/or TT. All the subjects had a lymphoproliferative response to PWM (mean =  $39 752 \pm 3893$  cpm).

The majority of subjects had the intestinal (31 patients) or hepatointestinal (17 patients) form of schistosomiasis and only 2 patients had the hepatosplenic form of the disease. There were no statistically significant differences in the lymphoproliferative responses among these different clinical forms of the disease.

Epidemiological and clinical studies have considered an infestation to be high when the number of eggs in the stool is greater than 400 eggs/g of stool [40, 41]. Twenty-four of the 50 subjects from the present study eliminated less than 400 eggs/g of stool and 26 eliminated more than 400 eggs/g of stool in the first parasitological examination at the beginning of the study. Table 1 shows a comparison of the mean age, the lymphoproliferative response to schistosomula, adult worm, PPD and TT antigens, and the percentages of CD11-, CD4- and CD8-positive cells in these 50

**Table 1.** Cellular immune response of patients infected by *S. mansoni* classified according to the degree of infection

| Variables<br>(mean $\pm$ SD) | Classification of patients<br>according to the number<br>of eggs/g of stool |                 | "p" <sup>a</sup> |
|------------------------------|-----------------------------------------------------------------------------|-----------------|------------------|
|                              | < 400<br>n = 24                                                             | > 400<br>n = 26 |                  |
| Age                          | 19 $\pm$ 11.6                                                               | 13 $\pm$ 4.7    | 0.005            |
| Schistosomula Ag (SI)        | 9 $\pm$ 6.1                                                                 | 4 $\pm$ 4.5     | 0.001            |
| Adult worm Ag (SI)           | 13 $\pm$ 8.9                                                                | 6 $\pm$ 5.4     | 0.001            |
| PPD (SI)                     | 18 $\pm$ 17.7                                                               | 12 $\pm$ 12.9   | 0.2              |
| T.toxoid (SI)                | 26 $\pm$ 36.9                                                               | 29 $\pm$ 27.5   | 0.7              |
| Tcell (%)                    | 84 $\pm$ 6.6                                                                | 79 $\pm$ 8.3    | 0.5              |
| CD4 (%)                      | 56 $\pm$ 7.3                                                                | 54 $\pm$ 8.8    | 0.5              |
| CD8 (%)                      | 28 $\pm$ 6.5                                                                | 31 $\pm$ 8.4    | 0.5              |

## a) Wilcoxon Rank Sum Test.

Similar immunological evaluations were performed on 10 healthy subjects not infected by *S. mansoni*. The SI to schistosomula and adult worm antigens were  $2 \pm 1.6$  and  $2 \pm 0.9$ , respectively and to PPD and TT antigens  $21 \pm 18$  and  $16 \pm 13$ . The percentage of total T cells, CD4<sup>+</sup> and CD8<sup>+</sup> cells in this control group was  $72 \pm 8\%$ ,  $54 \pm 8\%$  and  $24 \pm 6\%$ , respectively. No statistically significant difference ( $p > 0.05$ ) was observed when the lymphoproliferative response to PPD and TT as well as the percentage of T cell markers were compared with that of the schistosomiasis patients.

subjects classified according to the number of eggs per gram of stool. The mean age of the group of subjects with less than 400 eggs/g of stool was higher ( $19 \pm 11.6$  years) than the group of patients with more than 400 eggs/g of stool ( $13 \pm 4.7$  years,  $p = 0.005$ ). The SI of the lymphoproliferative responses to schistosomula and adult worm antigens in patients with a low degree of infection were  $9 \pm 6.1$  and  $13 \pm 8.9$ , respectively. These values were higher ( $p < 0.001$ ) than those observed in subjects with more than 400 eggs/g of stool ( $4.3 \pm 4.5$  and  $6 \pm 5.4$ , respectively). No statistically significant differences were found when the proliferative responses to PPD and TT were compared between these groups ( $p = 0.2$  and  $0.7$ , respectively). In addition, no differences were observed in the percentages of T cells,

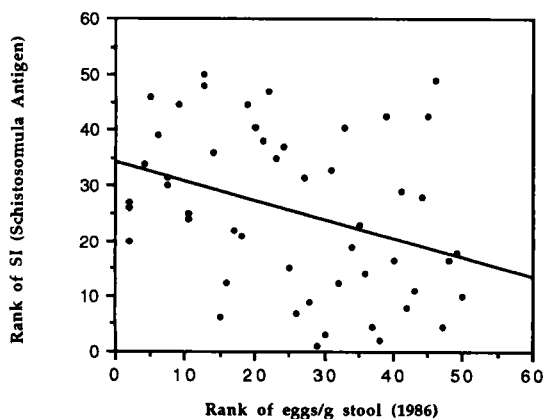


**Figure 3.** Inverse correlation between the stimulation index of the lymphoproliferative response to schistosomula antigen and the degree of infection (number of eggs/g of stool) in schistosomiasis patients. Kendall Rank correlation:  $p = 0.02$ .

CD4- and CD8-positive cells. An inverse correlation was observed between the SI and the number of eggs per gram of stool in 1986 from these 50 schistosomiasis subjects ( $p = 0.02$ , Fig. 3). When the number of eggs from the 1989 evaluation were analyzed in the same way a slight negative correlation was observed. However, this correlation was not statistically significant.

### 3.3 *In vitro* production of IFN- $\gamma$

IFN- $\gamma$  levels were determined in supernatants of lymphocyte cultures from eight patients of the studied population. Five of them with a significant lymphocyte blastogenesis to *S. mansoni* antigens (SI ranged from 16 to 22) and less than 400 eggs/g of stool plus three patients who did not respond to these antigens in the blastogenic assay and had more than 400 eggs/g of stool (Fig. 4). The mean IFN- $\gamma$  level in cultures from all eight subjects stimulated with PHA was  $169 \pm 106$  pg/ml with values ranging from 31 to 306 pg/ml. In cultures stimulated with *S. mansoni* adult worm antigen, the IFN- $\gamma$  levels were undetectable or low in both groups with values ranging from 0 to 20 pg/ml ( $4 \pm 6.8$ ). Five of these subjects were also tested with schistosomula antigen and the same results were observed. Additionally, three subjects cured of schistosomiasis and living outside the endemic area were also tested and showed a significant production of IFN- $\gamma$  (mean =  $112 \pm 26$  pg/ml) in supernatants of cultures stimulated with *S. mansoni* antigen.

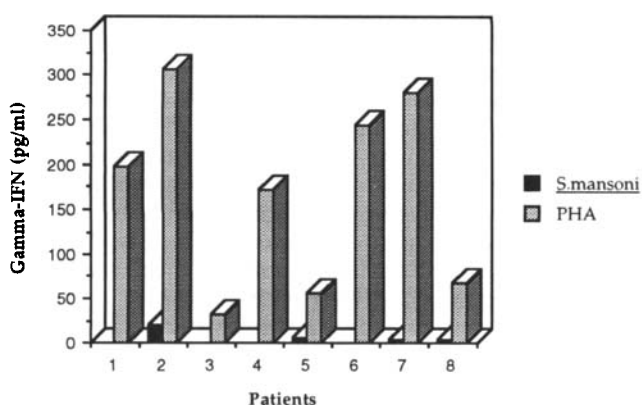


**Figure 4.** IFN- $\gamma$  levels (pg/ml) in supernatants of lymphocyte cultures stimulated *in vitro* with PHA and *S. mansoni* adult worm antigen from eight schistosomiasis patients. Three patients (1–3), had a blastogenic response to *S. mansoni* antigens ranging from 1.4–2.1 (SI) and five of them, patients 4–8, had SI ranging from 13.7–30.3.

### 3.4 Effect of treatment with Oxamniquine on the cellular immune response

Due to reinfection, the subjects were retreated with Oxamniquine in 1989 and a second evaluation of the lymphoproliferative response was performed beginning 60 days after this treatment, for 39 of the 50 patients. The mean SI in response to schistosomula and adult worm antigens after therapy was  $7 \pm 5.5$  and  $12 \pm 10.4$ , respectively. These responses did not differ from those observed in the first evaluation ( $p = 0.8$  and  $p = 0.9$ ).

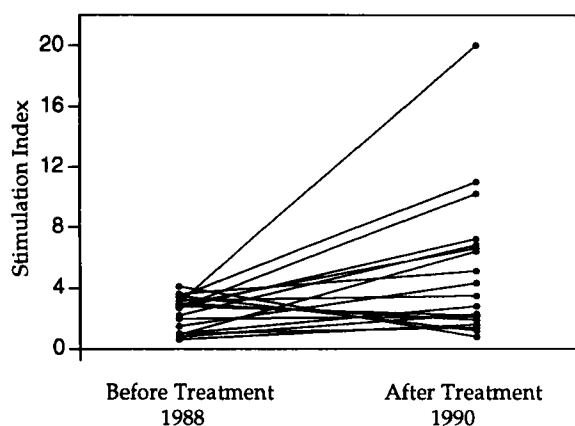


Figure 5. Lymphoproliferative response, expressed as stimulation index, to schistosomula antigen before (1988) and after treatment (1990) from 19 schistosomiasis patients with absence of lymphocyte blastogenesis to *S. mansoni* antigens in the first evaluation (1988).

However, when only subjects with a poor response to schistosomula antigen (SI < 5) were compared, 7 out of 19 showed an increase in their lymphoproliferative response (Fig. 5). Before therapy, the mean SI of lymphocyte cultures from these nineteen subjects was  $2 \pm 1.9$  while after therapy it was  $5 \pm 4.7$ , a statistically significant increase ( $p < 0.03$ ).

#### 4 Discussion

Subjects from a schistosomiasis endemic area, Caatinga do Moura, Bahia, Brazil, with similar degrees of water contact, showed a wide difference in degrees of infection. This finding is in agreement with previous studies of schistosomiasis endemic areas [18, 19] and suggests that these differences in the levels of infection cannot be explained by differences in exposure to *S. mansoni*. These findings were consistent at two different time points of the parasitological evaluation (1986 and 1989) with a positive correlation between the number of eggs per gram of stool in these two periods of evaluation. These data suggest that subjects in an endemic area have different degrees of resistance to infection by *S. mansoni*.

Attempts to correlate immunological response with the degree of infection in endemic areas of schistosomiasis have been performed previously. It has been demonstrated that serum from patients with a high degree of infection, assessed by the number of eggs/g of stool, had IgM-blocking antibodies to an *S. mansoni* antigen of 38 kDa [17]. Patients with a lower degree of infection had antibodies against specific parasite antigens of 27 and 25.5 kDa [18]. In addition, an association between lower degree of infection and the presence of antibodies to a 37-kDa antigen from schistosomula surface in the serum has been documented [19].

In the present study, the cellular immune response in subjects from an endemic area of schistosomiasis with similar degree of water contact was analyzed and an inverse correlation between the lymphoproliferative response to schistosomula and adult worm antigens and the degree of

infection from 1986 was observed. This finding suggests that cellular immune mechanisms can be involved in human resistance to *S. mansoni* infection. The absence of an inverse correlation between the blastogenic response to *S. mansoni* antigens and the number of eggs from the 1989 evaluation may be due to the lower levels of infection observed in this second evaluation because of the shorter interval of time between 1986 and 1989. A previous study did not document a correlation between cellular immune response to parasite antigens and the degree of infection [33]. A comparison between that observation and the data of the present study is, however, difficult to make; first, in the previous study, there was no control for exposure to *S. mansoni* and second, the degree of infection was not as variable as in the present study and most of the patients had a low degree of infection. In another study, the same group [48] observed a higher degree of lymphoproliferative response to egg, cercaria and adult worm antigens in subjects with more resistance to re-infection after treatment, but again in this study the investigators did not control for the degree of water contact. The degree of water contact is an important variable in the determination of the degree of infection [19] and in addition, the frequency of exposure to *S. mansoni* may be an important factor in the regulation of the immune response.

The role of cell-mediated immunity in protection against *S. mansoni* has been demonstrated in some studies using experimental models [16, 20, 24, 27–30]. Moreover, the role of the cellular immune response in the control of other immune mechanisms, cellular or humoral, that participate in the killing of schistosomula of *S. mansoni* is known. For instance, gamma-interferon can activate macrophages to destroy schistosomula [49]. This cytokine is also involved in the selection of B cell clones that produce IgG 2A [50], a class of antibody that is effective in the destruction of schistosomula by complement or antibody dependent cellular cytotoxicity. It is also known that IL-5 and granulocyte-macrophage CSF can activate eosinophils to destroy schistosomula [51–53] and IL-4 can stimulate the production of IgE antibodies [54, 55]. More recently, the resistance of mice to schistosomiasis has been associated with activation of Th1<sup>+</sup> cells [56]. In the present study, although lymphocyte proliferation, a phenomenon dependent on IL-2 production, has been documented in some subjects with low degree of infection, IFN- $\gamma$  was not detectable in supernatants of lymphocyte cultures stimulated with *S. mansoni* antigens. Since additional experiments have shown that subjects with a past history of schistosomiasis but living outside the endemic area have a good IFN- $\gamma$  production when stimulated by *S. mansoni* antigens, the absence of this response in subjects with high lymphoproliferative response from the endemic area can be explained by a regulation of this response due to the daily exposure to the infection.

In other studies, the absence of lymphocyte reactivity to *S. mansoni* antigens has been demonstrated mainly in patients with severe forms of the disease. The present study shows that in an endemic area, the majority of patients that have high contact with contaminated water do not respond to parasite antigens despite having a normal lymphocyte reactivity to PPD and TT. The observations that the mean age of the group with a low infection (< 400 eggs/g of stool) was higher than the group with a high infection and that the

cellular immune response is inversely correlated with the degree of infection, suggests that partial resistance to infection may be acquired over time.

Previous studies have shown an increase in cellular immune response after treatment, detected by blastogenic response [57] as well as IL-1, IL-2 and IFN- $\gamma$  production [58, 59]. In the present study, however, no significant change in lymphocyte blastogenesis was observed when the whole population was compared before and after therapy. An increase in lymphocyte reactivity after Oxamniquine therapy was observed in seven patients who previously were unresponsive to parasite antigens. Therefore, long-term evaluations of this population after therapy or subsequent treatments should be performed to determine the persistence of this increase in cellular immune response and if this increase is associated with protection against reinfection.

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