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The objective of this study was to evaluate the role of cytokines in hepatic fibrosis in the prehepatosplenic and early hepatosplenic stages of schistosomiasis mansoni. Hepatic fibrosis was classified by ultrasonography of 94 patients. Immunological evaluation was performed by the measurement of secreted cytokines (interleukin-5 [IL-5], IL-10, IL-13, gamma interferon, tumor necrosis factor alpha, and transforming growth factor β) in peripheral blood mononuclear cells (PBMC) stimulated by *Schistosoma mansoni* antigens. Significantly, higher levels of IL-5, IL-10, and IL-13 were found in supernatants of soluble egg antigen-stimulated PBMC from subjects with degree III hepatic fibrosis compared to patients with degree I or II fibrosis. Significant increases in IL-5 and IL-13 levels were also observed in some of the subjects who remained untreated for 1 year following initial assessment and developed more serious fibrosis during this period. The data suggest a role for type 2 cytokines in hepatic fibrosis in human schistosomiasis mansoni.

Schistosomiasis is a chronic and debilitating disease that affects over 200 million people worldwide (17). The pathology resulting from infection with the helminth parasite *Schistosoma mansoni* is predominantly caused by the host immune response to parasite eggs that are laid in the portal venous system and then become trapped in hepatic sinusoids and sequestered within granulomatous lesions (3, 8, 9, 22, 27). The fibrosis associated with granuloma formation can lead to portal hypertension, which causes much of the morbidity and mortality associated with schistosomiasis (14).

The formation of granulomas around schistosome eggs is mediated by $CD4^+$ T cells (28), and more recent studies have furnished numerous insights into the cytokine cascade that controls the development of these lesions (6, 8, 9, 18). In the murine model of schistosomiasis, type 2-associated cytokines, including interleukin-4 (IL-4), IL-5, and IL-13, contribute to granuloma formation and the presence of eosinophils in these lesions (9). However, in human schistosomiasis, studies have shown that high levels of tumor necrosis factor alpha (TNF-α) produced by peripheral blood mononuclear cells (PBMC) stimulated with schistosome antigen (Ag) are significantly associated with the presence of hepatosplenomegaly (4, 16, 19), while gamma interferon (IFN-γ) has a protective effect in severe fibrosis of the liver (4, 16). As hepatoesplenic disease is a long-term complication of schistosomiasis mansoni and is considered to be indicative of severe hepatic and periportal fibrosis, it is conceivable that the immune mechanisms responsible for this lesion occur much earlier during infection and precede the downstream development of hepatosplenomegaly. Consequently, it is important to evaluate the immune response in the early events of hepatic fibrosis. The present study measured the cytokine profiles in supernatants of soluble egg Ag-stimulated PBMC from schistosomiasis patients with different stages of hepatic fibrosis, and an association between type 2 cytokine production and progression to fibrosis was detected.

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MATERIALS AND METHODS

Endemic area and subject selection. This study included 94 patients with schistosomiasis; for each patient the degree of fibrosis was determined by ultrasonography. The majority of the patients ($n = 79$) were selected from Caatinga do Moura, a village where schistosomiasis mansoni is endemic, located 380 km from Salvador, the capital of the state of Bahia. Agriculture is the main economy of the village, and domestic use of the water contributes to constant aquatic exposure in a large part of the population. Because there were few patients with degree III hepatic fibrosis in Caatinga do Moura, more patients with degree III fibrosis were recruited from two other areas of schistosomiasis endemicity: Taquarendi ($n = 9$), another village with an agriculture-based economy, located 30 km from Caatinga do Moura, and Maruim ($n = 6$), a village from the state of Sergipe, north of Bahia state. None of the field sites was an area of endemicity for malaria, leishmaniasis, or Chagas' disease, but the sites have other intestinal parasitic infections such as *Ascaris lumbricoides*, *Trichuris trichiura*, *Ancylostoma duodenale*, *Entamoeba coli*, *Entamoeba histolytica*, and *Giardia lamblia*. All patients with degree III hepatic fibrosis had small spleen sizes (1 to 3 cm of the left costal ribs) and no past digestive bleeding or other signs of severe portal hypertension. They were all compensated forms of hepatosplenic disease that had not even received previous hospital care.

The inclusion criteria for subjects were as follows: positive Kato-Katz parasitological examinations for schistosomiasis, no history of therapy for schistosomiasis in the last 3 years, and hepatic fibrosis as assessed by ultrasonography. Exclusion criteria included being under 4 or above 60 years of age or having positive serology for human immunodeficiency virus, human T-cell leukemia

virus type 1, or hepatitis virus types B and C, as well as other conditions that could interfere in the immunological evaluation or classification by ultrasonography. Clinical and parasitological stool exams were performed in the study population every 3 years, followed by specific treatment for *S. mansoni* with oxaminiquine (20 mg/kg of body weight). All patients or the guardians of minors signed informed consent statements, and the study was approved by the Ethical Committee of the Hospital Universitário Professor Edgard Santos.

Ultrasonography. Ultrasonographic examination was performed by using World Health Organization criteria established in 1993 for the classification of hepatic fibrosis, according to a method described previously (1, 12). This classification defines hepatic fibrosis as degree 0 if the periportal tract thickness is <3 mm, as degree I if it is 3 to 5 mm thick, as degree II if it is >5 to 7 mm thick, and as degree III if it is >7 mm thick. The examinations were performed by two independent persons, and a correlation between periportal thickness and portal vein diameter and spleen size was demonstrated (12). Twenty-one subjects who went untreated, even after several attempts to locate them for treatment with schistosomicidal drugs, were reevaluated by ultrasonographic exam 1 year after the first evaluation. Nineteen of these patients showed an increase in the periportal tract thickness, but in only 12 had this increase induced a change in the degree of hepatic fibrosis. For three patients, the fibrosis had changed from degree 0 to I, and for nine patients, the fibrosis had changed from degree I to II. One year after treatment with oxaminiquine, all patients had improved and returned to degree 0 or I of fibrosis (12).

Antigens. The *S. mansoni*-specific Ags used were soluble extract of whole adult *S. mansoni* (SWAP) and soluble egg Ag (SEA), prepared as previously described (21).

Immunological methods. PBMC were isolated from heparinized blood by density gradient centrifugation by using Histopaque 1077 (Sigma Diagnostic, St. Louis, Mo.) as previously described (24). Briefly, 3×10^6 cells in 1 ml of RPMI 1640 (GIBCO-BRL) medium supplemented with 10% AB Rh-positive sera were either not stimulated or stimulated with *S. mansoni*-specific Ags at optimal concentrations determined previously by testing noninfected controls from Salvador. The Ag concentration of 10 μg/ml for both SEA and SWAP was chosen because it was able to induce a response in PBMC from schistosomiasis patients but not from the control subjects. After a 72-h incubation at 37°C in 5% CO_2, the supernatants were collected and stored at −20°C for the later measurement of the cytokine levels produced by the stimulated PBMC.

Cytokine level determination. Levels of the cytokines (IL-5, IL-10, IFN-γ, transforming growth factor β [TGF-β], IL-13, and TNF-α) were measured in supernatants from PBMC cultures stimulated with the two *S. mansoni* Ags. Cytokine concentrations were determined by using sandwich enzyme-linked immunosorbent assays (ELISAs), and the results were expressed as picograms of cytokine per milliliter of supernatant, based on comparisons with standard curves, according to a previously described technique (24, 25). Commercial kits from R&D Systems (Minneapolis, Minn.) were used for IFN-γ, IL-10, TNF-α, IL-13, and TGF-β. Duo Set antibodies (PharMingen, San Diego, Calif.) were used to test IL-5. Immulon 2 ELISA plates were from Dynatech. The techniques described by the manufacturers were followed, except that the diluent for establishing the standard curves was the same (RPMI plus 10% AB-positive sera) as for the tested samples. The sensitivity cutoffs for the ELISAs for each of the cytokines were as follows: 30 pg/ml for IFN-γ, IL-5, IL-10 and TNF-α; 14 pg/ml for IL-13; and 7 pg/ml for TGF-β. The results considered for analysis were the differences of cytokine concentrations from the stimulated culture supernatants versus the supernatants from the nonstimulated PBMC cultures.

Statistical methods. The differences in demographic data, infection levels, and cytokine levels in PBMC supernatants between the groups of subjects with degrees I, II, and III hepatic fibrosis were analyzed by the Mann-Whitney test. A nonparametric test was chosen because the cytokine data did not follow a Gaussian distribution. The differences in cytokine levels in the group of patients who increased the degree of fibrosis in two consecutive evaluations were compared by a Wilcoxon signed rank test for matched groups. These statistical analyses were performed by using the Instat program for Macintosh. An α value of 5% was considered for significance.

RESULTS

Overview of the subject groups. The 94 schistosomiasis patients selected for study had an age range of 5 to 55 years, with a mean and standard deviation (SD) of 16 ± 8 years (46 male and 48 female subjects). Their infection levels ranged from 24 to 1,784 eggs per gram (EPG) of feces. According to ultrasonographic examination, 8 subjects were classified as having degree 0 hepatic fibrosis, 50 had degree I, 22 had degree II, and 14 had degree III fibrosis. The age, sex, and infection levels (mean ± SD unless otherwise stated) in the subgroups of patients with different degrees of hepatic fibrosis are described in Table 1. Patients with degrees 0 and I hepatic fibrosis were included in the same subgroup. Differences in age were observed between patients with degree 0 to I (16 ± 11.5 years) and degree II (21 ± 10.5 years) hepatic fibrosis ($P = 0.01$ by Mann-Whitney test). Differences in infection levels were also observed between the patients with degrees I and III (379 ± 426 and 117 ± 98 EPG of feces, respectively) and between degrees II and III (412 ± 399 and 117 ± 98 EPG of feces, respectively) hepatic fibrosis ($P = 0.01$ by Mann Whitney test). The cytokine concentrations in Ag-stimulated PBMC cultures from schistosomiasis patients were determined. The levels of the cytokines IFN-γ, TNF-α, IL-10, IL-13, and TGF-β in SEA-stimulated PBMC supernatants were compared in schistosomiasis subjects with different degrees of hepatic fibrosis. The differences in the numbers of patients evaluated for each cytokine reflects the limited availability of culture supernatants, which itself reflects the limited numbers of PBMC obtained. Based on previous reports, we prioritized the evaluation of IFN-γ ($n = 67$), TNF-α ($n = 56$), and IL-5 ($n = 73$), leaving less supernatant for the measurement of IL-10 ($n = 49$), IL-13 ($n = 39$), and TGF-β ($n = 41$). Because patients were treated after donating PBMC samples, we were unable to perform repeat analyses. We were unable to assess IL-4 production due to the difficulty of measuring this cytokine in PBMC supernatants (26).

TABLE 1. Demographic data and infection intensity for schistosomiasis study subjects with different dgrees of hepatic fibrosis as classified by ultrasonography

Characteristic	Value for group with infection level of[a]:		
	Degrees 0 and I	Degree II	Degree III
No. of subjects			
Total	58	22	14
Male/female	31/27	11/11	8/6
Age (yr)			
Range	5–55	9–45	7–50
Mean ± SD	16 ± 11.5 A	21 ± 10.5 A	21 ± 13.3
No. of eggs/g of stool			
Range	24–1,784	40–1,616	24–216
Mean ± SD	379 ± 426 B	412 ± 399	117 ± 98 BC

[a] The same letter is placed next to combinations for which $P = 0.01$.

SEA-induced cytokine responses. A great variability in cytokine levels (given as mean ± SD unless otherwise stated) was observed in general, showing the heterogeneity of the response to *S. mansoni* Ags. No significant differences in IFN-γ levels were observed in supernatants from SEA-stimulated PBMC from the three groups of patients (79 ± 295.8, 3 ± 14.2, and 44 ± 79 pg/ml for patients with degrees I, II, and III fibrosis, respectively) (Fig. 1A). The levels of TNF-α in SEA-stimulated PBMC supernatants also did not show any differences between the groups (44 ± 124.2, 37 ± 93.3, and 19 ± 14.8 pg/ml for

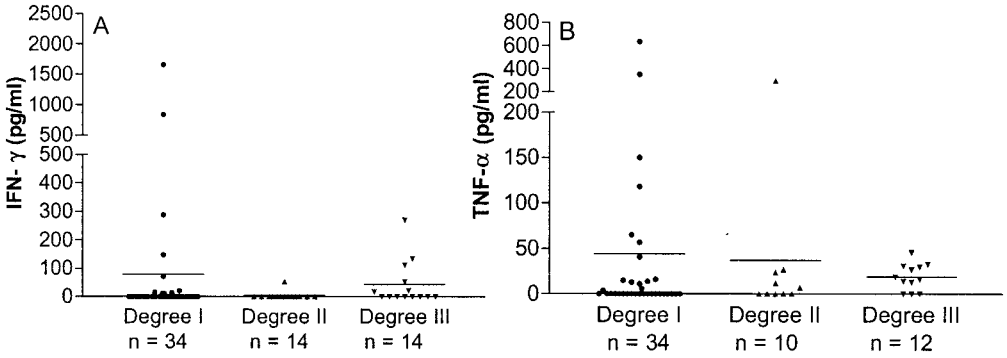

FIG. 1. IFN-γ (A) and TNF-α (B) levels in SEA-stimulated PBMC supernatants in schistosomiasis patients with different degrees of hepatic fibrosis. Differences between the groups were not statistically significant ($P > 0.05$ by Mann Whitney test).

patients with degrees I, II, and III fibrosis, respectively) (Fig. 1B).

Levels of IL-5 were significantly lower in supernatants from SEA-stimulated PBMC of subjects with degrees I (93 ± 248.4 pg/ml) and II (11 ± 29.8 pg/ml) of hepatic fibrosis than in supernatants from patients with degree III hepatic fibrosis (669 ± 815.6 pg/ml) ($P = 0.01$ and $P = 0.009$, respectively, by Mann-Whitney test) (Fig. 2A). IL-13 levels in supernatants of SEA-stimulated PBMC were also higher in subjects with degree III fibrosis (116 ± 120.9 pg/ml), compared to those with degrees I (24 ± 11.4 pg/ml) or II (86 ± 166.5 pg/ml) hepatic fibrosis ($P = 0.009$ and $P = 0.05$, respectively, by Mann-Whitney test) (Fig. 2B). Higher levels of IL-10 were observed in supernatants of SEA-stimulated PBMC from patients with degree III hepatic fibrosis (187 ± 110 pg/ml) compared to those with degree I (63 ± 62.9 pg/ml) or II (66 ± 53 pg/ml) hepatic fibrosis ($P = 0.01$ and $P = 0.03$, respectively, by Mann-Whitney test) (Fig. 3A). No differences were detected in TGF-β levels in SEA-stimulated PBMC supernatants between the groups of patients with degrees I, II, and III hepatic fibrosis (28 ± 18.7, 33 ± 40, and 26 ± 16 pg/ml, respectively; $P > 0.05$ by Mann-Whitney test) (Fig. 3B).

SWAP-induced cytokine responses. High levels of IL-5 were produced by SWAP-stimulated PBMC from all groups of patients (1043 ± 1844.5, 1319 ± 1706.7, and 2219 ± 2011 pg/ml for patients with degrees I, II, and III fibrosis, respectively), with significantly higher levels in patients with degree III versus degree I fibrosis ($P = 0.03$ by Mann-Whitney test). A significantly ($P = 0.03$ by Mann-Whitney test) higher level of IFN-γ was observed in supernatants of SWAP-stimulated PBMC from patients with degree II ($n = 23$) versus degree I ($n = 57$) versus degree III ($n = 14$) hepatic fibrosis (257 ± 354 versus 104 ± 189 versus 39 ± 58 pg/ml, respectively). There were no significant differences ($P > 0.05$) in the levels of TNF-α and IL-10 in cultures stimulated with SWAP (data not shown).

Responses of patients who remained untreated following initial assessment. Even after an active search, 21 patients missed treatment that should have been administered at the end of the study and remained untreated 1 year after their first ultrasonography examination. Ultrasonography was repeated on these individuals, and the periportal thickness measurements of 19 (90%) of the patients had increased, while the periportal thickness measurements of 2 of these patients were similar to initial measurements. Within this group, 12 of 19 (57%) patients exhibited more severe fibrosis compared to the previous exam. In three patients the fibrosis index had changed from degree 0 to degree I, and in nine patients the fibrosis index had increased from degree I to degree II. Immunological evaluation was repeated in these patients and compared with the first evaluation. An increase in the levels of some cytokines was observed in SEA-stimulated PBMC supernatants. Levels of IFN-γ were evaluated in 18 patients, and 2 patients presented a decrease; the others did not show any significant change in the second versus first evaluations (Fig. 4A). TNF-α

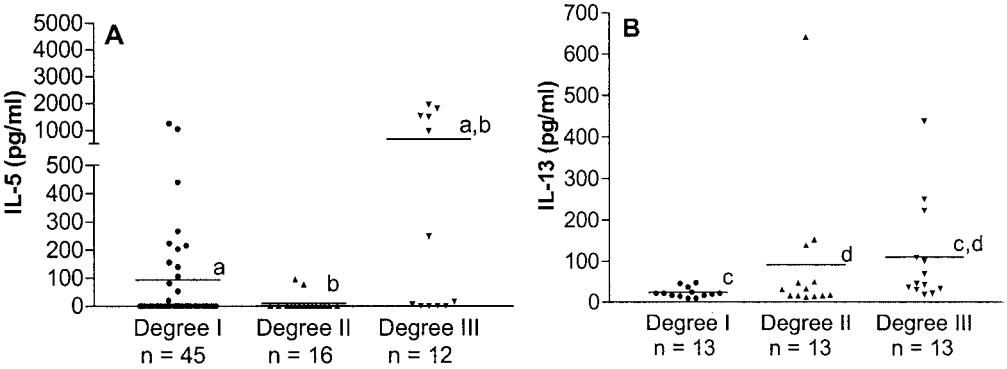

FIG. 2. IL-5 (A) and IL-13 (B) levels in SEA-stimulated PBMC supernatants in schistosomiasis patients with different degrees of hepatic fibrosis. a, $P = 0.01$; b, $P = 0.009$; c, $P = 0.0009$; d, $P = 0.05$ (Mann-Whitney test).

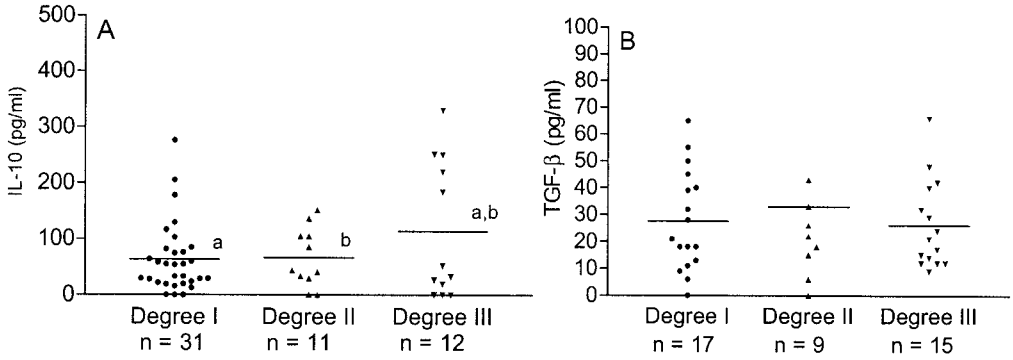

FIG. 3. IL-10 (A) and TGF-β (B) levels in SEA-stimulated PBMC supernatants in schistosomiasis patients with different degrees of hepatic fibrosis. a, $P = 0.01$; b, $P = 0.03$ (Mann-Whitney test).

levels increased in 5 patients and decreased in 2 patients and remained at similar levels in 8 of the 15 patients tested (Fig. 4B). IL-5 levels increased in 9 patients, decreased in 1 patient, and remained at similar levels in 8 of the 18 patients tested (Fig. 5A); IL-13 levels showed a major increase in 5 patients and a minor increase in 2 patients and decreased or remained at similar levels in 2 of the 9 patients tested (Fig. 5B). The levels of IL-5 and IL-13 were significantly higher in the second evaluation (206 ± 434 and 89.7 ± 56 pg/ml, respectively) in comparison to the first evaluation (19 ± 62 and 9.6 ± 11 pg/ml, respectively) ($P = 0.03$ and $P = 0.04$, respectively, by a Wilcoxon signed rank test). IL-10 levels also increased in 7 of 13 patients, decreased in 3 patients, and remained at similar levels in 3 other patients (Fig. 6A). TGF-β levels increased in 6 of 14 patients, decreased in 2 patients, and remained at similar levels in 2 other patients (Fig. 6B). No statistically significant differences were observed in the levels of IFN-γ, TNF-α, IL-10, and TGF-β in SEA-stimulated PBMC supernatants from the second to the first evaluations ($P = 0.4$, $P = 0.4$, $P = 0.3$, and $P = 0.4$, respectively, by Wilcoxon signed rank test). No significant differences were observed between the levels of cytokines in SWAP-stimulated PBMC supernatants from the second to the first evaluations.

DISCUSSION

A type 2 cytokine pattern dominates the immune response in mice and humans chronically infected by *S. mansoni*. In mice, IL-4 and IL-13 play a major role in egg granuloma formation, and IL-13 was shown to play a significant role in the development of hepatic fibrosis (8, 9, 10). In humans, although low IFN-γ and high TNF-α are associated with severe hepatic fibrosis (4, 16), it is still not clear which cytokines are involved in the progression of schistosomiasis mansoni pathology. The present study evaluated the cytokine profile in schistosomiasis patients developing hepatic fibrosis in prehepatosplenic and early hepatosplenic stages of the disease and showed an association between a type 2 cytokine profile (the production of IL-5, IL-10, and IL-13) and degree III hepatic fibrosis. IL-5 and IL-13 showed the strongest association with severe hepatic fibrosis and also increased significantly in patients who exhibited increased hepatic fibrosis after 1 year without treatment. Besides these Th2 cytokines, TGF-β levels also increased in the majority of the patients who exhibited increased hepatic fibrosis after 1 year without treatment. However, TGF-β levels decreased in two of these patients and remained at similar levels in two other patients, and no statistically significant differences were detected when the first and second evaluations were compared overall. Furthermore, TGF-β levels did not differ in the groups with different degrees of hepatic fibrosis.

Many variables may influence the magnitude of the immune response in human schistosomiasis, including age, intensity of infection, and the type of Ag used to assess immune responsiveness (e.g., whether the Ag is from schistosomula, adult worms, or eggs). Moreover, increasing age is associated with an

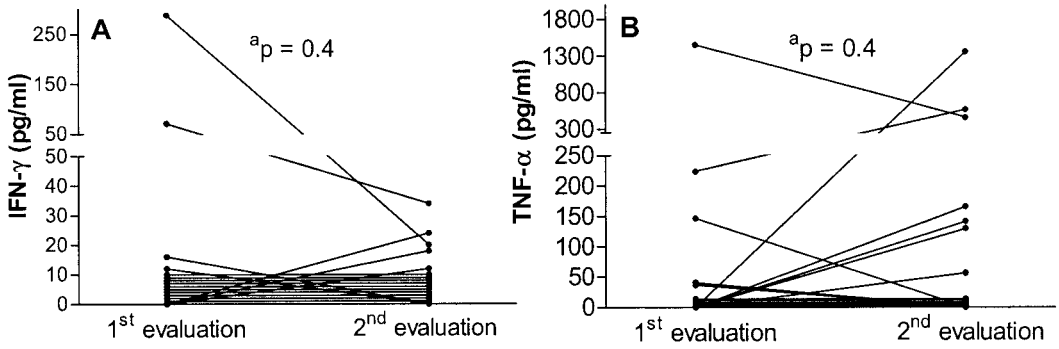

FIG. 4. IFN-γ (A) and TNF-α (B) levels in SEA-stimulated PBMC supernatants from subjects who increased the periportal tract thickness after one-year follow-up without treatment. a, *P* values determined by Wilcoxon signed rank test.

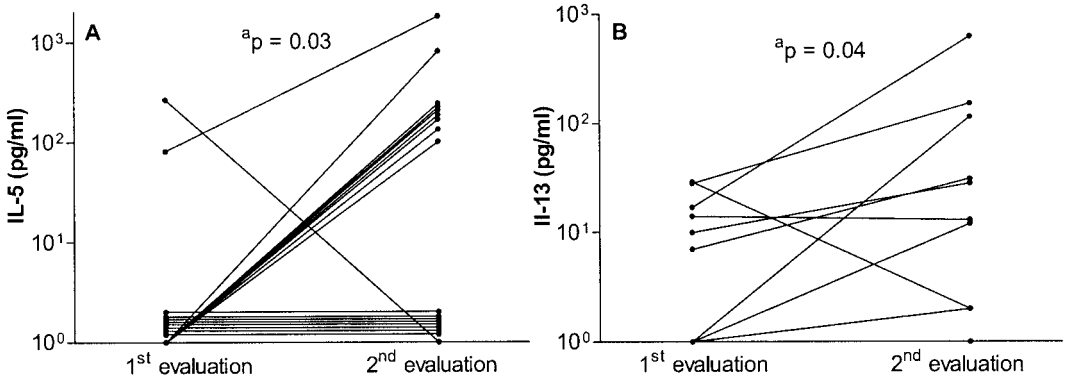

FIG. 5. IL-5 (A) and IL-13 (B) levels in SEA-stimulated PBMC supernatants from subjects in whom the periportal tract thickness had increased at follow-up after 1 year without treatment. IL-5 and IL-13 levels increased in 50% of the patients. a, *P* values determined by Wilcoxon signed rank test.

enhanced type 1 immune response (23), and heavy infections (more than 200 EPG of feces) are associated with an enhanced type 2 response (2). These variables did not explain the results of the present study, since patients with degree III hepatic fibrosis made more IL-5 and IL-13 despite the fact that their ages were higher than the ages of the other groups and that they had the lowest intensity of infection. Another important point is that the increase in IL-5 and IL-13 levels in patients with degree III liver fibrosis was only observed in SEA-stimulated cultures, indicating that the type 2 immunological response observed in these patients is directed primarily to egg Ag.

Recent studies have pointed out an important role for IL-13 in the development of liver fibrosis (8, 9). Although IL-4 and IL-13 share the same receptor and many biological activities, there are functional differences between the two cytokines. While granuloma formation was partially reduced in IL-4-deficient mice, blocking IL-13 and the IL-4 receptor in these animals almost completely eliminated granuloma development and tissue fibrosis (9). Indeed, IL-13 stimulates collagen production in fibroblasts and procollagen I and II mRNA expression. More recently, an IL-13 receptor α2 which blocks IL-13 function and hepatic fibrosis has been described (8). Our data, by showing a correlation between the production of high levels of IL-13 and the development of more severe fibrosis, suggest that this cytokine might also play a significant role in human schistosomiasis fibrosis. A recent study has found a protective effect of IFN-γ in liver fibrosis in Sudanese *S. mansoni*-infected subjects (16). The same group has also described an association between severe hepatic fibrosis with a polymorphic allele of the IFN-γ gene involved in reduced IFN-γ production (7). Another recent study has also associated low IFN-γ production with liver fibrosis (4). In fact, earlier studies on schistosomiasis pathology in mice demonstrated that IFN-γ reduces the cellularity of granulomas and downmodulates granuloma size (5). These data are in agreement with the data from the present study, because IFN-γ is the major type 1 cytokine involved in the downmodulation of T helper type 2 cells.

In contrast to our data that showed increased levels of IL-5 and IL-13 associated with liver fibrosis, studies of schistosomiasis patients from Africa showed that patients with more severe hepatic fibrosis had higher levels of TNF-α in PBMC supernatants (4, 16, 19). The contradictory results might be explained by the differences in the stages of the disease evaluated in these two studies: while the present study evaluated patients in the prehepatosplenic and early hepatosplenic stages

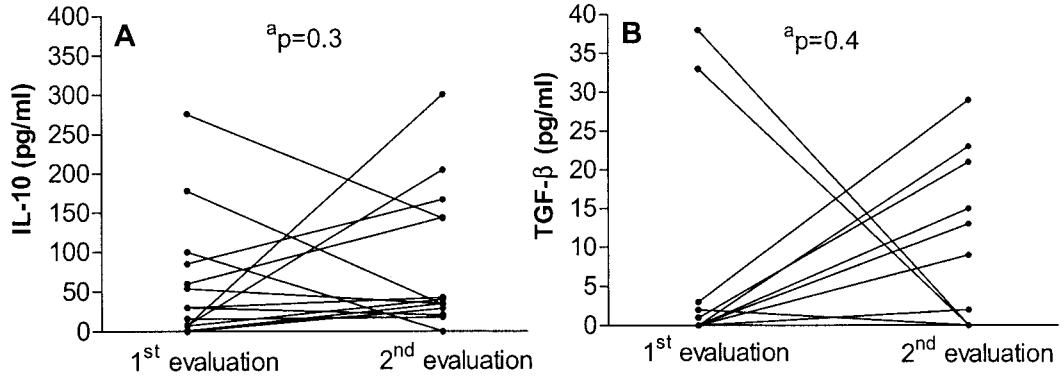

FIG. 6. IL-10 (A) and TGF-β (B) levels in SEA-stimulated PBMC supernatants from subjects in whom the periportal tract thickness had increased at follow-up after 1 year without treatment. IL-10 levels increased in 39% of patients and TGF-β increased in 43% of patients. a, *P* values determined by Wilcoxon signed rank test.

of schistosomiasis mansoni, these studies evaluated hepatosplenic patients. Since the degree III hepatic fibrosis patients are in the early hepatosplenic phase of disease evolution, it is possible that TNF-α is inducing the Symmers fibrosis that occurs in later stages of the disease. Another possible explanation for the contrasting results of these two studies is that the African population has a high prevalence of malaria, a protozoan infection that is known to cause hepatosplenomegaly and also to induce high levels of TNF-α (11). In fact, a more recent study from one of these groups of investigators (19), evaluating the serological response in the same population described in the previous study, confirmed that hepatosplenic patients had significantly higher levels of anti-*Plasmodium falciparum* antibodies compared to levels of nonhepatosplenic patients (20). Further studies are necessary to better clarify the cytokines involved in advanced stages of hepatosplenic schistosomiasis and the role of coinfections with malaria and other pathogens.

The documentation that levels of IL-10 were found to be higher in subjects with degree III compared to degrees I and II hepatic fibrosis was unexpected. IL-10 is a modulatory cytokine which downregulates macrophage activation and major histocompatibility complex class I and class II expression and reduces activation of both Th1 and Th2 cells (13). Previous studies have shown that IL-10 is important to reduce the pathology of acute schistosomiasis and that the level of Il-10 decreases in patients with hepatosplenomegaly (15). Since the patients in the present study were in the prehepatosplenic and early stages of hepatosplenomegaly, it is possible that the documentation herein of increased levels of IL-10 in patients developing liver fibrosis may represent an attempt of this cytokine to downregulate the high levels of IL-5 and IL-13. Furthermore, in the group of patients who presented an increase in hepatic fibrosis, IL-10 levels increased in seven patients but decreased in three and remained at similar levels in three other patients, and no statistically significant changes were demonstrated when the first and second evaluations were compared overall. Another interesting finding from the present study is the increased levels of TGF-β in supernatants from SEA-stimulated PBMC in 43% of subjects who had a higher degree of hepatic fibrosis after 1 year without treatment. Although there were no statistically significant differences between the levels of this cytokine from the first to the second evaluation, the small increase in the TGF-β levels might have a biological role in the induction of hepatic fibrosis. TGF-β is known to be involved in fibrosis due to its ability to induce collagen deposition (29). The increase in TGF-β levels during the evolution of hepatic lesions and the lack of differences in subjects with degrees I, II and III hepatic fibrosis could be due to an intermittent induction of fibrosis.

The present study presents evidence for a role of type 2 immune response in the development of liver fibrosis in human schistosomiasis. The expression of IL-5, IL-10, and IL-13 were associated with degree III hepatic fibrosis and the levels of IL-5 and IL-13 increased significantly in patients who developed more serious hepatic fibrosis over the course of the study.

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