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Cytokine Regulation of Periportal Fibrosis in Humans Infected with *Schistosoma mansoni*: IFN-γ Is Associated with Protection Against Fibrosis and TNF-α with Aggravation of Disease[1]

Sandrine Henri,* Christophe Chevillard,* Adil Mergani,† Patricia Paris,* Jean Gaudart,* Christophe Camilla,‡ Hélia Dessein,* Felix Montero,‡ Nasr-Eldin M. A. Elwali,† Osman K. Saeed,§ Mubarak Magzoub,† and Alain J. Dessein[2]*

Hepatic periportal fibrosis, which affects 5–10% of subjects infected by *Schistosoma mansoni*, is caused by the T cell-dependent granuloma that develop around schistosome eggs. Experimental models of infection have shown that granuloma and fibrosis are tightly regulated by cytokines. However, it is unknown why advanced periportal fibrosis occurs only in certain subjects. The goal of the present study was to evaluate the cytokine response of *S. mansoni*-infected subjects with advanced liver disease in an attempt to relate susceptibility to periportal fibrosis with an abnormal production of cytokines that regulate granuloma and fibrosis. Fibrosis was evaluated by ultrasound on 795 inhabitants of a Sudanese village in which *S. mansoni* is endemic: advanced periportal fibrosis was observed in 12% of the population; 35% of the affected subjects exhibited signs of portal hypertension. Age (odds ratio (OR), 11.5), gender (OR, 4.2), and infection levels (OR, 2.2) were significantly ($p \leq 0.01$) associated with hepatic fibrosis. Cytokines produced by egg-stimulated blood mononuclear cells from 99 subjects were measured (75 with no or mild fibrosis; 24 subjects with advanced fibrosis). Multivariate analysis of cytokine levels showed that high IFN-γ levels were associated with a marked reduction of the risk of fibrosis ($p = 0.01$; OR, 0.1); in contrast, high TNF-α levels were associated with an increased risk ($p = 0.05$; OR, 4.6) of periportal fibrosis. Moreover, infection levels were negatively associated with IFN-γ production. These results with observations in experimental models strongly suggest that IFN-γ plays a key role in the protection of *S. mansoni*-infected patients against periportal fibrosis, whereas TNF-α may aggravate the disease. *The Journal of Immunology*, **2002, 169: 929–936.**

Millions of people in subtropical countries are infected by *Schistosoma mansoni*, which is the most common human schistosome (1). Schistosome worms live in mesenteric and portal veins of their human host, and schistosome eggs trapped in hepatic sinusoids induce an inflammatory granuloma that prevent toxic substances from diffusing from the eggs to the surrounding hepatic tissue. Products of the inflammation, including molecules released by damaged cells, stimulate the differentiation of stellate cells into myofibroblasts that secrete extracellular matrix proteins (ECMP)[3] in the space of Disse (2). The disease is the consequence of the excessive accumulation of these matrix components in the periportal space. Fortunately, most infected subjects living in highly endemic areas control the infection with only minor pathological manifestations. However, in a small percentage of infected subjects, especially in those with high infections, extended periportal fibrosis (PPF) leads to portal hypertension, varices, and ascites.

The production of ECMP at the site of an inflammation is part of the normal repair process. It is initiated by molecules released by insulted tissues and is regulated by a number of cytokines, among which TGF-β, IL-1β, IL-6, IL-4, IL-5, IL-10, IL-13, TNF-α, and IFN-γ are the most important (3, 4). In particular, in vitro work has shown that IFN-γ is a strong antifibrogenic cytokine. It inhibits the production of ECMP by stellate cells and increases the collagenase activity of the liver by stimulating metalloprotease (MP) synthesis and by inhibiting tissue inhibitors of MP (TIMP) synthesis (5–9). TGF-β, IL-1, and IL-4 are fibrogenic; they stimulate the differentiation of stellate cells into myofibroblasts and they exert effects opposite to those of IFN-γ on the synthesis of ECMP and TIMPs (10–12). The roles of IL-4, IL-5, IL-10, IL-13, TNF-α, and IFN-γ in granuloma and in fibrosis have been evaluated in experimental models of schistosomiasis. IFN-γ was confirmed as the major down-regulator of fibrosis (13), whereas IL-4 was shown to be strongly proinflammatory (14–16) and IL-13 was reported to be fibrogenic (17–19). Recent observations indicate that IL-10 could have the key regulatory role of controlling excessive Th1 and Th2 polarization of the granulomatous response (20, 21). IL-12, when administered with egg Ags, stimulates protection against fibrosis by increasing IFN-γ and TNF-α (22). Finally, TNF-α could have protective (23) as well as proinflammatory and profibrogenic effects (24–26).

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*Immunologie et Génétique des Maladies Parasitaires, Institut National de la Santé et de la Recherche Médicale, Unité 399, Marseille, France; †Institute of Nuclear Medicine and Molecular Biology, University of Gezira, Wad Medani, Sudan; ‡Immunotech, Marseille, France; and §Blue Nile Research Institute, Wad Medani, Sudan

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[2] Address correspondence and reprint requests to Dr. Alain J. Dessein, Institut National de la Santé et de la Recherche Médicale, Unité 399, Faculté de Médecine, Université de la Méditerranée, 27, bd J. Moulin 13385 Marseille Cédex 5, France. E-mail address: alain.dessein@medecine.univ-mrs.fr

[3] Abbreviations used in this paper: ECMP, extracellular matrix protein; MP, metalloprotease; OR, odds ratio; PPB, peripheral portal vein branch; PPF, periportal fibrosis; PV, portal vein diameter; SEA, soluble egg Ag; TIMP, tissue inhibitor of MP; US, ultrasound.

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Our group has recently demonstrated in a Sudanese population in a region endemic for *S. mansoni* that severe PPF was under the control of a major genetic locus that is closely linked to *IFNGR1* (27), the gene that encodes the γ-chain of the IFN-γ receptor. This finding together with the results of various studies that have shown the key role of certain cytokines in regulating hepatic fibrosis in experimental schistosomiasis led us to test whether an imbalance in the production of the above mentioned cytokines was associated with advanced PPF in humans. The data strongly support a critical role of IFN-γ in protection against PPF, whereas TNF-α is shown to be associated with disease.

Materials and Methods

Study area and study subjects

Study subjects were from Al Taweel, a Sudanese village (900 individuals) in the Gezira irrigated scheme region that is highly endemic for *S. mansoni*. The villagers are migrants who settled 15–20 years ago in the village. They all came from the same region of west Sudan in which schistosomiasis is not endemic. The only source of water for the villagers is the canal water, which is used for drinking, domestic use (baths, washing), and irrigating the fields. The canal is densely populated by infected snails, and all study subjects had frequent contacts with contaminated waters for the past 15–20 years. A total of 795 subjects was studied by ultrasound (US).

Selection of subjects for the cytokine study

All subjects with FII or FIII fibrosis were invited to participate in the study. Only, one case, chosen randomly, was included per nuclear family.

A list of 150 subjects with no fibrosis (F0) or mild fibrosis (FI) was prepared using the following criteria: 1) 50% of the subjects should be >25 years to insure that these unaffected subjects were unlikely to develop FII or FIII fibrosis years later. FII peaks sharply in that population at ~20–25 years of age; 2) only one case per nuclear family (chosen randomly).

A total of 78 subjects with F0-FI and 25 with FII-FIII accepted to donate blood. The others either refused or were absent, working in the field, or could not spend a day in the city in which the laboratory work was performed.

Among these 103 subjects, 45 were 10–20 years, 30 were 21–35 years, and 28 were >35 years; 52% of the subjects were males. The proportion of subjects with advanced fibrosis was 8.8, 40, and 46% in the 10- to 20-year, 21- to 35-year, and >35-year age groups, respectively.

Of these 103 blood samples, complete cytokine data were obtained on 99 samples (75 FO-FI and 24 FII-FIII).

US evaluation

PPF was evaluated by US using an Aloka SSD 500 Echo camera and a 3.5 MHz convex probe according to World Health Organization guidelines (28). These guidelines were used rather than more recent ones because they allowed us to demonstrate that a discrete step in FFP was controlled by a major genetic locus (27). One objective of this study was to find out whether differences in cytokine production were associated with these genetically controlled phenotypes. Liver size, peripheral portal vein branches (PPB), the degree of PPF, thickness of the walls of PPB, spleen size, and splenic vein diameter were assessed. Portal vein diameter (PV) was measured at its entrance to the porta hepatis at the lower end of the caudete lobe, on subjects who had fasted for 8–10 h. Thickening of secondary periportal branches was observed for all subjects with FI to FIII, and the thickness tended to increase with fibrosis grade.

PPF was graded 0-III. Grade 0 (F0) corresponds to normal liver with no thickening of the wall of PPB. PPB diameter (outer to outer) is ~2–3 mm. Grade I (FI) corresponds to a pattern of small stretches of fibrosis around secondary portal branches. This patchy fibrosis usually yields a "fishes in the pond" appearance. PPB diameter is ~4 mm. Grade II (FII) corresponds to continuous in addition to patchy thickening of PPB. Most second order branches appear as long segment of fibrosis; PPB diameter is ~5–6 mm. Gallbladder wall thickness may be >4 mm. Grade III (FIII) corresponds to wall thickening of almost all PPB. Fibrosis reaches the surface of the liver; in some branches, the lumen is occluded. Gallbladder wall thickness is usually above normal (2–4 mm).

Parasitological procedures and treatment

Egg counts were performed by Kato's method on at least four stools collected on different days. All subjects were treated with Praziquantel. This treatment was repeated once to improve the cure, as assessed by three stool exams 3 mo after treatment. This second treatment was sufficient to cure all study subjects, as assessed by egg excretion in the feces. *Plasmodium falciparum* infections monitored by blood smears showed that malaria was endemic in that village. Treatment of malaria was given by local doctors at the local outpatient clinic. Transmission was seasonal. The study was conducted during the dry season outside the transmission period. At the time of examination (clinical and US), none of the patients showed signs of malaria. Note: 1) in these subjects, splenomegaly correlated with PPF (29); 2) neither splenomegaly nor hepatomegaly entered in the definition of the clinical phenotype that was studied in the present study.

Ag preparation

Frozen pellets of *S. mansoni* eggs were suspended in PBS and sonicated twice for 10 min in PBS, on ice. Insoluble material was removed by ultracentrifugation at $10^5 \times g$ for 1 h at 4°C. Supernatants (soluble egg Ags (SEA)) containing 1–1.5 mg/ml protein were stored at −70°C until use.

Cytokine production and titration

PBMC were isolated from heparinized venous blood by Ficoll-Hypaque gradient sedimentation (400 × *g*, 30 min, 18°C). PBMC were washed twice in RPMI medium containing 10 μg/ml gentamicin, and then suspended in the same medium supplemented with 50 μM 2-ME, 2 mM L-glutamine, 10% FBS, 10 mM HEPES, and 100 μg/ml sodium pyruvate and distributed at 5×10^6 cells/well in 24-well culture plates. After the addition of Ag (2.5 μg/ml SEA), cells were incubated at 37°C in a 5% CO_2 atmosphere in a humidified incubator. Supernatants were collected on days 2 and 5, and cytokines were titrated by ELISA (IFN-α, IFN-γ, IL-5, TNF-α) or by the Simultaneous Multianalyte Reagent Technology method (IL-1β, IL-4, IL-6, IL-10, and IL-12p40). For ELISA, titration plates were coated overnight at 4°C with 4 μg/ml anti-human IFN-γ mAb (Mabtech, Nacka, Sweden), 2 μg/ml anti-human IL-5 mAb (BD PharMingen, San Diego, CA), 2 μg/ml anti-human TNF-α mAb (R&D Systems, Abingdon, U.K.) diluted in carbonate buffer 0.1 M, pH 9.6. Then plates were incubated for 2 h with PBS-3% BSA, and washed twice with PBS-0.05% Tween 20. Culture supernatants were added and incubated overnight at 4°C. After three washes, plates were incubated for 2 h at room temperature with 0.5 μg/ml biotinylated anti-human IFN-γ Ab (Mabtech), 2 μg/ml biotinylated anti-human IL-5 Ab (BD PharMingen), or 100 ng/ml biotinylated anti-human TNF-α Ab (R&D Systems) diluted with PBS-3% BSA. After three washes, plates were incubated for 2 h at room temperature with 1 μg/ml streptavidin-alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted with PBS-3% BSA. After four washes, 200 μl 1 mg/ml *p*-nitrophenyl solution was added. OD was read at 405 nm. Standards were recombinant proteins. IFN-α was titrated using a kit (Immunotech, Marseille, France).

The Simultaneous Multianalyte Reagent Technology method is a flow cytometric immunoassay performed on fluorescent and Ab-coated microspheres (30), allowing the simultaneous titration of IL-1β, IL-4, IL-6, IL-10, and IL-12p40, with a sensitivity of 0.5–1.5 pg/ml. It has been described extensively previously (31). The flow cytometer microsphere-based assay uses green fluorescence intensity measurement to discriminate between microspheres. Microspheres in each category are coated with a specific anticytokine mAb. The red fluorescent intensity allows the sensitive quantitation of the immune complexes formed at the surface of each microsphere. The reliability of the assay has been improved with an internal standard for the adjustment of the fluorescent signal from anticytokine microspheres in each sample. The analytical performance of the assay has been described in an investigation on the cytokine profiles (IL-4, IL-6, IL-10, IL-12, IFN-γ, and TNF-α) of in vitro activated whole blood from atopic and nonatopic patients (31). A total of 50 μl sample was incubated for 2 h with 10 μl mixture of coated microspheres (100 μg/ml) and 50 μl mixture of biotinylated Abs (1 μg/ml) at room temperature with shaking. After two washes, 100 μl (0.5 μg/ml) of streptavidin-PE-Cy5 conjugate was incubated with the microspheres for 30 min at room temperature with shaking, and washed twice. The microspheres were then analyzed on a Coulter EPICS XL/MCL flow cytometer (Beckman Coulter, Miami, FL). The instrument was carefully set to provide optimal discrimination for FL1-coded microspheres and the optimal range for FL4 binding. FCS file processing and subsequent calculation of the immunoassay data were performed automatically with a postanalysis software package developed in-house.

Statistical analysis

The phenotypes under study (advanced PPF and advanced PPF + enlarged PV) depend on several covariates; some of these covariates could be confounder for the effect of others, and their effect on the phenotype must be tested simultaneously (multivariate analysis). We therefore first tested independently (nonparametric Wilcoxon ranking test) the association of the various covariates that may influence the phenotype. The results are presented (see Table II) that shows p values <0.2 because p values between 0.05 and 0.2 may indicate a trend for association that may be suggestive for other studies. In the multivariate analysis, we tested simultaneously all covariates. The multivariate method used in this study is logistic regression that specifies a regression relationship between the probability of an individual to develop advanced fibrosis and various covariates, as follows:

$$P(M^+/X_1, X_2, \ldots X_p) = (1 + \exp(-(\alpha + \Sigma\beta_i X_i)))^{-1}$$

where $P(M^+/X_1, X_2, \ldots X_p)$ is the probability of being affected knowing X_1 to X_p covariates; α and β are constants and estimated in the analysis. The analysis tests whether β_i is significantly different from zero. Note that $\exp\{\beta_i\}$ is the odds ratio (OR) associated with the covariate X_i, which measures the strength of the association between X_i and the phenotype, taking into account (adjusted to) the other covariates. With the stepwise procedure, one can select the covariates significantly ($p < 0.05$) associated with the risk of being affected.

Univariate analysis of the data, performed by the Wilcoxon test, is shown (see Table II). Data analysis in other tables was performed by ascendant stepwise (likelihood ratio procedure) logistic regression on the probability of being affected by PPF. The affected phenotypes were FII-III or FII-III associated with signs of portal hypertension (PV > 13 mm in females and PV > 14 mm in males). The nonaffected phenotype was F0-I with normal PV.

The regression analysis classes were defined as follows: gender (female, male), ethnic group (Tama-Messeria; Rawashda), and infection levels: three classes that were defined to include all negative subjects in the same groups (<6 eggs/g) and to have an equal number of subjects in the two other groups (7–48 eggs/g and 48–500 eggs/g) (see Table II). Cytokine classes were defined with the median value of the cytokine titer to have two classes (low and high) of equal size. Age classes were based on the U.S. evaluation of this population, which showed a marked difference in fibrosis prevalence between the classes of age: 10–20, 21–35, and >35 years (29). The same classes were used throughout this work in tables and figures. The statistical SPSS software (version 10.0; Chicago, IL) was used for this analysis.

Results

Pathological manifestations in study subjects

Previous studies have shown that PPF in human schistosomiasis can be accurately evaluated by US, and guidelines have been edited by World Health Organization to increase consistency of observations between observers (28, 32). PPF (FII or FIII) was observed in 12% of the 795 subjects; the other subjects had normal liver or mild hepatic fibrosis. Signs of portal hypertension were observed in 35% of FII and in all FIII subjects. Splenomegaly was observed in all groups (F0, I, II, III), but it was more frequent in subjects with PPF. FII and FIII were associated with a smaller left lobe of the liver, as determined by US (29).

Table I. *Stepwise regression analysis of the effects of gender, age, and infection levels on the probability of a given individual to be affected by advanced PPF (FII–III) or advanced PPF and portal hypertension*

		Gender	Age	Infection levels
Fibrosis II or III[a]		$p < 10^{-4}$	$p < 10^{-4}$	$p = 0.01$
	OR[b]=	4	14.06	
	CI:	2.1–7.4	6.7–29.4	
Fibrosis II or III and portal hypertension		$p < 10^{-4}$	$p < 10^{-4}$	NS
	OR[b]=	12.5	21.7	
	CI:	2.1–72	2–239	

[a] A total of 702 subjects had no or mild fibrosis; 61 had advanced (FII or FIII) fibrosis; and 39 had advanced fibrosis with enlarged portal vein.
[b] OR compare males with females and the >35-year with the 10- to 20-year age group.
[c] Confidence interval.

Stepwise logistic regression analysis was performed with the variables age, gender, and infection levels, which could influence disease development (this work and Ref. 29), and the probability of developing PPF (FII-III) in the whole population (Table I). All three variables were significantly ($p < 0.01$) associated with the probability of developing PPF. OR measure the strength of the association of covariates with fibrosis; they are adjusted on other covariates with significant association with disease. Thus, OR for infection levels are adjusted on age and gender. OR are a good approximation of the relative risk associated with the covariates. Thus, the relative risk of PPF was 4 times higher in males than in females, 14 times higher in the >35 years of age than in the 10–20 years of age, and higher in subjects with the highest infections than in subjects with the lowest infections. Contrary to infection levels, age and gender were important explicative variables for a phenotype combining PPF and portal hypertension (Table I). There was no significant association between either one of the two phenotypes and ethnic groups.

Cytokine production by PBMCs

The levels of cytokine produced by SEA-stimulated PBMC of FII-III subjects and F0-I subjects are shown in Table II. IL-12 and IFN-α levels were under detection levels in most cultures and are not presented. At the time this study was performed, there was no evidence that IL-13 had a fibrogenic effect in schistosomiasis; for this reason, IL-13 was not evaluated in this study. Table II gives the cytokine titers in 48- and 120-h cultures of cells stimulated with SEA and in 48-h cultures of unstimulated cells. Data are shown by age classes for both clinical groups. Differences between the two clinical groups were analyzed by the Wilcoxon ranking test that included either all age groups (p1 value) or subjects older than 20 years (p2 value). The 75% values are given only when the statistical test gave a significant (p1 or p2 <0.05) or a suggestive (p1 or p2 <0.2) p value. In SEA-stimulated cultures, only IFN-γ levels were different between the two clinical groups; this difference was greater when the analysis was performed on data from adults >20 years. Subjects with advanced fibrosis produced less IFN-γ than subjects with mild disease. TNF-α, IL-1β, IL-4, IL-5, IL-6, and IL-10 levels were not significantly different in the two study groups; there was, however, a trend for an association of fibrosis with higher levels of IL-1β ($p = 0.1$) and lower levels of IL-10 ($p = 0.13$) in SEA-stimulated cultures. In unstimulated cultures, low levels of IFN-γ ($p = 0.05$) and high levels of IL-1β ($p = 0.013$) were significantly associated with fibrosis. Note that there was also a trend for association of high IL-6 levels in unstimulated cultures of PBMC from subjects with disease; however, this observation was not duplicated in the SEA-stimulated cultures. Note that TNF-α showed no association with fibrosis in the univariate analysis, but it did so with logistic regression analysis (see below). IFN-γ levels in individual cultures of subjects of the two clinical groups are shown in Fig. 1.

Association of IFN-γ and TNF-α production with PPF

Because various covariates could be confounders for the effects of other covariates, the association of the covariates with the phenotype had to be tested simultaneously for all covariates that showed a significant association with the clinical phenotype (age, gender, infection levels, IFN-γ) or a trend for an association (IL-1β, IL-4, IL-10, IL-6). Moreover, covariates (IL-4, IL-5, TNF-α) reported by others to be associated with the disease phenotype or a related phenotype were also tested in the analysis, although they showed no evidence for an association in the univariate analysis. Of all cytokines tested, only IFN-γ and TNF-α showed a significant association with fibrosis: the best model included IFN-γ, TNF-α,

Table II. *Cytokines produced in cultures of SEA-stimulated blood mononuclear cells from subjects with no or mild fibrosis (F0-I,* n = *75) and subjects with advanced periportal fibrosis (FII–FIII,* n = *24)*

			No or Mild Fibrosis			Fibrosis FII or FIII				
Cytokines[a]	Culture[b] Condition	Hours	10–20 years	21–35 years	>35 years	10–20 years	21–35 years	>35 years	p1	p2
IL-1β		48	46.5	1.6	1.6	ND	16.0	142.5		0.02
			144[c]	*24*	*30*		*180*	*325*		
	SEA	48	7.0	9.0	5.0	8.3	27.0	23.0		
	SEA	120	67.0	12.5	44.0	1.6	13.5	59.0	0.1	
			135	*102*	*127*	*16*	*68*	*199*		
IL-4	—	48	1.4	1.4	1.4	1.4	1.4	4.7		0.13
			1.5	*1.5*	*1.6*	*1.4*	*5.8*	*21.5*		
	SEA	48	29.0	34.0	52.0	28.0	35.0	33.0		
	SEA	120	1.5	1.5	3.0	1.5	2.3	4.0		
IL-5	SEA	120	3719.3	4501.1	6895.1	4057.1	7458.1	2849.6		
IL-6	—	48	933.0	414.0	381.0	ND	508.0	2524.0		0.14
			2059	*958*	*1030*		*2654*	*4976*		
	SEA	48		905.0	765.5	739.0	396.5	895.5	958.0	
	SEA	120	1214.0	777.5	791.0	409.0	681.5	721.0	0.2	
			2955	*2387*	*1463*	*5753*	*1017*	*3210*		
IL-10	—	48	54.0	55.0	26.0	—	45.5	57.5		
	SEA	48	298.0	373.5	331.0	96.5	186.0	159.0	0.13	
			543	*663*	*389*	*306*	*447*	*745*		
	SEA	120	705.0	557.0	570.0	406.0	664.0	465.5	0.13	
			1094	*813*	*909*	*626*	*855*	*830*		
TNF-α	—	48	93.5	97.0	52.0	ND	58.0	199.0		
	SEA	48	134.0	171.5	175.0	200.5	158.5	207.0		
	SEA	120	290.5	414.5	382.0	273.5	391.0	339.5		0.2
			420	*590*	*549*	*1152*	*441*	*407*		
IFN-γ	—	48	0.2	0.2	0.2	ND	0.2	30.1		0.05
			2.4	*0.2*	*0.2*		*58*	*199*		
	SEA	48	0.8	14.3	21.0	7.6	0.2	24.0		
	SEA	120	61.5	605.0	350.0	111.5	40.0	83.0	0.05	0.004
			322	*919*	*1129*	*337*	*78*	*372*		

[a] Cytokines were measured in 48- and 120-h supernatants of single cultures of SEA-stimulated cells and in 48-h cultures of unstimulated cells. Cytokine production is the total production (production in unstimulated cultures was not subtracted). Cytokine levels in cultures of cells from both study groups were compared by the nonparametric Wilcoxon test. The test was performed either on data from all three age groups and yield p1 values or on data from the 21- to 35-year and >35-year age groups and yield p2 values. p1 and p2 values are indicated when they are ≤0.2. Cytokine levels are median values.

[b] Culture conditions. Cells were cultured in medium without (−) additional stimulus or in the presence of SEA. Unstimulated culture supernatants were collected at 48 h only.

[c] The 75% upper percentile values are indicated in smaller italic numbers when $p \leq 0.2$.

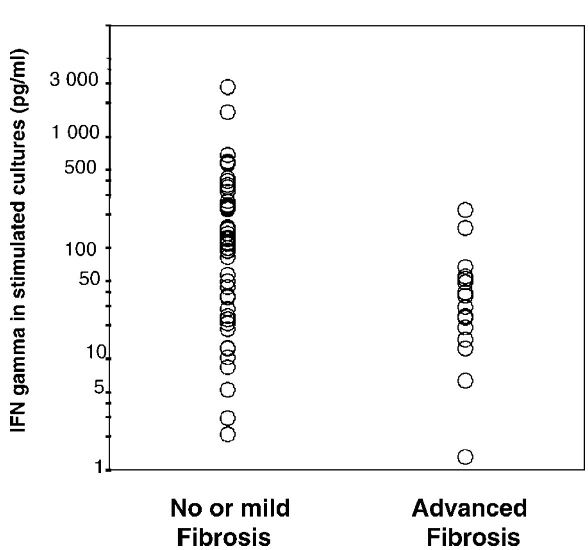

FIGURE 1. IFN-γ production in SEA-stimulated cultures of PBMC from subjects with no or mild fibrosis or with advanced fibrosis. Squares represent individual data. The figure are the data from the 99 subjects (studied in Tables II and III).

age, and gender as covariates (Table III). Thus, in that model, the associations of IFN-γ and TNF-α were adjusted on age and gender because the association of these cytokines with the disease phenotype varies (confounder effect) in the different age and gender classes and in males vs females. Note that this model does not adjust for intensity of infection because this covariate does not improve the likelihood of the model and it was rejected from the model by stepwise procedure.

Thus, in conclusion, IFN-γ showed a significant (inverse) association ($p = 0.01$) with PPF; high levels of IFN-γ were associated with a reduced risk of PPF. The OR that measures the strength of the association of IFN-γ with fibrosis after adjustment on other covariates with significant association with disease (age, gender, and TNF-α) is 0.11 (confidence interval, 0.03–0.6). This grossly indicates that the risk of developing severe disease is on average 9 times higher among low IFN-γ producers than among high IFN-γ producers. Because the association of infection levels with fibrosis was not significant when tested in the presence of IFN-γ, OR were not adjusted on infection levels. Forcing this covariate in the analysis had no effect on the results of the analysis. The association of low IFN-γ levels and advanced disease was also observed ($p =$

Table III. *Stepwise regression analysis of the effects of cytokine levels on the probability of a given individual to be affected by advanced PPF (FII–III) or advanced PPF and portal hypertension*

		Gender	Age	IFN-γ	TNF-α
Fibrosis II or III[a]		$p = 0.003$	$p < 0.01$	$p = 0.01$	$p = 0.05$
	OR[b]=	6.7	12.5	0.11	4.6
	CI:	*2–40*[c]	*2.3–66*	*0.03–0.6*	*1–22*
Fibrosis II or III, and PV > 14mm	OR[b]=	$p = 0.015$	$p = 0.004$	$p = 0.003$	$p > 0.1$
	CI:	10.5	79	0.01	
		2–69[c]	*2–300*	*<0.001–0.4*	

[a] Number of subjects in the study: 75 subjects with mild or no fibrosis, 24 subjects with FII or FIII, and 13 subjects with FII, FIII and PV > 14 mm.
[b] OR males with females, the 21- to 35-year age group with the 10- to 20-year age group, and the high with the low TNF-α, and IFN-γ level groups. As described in *Materials and Methods*, low and high cytokine groups are defined by the median value of TNF-α and IFN-γ levels defined on the ≥10 years of age subjects, as in figures. Age groups are 10–20, 21–35, and >35 years, as in figures.
[c] Confidence interval. Infection levels were not significantly associated with the probability of developing advanced disease in this model. Thus, OR are not adjusted on infection levels. Forcing infection levels in the model did not change the results.

0.003; adjusted OR = 0.01) when the affected phenotype combined PPF with portal hypertension (Table III).

TNF-α was positively associated with PPF (Table III): high TNF-α levels were associated with a risk of FII-III on average 4 times higher in the high TNF-α producers than in low TNF-α producers ($p = 0.05$; OR = 4.6; confidence interval, 1–22). As for other covariates in the analysis, the OR for TNF-α was adjusted on age, gender, and IFN-γ levels to take into account other covariates significantly associated with advanced fibrosis. The association of TNF-α with disease was not significant ($p = 0.065$) when fibrosis was combined with signs of portal hypertension probably because of the smaller size of the PPF group. No other cytokines, including IL-10 and IL-1β, showed a significant association with disease in the regression analysis.

The association of PPF with low IFN-γ production is illustrated on Fig. 2, which shows the percentage of subjects with FII-III in high and low IFN-γ classes, for the three classes of age used in the multivariate analysis. Fig. 3 shows the percentage of subjects with FII-III in low and high TNF-α classes for the different high and low IFN-γ classes.

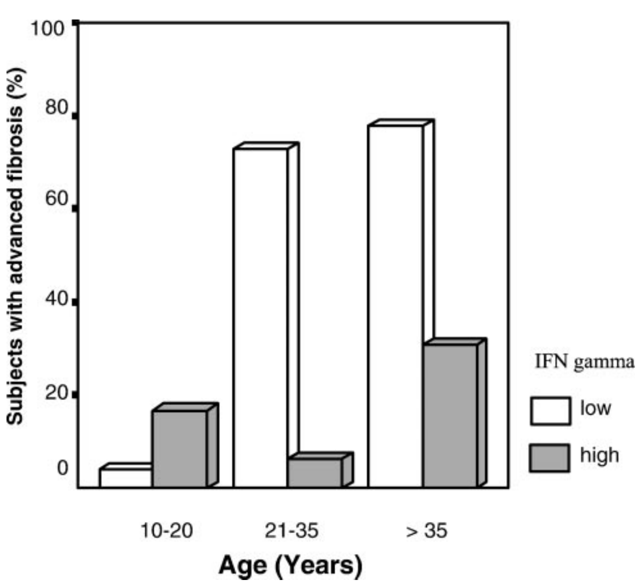

FIGURE 2. Proportion of grade II or III fibrosis in high and low IFN-γ classes in the three age groups. Age and IFN-γ classes were defined in *Materials and Methods*. The figure are the data from the 99 subjects studied in Tables II and III. Classes were defined in *Materials and Methods*. IFN-γ data are from 120-h cultures stimulated with SEA.

High infections that are associated with PPF are associated with a reduction of IFN-γ production

Because IFN-γ was strongly associated with protection against PPF, we evaluated which factors could modulate IFN-γ levels. IFN-γ levels were negatively correlated with infection intensities ($p = 0.01$; OR = 0.15). This result is illustrated in Fig. 4. Introducing infection levels as one of the covariates tested in Table III did not yield a better model, as discussed above, and IFN-γ was still strongly associated with fibrosis. There were also statistically significant differences in IFN-γ levels between Tama-Messeria and Rawashda ($p = 0.05$; OR = 0.15); this result is to be related to our previous report of a trend for more severe disease in the Rawashda (which produced on average less IFN-γ) than in the Tama-Messeria (29).

Discussion

In 5–10% of infected subjects, *S. mansoni* causes a severe and often lethal hepatic disease characterized by massive PPF that leads to portal hypertension, esophageal varices, and ascites. In some subjects, the liver and spleen are much enlarged. How hepatosplenomegaly and PPF are related is ill defined. Hepatosplenomegaly is not always associated with PPF, and conversely PPF can occur in subjects without hepatosplenomegaly. This was shown by

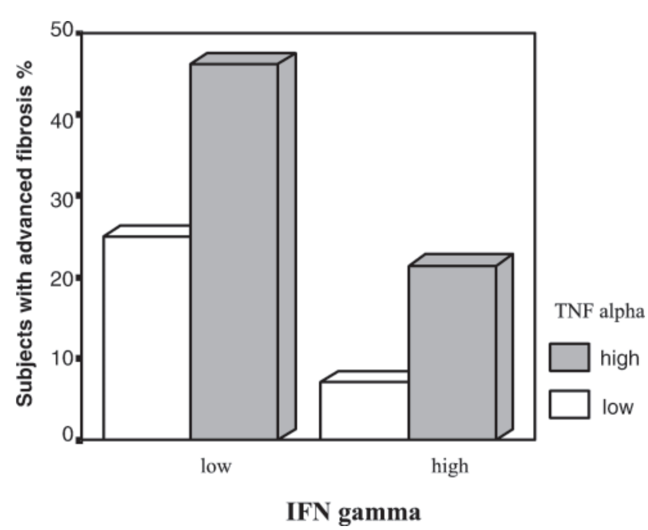

FIGURE 3. Proportion of grade II and III fibrosis in high and low TNF-α classes in the two low and high IFN-γ groups. Classes were defined in *Materials and Methods*. IFN-γ and TNF-α were measured in 48-h (TNF-α) and in 120-h (IFN-γ) cultures of PBMC stimulated with SEA.

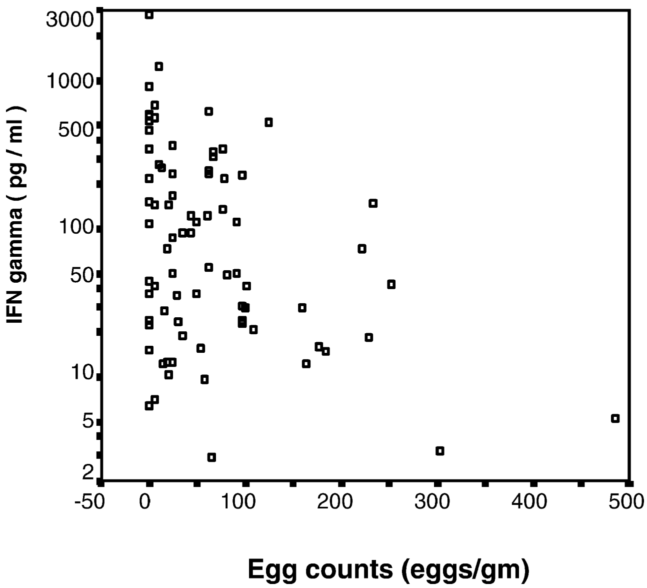

FIGURE 4. IFN-γ produced in SEA-stimulated cultures of PBMC of subjects with different infection levels. Squares indicate individual data or identical data from different subjects. Horizontal bars represent median value (picograms per milliliter).

others (29, 33) and also by our Taweela's study: the distribution of the subjects with advanced fibrosis (FII-III) in the four quartiles of spleen size (smallest to largest size) was 15.8, 26.3, 29.4, and 33.3%; only the two upper quartiles could be considered as indicating splenomegaly.

The factors that determine PPF in humans infected by *S. mansoni* are poorly understood, except for the role of some genetic and epidemiological factors. In contrast, the egg-induced pathology has been extensively investigated in experimental models of infection. Studies in mice, especially in animals deficient in certain genes such as those of TGF-β1, IL-4, IL-10, IL-12, Stat4, and Stat6 (15, 20, 21, 34, 35), have shown that the egg granuloma and hepatic fibrosis are markedly dependent on cytokine regulation. How much of this work can be extended to the human disease is unknown. Analysis of the role of cytokines in hepatic fibrosis in infected humans is difficult for several reasons: such studies must be conducted in the field on subjects with similar exposure to the pathogen and with similar living habits; the analysis must be performed on patients with active disease rather than subjects with end stage disease. These studies must also evaluate nonimmunological covariates that are cofounders in the analysis. In addition, immunological studies in infected humans can be performed only on blood leukocytes, while sinusoidal Kupffer cells, stellate cells, and endothelial cells are also important players in fibrosis (reviewed in Ref. 36). Nevertheless, there were several reasons to believe that evaluating cytokine production on blood leukocytes was a useful approach: 1) experimental studies conducted with lymphocytes from various tissues have identified the principal cytokines involved in the hepatic granuloma; 2) the regulation of mouse schistosome egg granuloma is dependent on $CD4^+$ T lymphocytes; and 3) if polymorphisms in cytokine gene(s) account for increased susceptibility to PPF, the effects of these mutations should be observable on blood leukocytes.

Then the present study was performed to evaluate the cytokine response of subjects with advanced liver disease in an attempt to relate susceptibility to disease with an abnormal production of cytokines that regulate granuloma and/or fibrosis in mice.

This work showed that production of IFN-γ in cultures of leukocytes from subjects with FII-III is much lower than levels in cultures from subjects with mild or no fibrosis. This association between low IFN-γ levels and PPF was also confirmed after taking into account the effects of important covariates such as gender and age. Because both study groups have been living in similar conditions for many years, including 15–20 years of frequent exposure to schistosome infections, it is unlikely that differences in unidentified environmental factors could explain these differences in IFN-γ production. This result on the association between low IFN-γ production and susceptibility to PPF must be analyzed in light of the large body of evidence showing that IFN-γ is certainly the most powerful and most active antifibrogenic cytokine in the experimental schistosome egg granuloma (13–16, 37) and in many injury-induced hepatic fibrosis (5–9, 12). IFN-γ acts at various levels of fibrogenesis to limit accumulation of ECMP: it inhibits the differentiation/activation of stellate cells, it inhibits production of ECMP by stellate cells, it increases ECMP degradation by inducing MPs, and it inhibits TIMPs. The association of low IFN-γ production with fibrosis, added to observations in experimental schistosomiasis and in studies on the regulation of ECMP production, accumulation, and degradation, strongly suggests that PPF is related to a decreased production of IFN-γ. In addition, the observations that IFN-γ levels are inversely related to infection and account for the association of infection with PPF (Table III) also suggest that high infections may contribute to PPF by down-modulating IFN-γ. Several studies (29, 38) including this one have shown that high infection levels are associated with advanced fibrosis, especially in adolescents. High infections could contribute in several ways to PPF, i.e., a higher number of eggs could increase tissue inflammation or modulate cytokines that regulate fibrosis. An interesting finding is this effect is not dependent on patient age because age was taken into account in the multivariate analysis that tested the association between IFN-γ and infection levels. The key role of IFN-γ in PPF was also suggested by the existence of a major susceptibility locus for PPF closely linked to *IFNGR1* (27). A study in progress by our group has uncovered various polymorphisms in *IFNGR1* of these subjects. These polymorphisms are being tested for their association with PPF. It is, however, too early to speculate more on the identity of the susceptibility alleles. Finally, the existence of a major gene does not rule out other gene(s) in the genetic control of fibrosis; the relative importance of these different genes in fibrosis will depend on the study population. The present study suggests that susceptibility alleles might also be found in the IFN-γ and TNF-α pathway, including in IFN-γ and TNF-α genes.

A previous study in subjects with acute and chronic schistosomiasis (39) has suggested that IFN-γ and IL-10 cross-regulate each other and that IL-10 is beneficial in patients with acute infections or with hepatosplenomegaly. The present study did not detect an association of IL-10 with fibrosis in the presence or in the absence of either IFN-γ or TNF-α. It should be noted, however, that there was a trend ($p = 0.13$) for lower IL-10 production in both unstimulated and SEA-stimulated cultures of subjects with FII-FIII. The study also failed to detect, in the regression analysis, a regulatory influence of IL-10 on IFN-γ production in culture of blood mononuclear cells. This question, however, would be better addressed by inhibiting IL-10 production in cultures with mAb, as done by others (39). This was not an objective of our study.

The association of TNF-α with disease was detected in the presence of IFN-γ in the regression analysis; TNF-α alone showed no association with fibrosis. This and experimental results discussed above suggest that TNF-α could act on fibrosis by balancing the protective effect of IFN-γ. TNF-α has pleiotropic effects on the immune response against schistosomes: it restores the ability of T cell-deficient mice to mount a granuloma around schistosome eggs (24); it increases ECMP production by Kupffer cells (36); it stimulates MP gene expression (40) and protects IL-12-vaccinated mice against the deleterious effects of the granuloma (23); TNF-α also increases the production of NO, whose hypotensive effects might benefit subjects with portal hypertension (41). Then, the primary role of TNF-α in schistosomiasis is a protective one. However, as in various pathologies, an imbalance between TNF-α and other regulatory cytokines may cause tissue damage. A possible mechanism for this damage is an exacerbation of the granuloma by overproduction of reactive oxygen species (4, 25, 26), as suggested in experimental schistosomiasis (42). The association between TNF-α and clinical disease in schistosomiasis has also been found in the sera and in the culture of blood mononuclear cells from subjects with hepatosplenomegaly (43, 44).

That we did not observe an association between other cytokines and PPF does not mean that in other conditions such an association could not be uncovered. Table II indicates that IL-1β, IL-10, and IL-4 showed a trend ($0.2 \leq p < 0.05$) for an association with fibrosis. Note, however, that these cytokines were rejected from the regression analysis although the threshold value for inclusion in the model was set up to 0.1. IL-1β data were the most suggestive. IL-1β is a strong proinflammatory cytokine. It could aggravate fibrosis by increasing chronic hepatic inflammation due to eggs and worm Ags; IL-1β has not been reported to have direct effect on fibrosis. As mentioned above, there was also a suggestion that IL-10 was reduced in advanced fibrosis. It is unlikely that protection against PPF is associated with Th2 cytokines, as reported by Mwatha and colleagues for hepatosplenomegaly (43), because of the strong association of this phenotype with IFN-γ. Note also that the data in Table II ($p = 0.13$) suggest that IL-4 was augmented in unstimulated cultures of FII-FIII subjects. This may relate to the likelihood that hepatosplenomegaly and PPF are distinct clinical phenotypes (33, 45).

In conclusion, this study shows that low production of IFN-γ is associated with severe PPF in subjects living in an area endemic for schistosomiasis and that a reduction in IFN-γ might account for the higher risk of disease in subjects with high infections. Results also indicate that TNF-α might aggravate PPF in chronically infected subjects.

It is now essential to determine whether these observations are related to our previous work showing the existence of a strong genetic control of PPF in certain subjects.

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