No Evidence for a Major Effect of Tumor Necrosis Factor Alpha Gene Polymorphisms in Periportal Fibrosis Caused by Schistosoma mansoni Infection

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No Evidence for a Major Effect of Tumor Necrosis Factor Alpha Gene Polymorphisms in Periportal Fibrosis Caused by *Schistosoma mansoni* Infection

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Hepatic periportal fibrosis (PPF), associated with portal hypertension, is a major pathological consequence of infections with *Schistosoma mansoni* and *Schistosoma japonicum*. Indeed, affected subjects may die from portal hypertension. Previous studies have indicated that tumor necrosis factor alpha (TNF-α) may aggravate fibrosis. We therefore investigated whether PPF was associated with certain polymorphisms of the TNF-α gene. Four polymorphisms (TNF-α −376 G/A, −308 G/A, −238 G/A, and +488 G/A) were investigated in two Sudanese populations living in an area in which *S. mansoni* is endemic. These polymorphisms were analyzed for 105 Sudanese subjects with various grades of PPF, from mild to advanced; all subjects were from two neighboring villages (Taweela and Umzukra). They were then analyzed for 70 subjects with advanced liver disease and for 345 matched controls from the Gezira region. We found no evidence of associations between these four polymorphisms and PPF in both of these studies. Thus, these four polymorphisms, two of which (TNF-α −376 and −308) were found to increase TNF-α gene transcription, are unlikely to have a major effect on PPF progression in these populations. However, this result does not exclude the possibility that these polymorphisms have a minor effect on PPF development.

Schistosomiasis is a serious public health problem affecting over 200 million people in developing countries (15, 41). Most of the infections occurring in areas where schistosomes are endemic are asymptomatic. However, 5 to 15% of infected individuals develop severe disease with Symmers fibrosis. Schistosomes (*Schistosoma mansoni*) produce several hundred eggs per day, and a proportion of these eggs are trapped in hepatic tissues and in presinusoidal venules. There, they induce a granulomatous inflammation that leads, in certain subjects, to the accumulation of scar tissue in the periportal spaces. Periportal fibrosis (PPF) causes venous obstruction and portal blood hypertension and contributes to the development of splenomegaly. Severely affected patients develop esophageal varices, ascites, and cachexia, resulting in death in the absence of treatment. Fibrosis, which involves stellate (Ito) cells derived from fibroblasts, results from an imbalance between the positive and negative regulatory mechanisms controlling the production and degradation of extracellular matrix proteins (ECMP). The production of collagen and ECMP is stimulated by a variety of cytokines and growth factors (interleukin-1 [IL-1], IL-4, IL-13, tumor necrosis factor alpha [TNF-α]), monocyte chemotactic protein 1, platelet-derived growth factors, and transforming growth factor β), which stimulate fibroblast differentiation and the production of ECMP by fibroblasts, Kupffer cells, and endothelial cells (14, 16, 22, 30). Other cytokines, such as alpha interferon (IFN-α) and IFN-γ, have the opposite effect (6). ECMP turnover depends on matrix metalloproteases and their inhibitors, the tissue inhibitors of metalloproteases, the synthesis of which is also regulated by various cytokines, including TNF-α, transforming growth factor β, and IFN-γ (33, 34).

We have studied the development of PPF in a Sudanese population from the village of Taweela, which is located in a region where *S. mansoni* is endemic (36). We have shown that advanced PPF associated with portal hypertension is under the control of a major genetic locus (SM2) that is closely linked to *IFNGR1*, the gene encoding the alpha chain of the IFN-γ receptor (18). Analysis of the production of cytokines by the mononuclear cells present in the blood of these subjects showed that high IFN-γ levels were associated with a much lower risk of PPF and that high TNF-α levels were associated with a high risk of PPF (25). These findings are consistent with the known antifibrogenic properties of IFN-γ (see reference 26 for a review). TNF-α has been reported to modulate the granulomatous reaction induced by schistosome eggs (5, 29). Several reports have also suggested that TNF-α may increase hepatic fibrosis and severe disease in *S. mansoni* infections: in chronically infected CBA/J mice, TNF-α mRNA levels increase with the collagen content of the liver and with splenomegaly (3), and similar results have been obtained in BALB/c mice infected with *S. mansoni* (32). Furthermore, TNF-α increases superoxide production and downregulates the chemotactic response of human neutrophils, thereby probably increasing inflammation (7, 42). Finally, in diseases caused by parasites, such as cerebral malaria and cutaneous leishmaniasis, in which there is an exaggerated inflammatory reaction to parasite antigens, aggravation of the clinical status of the pa-

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TABLE 1. Distribution of TNF-α polymorphisms in the populations of Taweela and Umzukra[a]

Polymorphism	Genotype	No. with genotype/no. genotyped (% with genotype) in:				
		Whole population	Clinical group:			
			F0	FI	FII	FIII
TNF-α −376	G/G	96/98 (97.96)	30/30 (100)	23/23 (100)	18/18 (100)	25/27 (92.59)
	G/A	2/98 (2.04)	0/30 (0)	0/23 (0)	0/18 (0)	2/27 (7.41)
	A/A	0/98 (0)	0/30 (0)	0/23 (0)	0/18 (0)	0/27 (0)
TNF-α −308	G/G	81/97 (83.5)	23/30 (76.67)	21/23 (91.3)	16/17 (94.12)	21/27 (77.78)
	G/A	14/97 (14.43)	5/30 (16.67)	2/23 (8.7)	1/17 (5.88)	6/27 (22.22)
	A/A	2/97 (2.06)	2/30 (6.66)	0/22 (0)	0/17 (0)	0/27 (0)
TNF-α −238	G/G	89/99 (89.9)	30/31 (96.8)	20/24 (83.3)	16/17 (94.1)	23/27 (85.2)
	G/A	8/99 (8.08)	1/31 (3.2)	3/24 (12.5)	1/17 (5.9)	3/27 (11.1)
	A/A	2/99 (2.02)	0/31 (0)	1/24 (4.2)	0/17 (0)	1/27 (3.7)
TNF-α +488	G/G	80/97 (82.47)	25/30 (83.3)	22/24 (91.7)	14/19 (73.7)	19/24 (79.2)
	G/A	16/97 (16.5)	5/30 (16.7)	2/24 (8.3)	4/19 (21.0)	5/24 (10.8)
	A/A	1/97 (1.03)	0/30 (0)	0/24 (0)	1/19 (5.3)	0/24 (0)

[a] PPF was evaluated by ultrasound scan (portable Aloka SSD 500 Echo camera and 3.5-MHz convex probe) for all of the residents of Taweela (n = 770) and for one-third of the population of Umzukra (n = 700). Family relationships were determined for the populations of both villages. For the study group, we included all unrelated subjects from these two villages. If several choices of individual were possible, we systematically selected the oldest subject because PPF develops mainly in individuals over 20 years old in this population (17). Based on these criteria, we selected 105 unrelated individuals from those who volunteered to give blood samples. TNF-α genotypes were determined as previously described (16, 19, 24, 38, 48).

tient has been shown to be associated with polymorphisms that increase the levels of transcription of the TNF-α and TNF-β genes (11, 26, 31, 35, 46). We therefore investigated whether progression to severe PPF in schistosomiasis was associated with polymorphisms in the TNF-α gene. We began by investigating the possible association of PPF with polymorphisms in the TNF-α promoter (−376 G/A, −308 G/A, and −238 G/A) and in exon 1 (+488 G/A) in residents of the Sudanese villages of Taweela and Umzukra in the Gezira region and in another cohort selected from the same region, in which *S. mansoni* is endemic.

MATERIALS AND METHODS

Blood samples. Ten to 15 milliliters of venous blood was collected on Na citrate. Genomic DNA was prepared by a classical standard salting-out method.

PCR amplification. Four different mutations were screened for samples from the village population and the case-control cohort. Oligonucleotides were obtained from MWG Biotech, Courtaboeuf, France, and restriction enzymes were purchased from Life Technologies, Cergy-Pontoise, France. TNF gene sequences from all individuals included in this study were amplified by PCR. The reaction mixtures (30 μl) contained 100 ng of genomic DNA, a 1 mM concentration of each primer, a 0.25 mM concentration of each nucleotide, 1.5 mM $MgCl_2$, and 1.5 U of *Taq* polymerase in 1× commercial buffer (Appligene, Illkirch, France). The temperature cycles were 1 min at 94°C for denaturation, 45 s at the specific annealing temperature of each primer, and 45 s at 72°C for elongation for 35 cycles in Stratagene Robocycler 96. Amplified products were visualized on a 1.5 or 2% agarose gel with ethidium bromide (1 mg/ml) staining.

Primer sequences and analysis of TNF allele frequency. The TNF-α −238 (G/A) polymorphism in the promoter of the TNF-α gene was determined by PCR amplification (17) with the primers 238F (5′ GGT CCT ACA CAC AAA TCA GT 3′) and 238R (5′ CAC TCC CCA TCC TCC CTG GTC 3′). At the 3′ end of the reverse primer, C has been changed to G (underlined base) in order to facilitate detection of the mutation by restriction enzyme digestion. The 71-bp fragment generated by PCR was digested with the *Ava*II enzyme under the conditions described by the manufacturer. Digestion products were analyzed on a 12% acrylamide gel (37.5:1) stained with ethidium bromide (1 mg/ml). The PCR products from patients who were homozygous for allele −238A (−238A/A) gave a single 71-bp fragment after digestion, whereas those from patients who were homozygous for allele −238G (−238 G/G) were completely digested (51- and 20-bp fragments). Three fragments of 71, 51, and 20 bp were present in the patients who were heterozygous.

The TNF-α −308 (G/A) polymorphism in the promoter of the TNF-α gene was determined by PCR amplification with the primers −308F (5′ AGG CAA TAG GTT TTG AGG GCC AT 3′) and −308R (5′ TCC TCC CTG CTC CGA TTC CG 3′) (47). A single base change at the 3′ end of the forward primer (underlined) was required for the formation of a *Nco*I recognition sequence. The 107-bp fragment was digested with the *Nco*I enzyme, and digestion products were analyzed on 10% acrylamide gel (37.5:1) stained with ethidium bromide solution (1 mg/ml). The PCR products from patients who were homozygous for allele −308A (−308 A/A) gave a single 107-bp fragment, whereas those from patients who were homozygous for allele −308G (−308 G/G) were completely digested (87- and 20-bp fragments).

The TNF-α −376 (G/A) polymorphism in the promoter of the TNF-α gene was previously determined by PCR amplification (24) with the primers −376F (5′ CCC CGT TTT CTC TCC CTC AA 3′) and −376R (5′ TGT GGT CTG TTT CCT TCT AA 3′). The 106-bp PCR fragment was digested with the *Tsp*509I enzyme. Digestion products were analyzed on a 10% acrylamide gel (37.5:1) stained with ethidium bromide solution (1 mg/ml). The PCR products from homozygotes for allele −376G (−376 G/G) were not digested and gave a single 106-bp fragment, whereas in homozygotes for allele −376A (−376 A/A) fragments were completely digested (86 and 20 bp).

The TNF-α +488 (G/A) polymorphism in intron 1 of the TNF-α gene was associated with granulomatous common variable immunodeficiency (20, 37). PCR amplifications were performed with the primers +488F (5′ CAG AGG GAA GAG GTG AGT GC 3′) and +488R (5′ GCC AGA CAT CCT GTC TCT CC 3′). A 220-bp PCR fragment was digested with the *Nla*III enzyme. Digestion products were analyzed on a 10% acrylamide gel (37.5:1) stained with ethidium bromide solution (1 mg/ml). The PCR products from homozygotes for allele +488G (+488 G/G) were not digested and gave a single 220-bp fragment, whereas in homozygotes for allele +488A (+488 A/A) fragments were completely digested (82 and 138 bp).

Statistical analysis. Fisher's exact test and the chi-square test were performed with SPSS software.

RESULTS AND DISCUSSION

The first part of the study concerned the populations of two villages (Taweela and Umzukra) in the Gezira region of Sudan (Table 1). PPF was studied by ultrasound, making it possible to grade the disease (F0, FI, FII, and FIII), as described elsewhere (12). The study population consisted of 105 subjects: 56 had no fibrosis (F0) or mild fibrosis (FI), and 49 individuals

TABLE 2. TNF-α genotype distribution and genotype frequencies in clinical groups[a]

Polymorphism	Genotype	No. with genotype/no. genotyped (% with genotype) in clinical group(s):		*P* (Fisher's exact test)[b]
		F0, FI, and FII[a]	FIII	
TNF-α −376	G/G	71/71 (100)	25/27 (92.6)	0.074
	G/A or A/A	0/71 (0)	2/27 (7.4)	1
TNF-α −308	G/G	60/70 (85.7)	21/27 (77.8)	1
	G/A or A/A	10/70 (14.3)	6/27 (22.2)	
TNF-α −238	G/G	66/72 (91.7)	23/27 (85.2)	1
	G/A or A/A	6/72 (8.3)	4/27 (14.8)	
TNF-α +488	G/G	61/73 (83.6)	19/24 (79.2)	
	G/A or A/A	12/73 (16.4)	5/24 (20.8)	1

[a] The genotype distributions for the four polymorphisms did not deviate from Hardy-Weinberg equilibrium within the F0-FI-FII group ($\chi^2 = 0$, 5.07, 4.50, and 0.37 for TNF-α −376, −308, −238, and +488, respectively).
[b] Fisher's exact test was carried out with SPSS software to compare the two study groups (F0-FI-FII versus FIII).

displayed advanced fibrosis (FII or FIII), which was associated with portal hypertension in a third of the FII subjects and in all FIII subjects. These individuals were genotyped for the TNF-α (−376, −308, −208, and +488) polymorphisms. The frequencies of these polymorphisms in each clinical group are indicated in Table 1. Statistical analysis of the frequencies of the various phenotypes in F0, FI, and FII subjects showed that these polymorphisms were in Hardy-Weinberg equilibrium (Table 2) ($\chi^2 \leq 5.99$).

We then increased the sizes of the groups to be compared by assigning study subjects to one of two groups: one group was composed of the F0, FI, and FII subjects, and the other consisted of the subjects with the most severe fibrosis (FIII). No statistically significant difference in genotype distribution between these two clinical groups was observed in studies of the TNF-α −238, −308, and +488 polymorphisms. However, there was a trend for an association of TNF-α −376A and FIII ($P = 0.074$, not taking into account multiple comparison) (Table 2). The frequency of the TNF-α −376 G/A genotype was higher (7.4 versus 0%) in FIII subjects than in subjects with less severe fibrosis. This polymorphism may increase the level of transcription of the TNF-α gene (31). As some FII subjects may progress to FIII, we repeated the analysis, comparing FIII with F0 and FI subjects. This additional analysis showed that there was no statistically significant association between any of the TNF-α polymorphisms tested and either of the two clinical groups (not shown).

The second part of the study was carried out with subjects recruited at the endoscopy unit of Wad Medani Teaching Hospital and with controls recruited from contributors to the Wad Medani blood bank or from nearby villages (Table 3). Seventy patients with PPF and portal hypertension and 345 unrelated matched controls were included in this part of the study. Genotype frequencies (TNF-α −376, −308, and −238 polymorphisms) are indicated in Table 3. None of these polymorphisms displayed significant deviation from Hardy-Weinberg equilibrium in the control group (Table 3) ($\chi^2 \leq 5.99$). We used the chi-square test to assess the association between PPF and polymorphisms in TNF-α promoter region (−308, −376, and −238). None of these polymorphisms, including TNF-α −376, was associated with severe disease (Table 3) ($P \geq 0.5$).

In the Taweela population, PPF has been shown to be associated with an increase in TNF-α production (25). Other studies have also indicated that advanced hepatic disease is more likely to occur in human schistosomiasis patients with higher levels of TNF-α production (38, 48). Studies in vitro and with experimental models have shown that TNF-α is required for granuloma formation and the development of hepatic fibrosis (9, 13, 19, 32). Nevertheless, the role of TNF-α in the aggravation of fibrosis remains unclear, because TNF-α may protect against PPF (4, 27) or aggravate this condition (5, 8, 39, 45) in schistosomiasis. TNF-α protects IL-12-vaccinated mice against the deleterious effects of the granuloma (27) and increases the production of NO, the hypotensive effects of which may be beneficial in patients with portal hypertension (4). However, TNF-α also increases ECMP production by Kupffer cells (21), stimulates matrix metalloprotease gene expression (40), and may exacerbate hepatic inflammation by stimulating the production of reactive oxygen species (39).

PPF and advanced liver disease are more severe in Egyptians infected with *S. mansoni* who carry certain HLA haplotypes. This indicates that HLA alleles or TNF-α and TNF-β alleles in linkage disequilibrium with HLA haplotypes may affect disease development (1, 2, 10, 43, 44). Polymorphisms at the HLA locus and hepatosplenomegaly (HS) have also been observed in schistosome-infected patients (1, 10, 43). However, HS is not identical to the clinical phenotypes studied here, because PPF may occur in the absence of HS and HS may develop in the absence of advanced PPF (23, 25, 28). Two of the alleles studied, TNF-α −376A and −308A, increase transcription of the TNF-α gene (3, 31). The lack of a significant

TABLE 3. TNF-α genotype distribution and genotype frequencies in the Gezira cohort[a]

Polymorphism	Genotype	No. with genotype/no. genotyped (% with genotype)		*P* (Fisher's exact test)[c]
		Controls[b]	Cases	
TNF-α −376	G/G	321/344 (93.3)	66/70 (94.3)	
	G/A	22/344 (6.4)	3/70 (4.3)	1
	A/A	1/344 (0.3)	1/70 (1.4)	
TNF-α −308	G/G	275/342 (80.4)	58/70 (82.9)	
	G/A	63/342 (18.4)	12/70 (7.1)	0.636
	A/A	4/342 (1.2)	0/70 (0)	
TNF-α −238	G/G	288/341 (84.5)	58/70 (82.9)	
	G/A	52/341 (15.2)	11/70 (15.7)	0.738
	A/A	1/341 (0.3)	1/70 (1.4)	

[a] A case-control study was performed with patients recruited at the endoscopy unit of Wad Medani Teaching Hospital (Sudan). Healthy controls were recruited from contributors to the Wad Medani blood bank or from nearby villages. All patients with evidence of PPF and varices attending the hospital during the study period were included. Thus, the inclusion criteria were PPF diagnosed by ultrasound and esophageal varices diagnosed by endoscopy. The patients gave informed consent for participation in the study. Control subjects were matched with the patients (cases) for age and sex. All controls lived in the area of the Gezira region in which *S. mansoni* is endemic.
[b] The genotype distributions for the three polymorphisms did not deviate from Hardy-Weinberg equilibrium within the control group ($\chi^2 = 1.05$, 0.03, and 0.71 for TNF-α −376, −308, and −238, respectively).
[c] Fisher's exact test was carried out with SPSS software to test the association between TNF-α polymorphisms and disease.

association between these TNF-α alleles conferring higher levels of transcription and PPF is not consistent with the hypothetical association between higher levels of TNF-α production and PPF. In conclusion, our results show that the TNF-α polymorphisms tested here are unlikely to play an important role in the progression of the disease from mild to severe PPF. However, our data do not exclude the possibility that these polymorphisms play a minor role in disease development or that other polymorphisms (in the TNF-α–HLA locus), not in linkage disequilibrium with the polymorphisms studied here, are associated with PPF. This hypothesis, which needs to be confirmed, is consistent with previous results described by Hirayama (26), showing that in *Schistosomiasis japonica* infection (another form of hepatic schistosomiasis) the frequencies of several HLA class II alleles were significantly increased or decreased in patients with or without fibrosis. The HLA-DRB1*1101-DQB1*0301-DQA1*0501 haplotype decreases susceptibility to grade I, II, and III fibrosis, whereas the HLA-DRB1*1501-DRB5*0101 haplotype increases this susceptibility.

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