Specific Cellular Immune Response and Cytokine Patterns in Patients Coinfected with Hepatitis C Virus and *Schistosoma mansoni*

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Patients coinfected with hepatitis C virus (HCV) and *Schistosoma mansoni* show high incidence of viral persistence and accelerated fibrosis. To determine whether immunological mechanisms are responsible for this alteration in the natural history of HCV, the HCV-specific peripheral $CD4^+$ T cell responses and cytokines were analyzed in patients with chronic hepatitis C monoinfection, *S. mansoni* monoinfection, or HCV and *S. mansoni* coinfection. An HCV-specific $CD4^+$ proliferative response to at least 1 HCV antigen was detected in 73.3% of patients infected with HCV, compared with 8.6% of patients coinfected with HCV and *S. mansoni*. Stimulation with HCV antigens produced a type 1 cytokine profile in patients infected with HCV alone, compared with a type 2 predominance in patients coinfected with HCV and *S. mansoni*. In contrast, there was no difference in response to schistosomal antigens in patients infected with *S. mansoni* alone, compared with those coinfected with HCV and *S. mansoni*. These findings suggest that the inability to generate an HCV-specific $CD4^+$/Th1 T cell response plays a role in the persistence and severity of HCV infection in patients with *S. mansoni* coinfection.

Hepatitis C virus (HCV) infection often is characterized by a protracted clinical course with viral persistence, which leads to liver cirrhosis and hepatocellular carcinoma in some individuals [1–3]. Mechanisms underlying viral persistence and liver damage in chronic HCV are not yet clarified, but a complex interplay of virological and immunologic factors is implicated [4].

Virological factors that may be important are virus load, genotype and quasi-species complexity [5–7]. The host immune response probably plays a critical role in the control of both HCV replication and liver injury [4]. HCV infection evokes $CD4^+$ HLA class II–restricted [8–10] and $CD8^+$ (cytotoxic T lymphocytes) HLA class I–restricted T cell responses [11–13]. $CD4^+$ T lymphocytes have been shown to be crucial for recovering from acute HCV infection and for maintaining such recovery, since the loss of HCV-specific CD4 responses correlates with relapsing disease [14]. However, the role of CD4 cells in the natural history of chronic HCV is not yet well established.

Schistosomiasis is a chronic helminth disease that infects >200 million people worldwide [15]. Infection with *Schistosoma mansoni* is endemic in Egypt, with a prevalence of 17.5%–42.9% [16]. Morbidity in humans infected with *S. mansoni* results primarily from the deposition of parasite ova in the portal areas that induces a T cell–dependent granulomatous response, which progresses to irreversible fibrosis and severe portal hypertension in >60% of cases [17]. *S. mansoni* infection in mice is characterized by a strong Th2-associated immune response that is coupled with a defect in Th1–cell effector function [18–20].

Schistosomiasis and HCV coinfection is common in Egypt [21–24] and other developing countries [25]. Patients coinfected with HCV and schistosomiasis exhibit a unique clinical, virological, and histological pattern manifested by viral persistence with high HCV RNA titers, as well as higher necroinflammatory and fibrosis scores in their liver biopsy samples [23, 24]. However, no study to date has investigated the influence of *S. mansoni* on the HCV-specific cellular immune response in this pattern of coinfection. We hypothesized that the changes of the immune response that are induced by infection with schistosomiasis might influence the HCV-specific immune

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All patients participating in the study presented oral or written informed consent. The protocol and all the procedures of the study were conducted in conformity with the ethical guidelines of the declaration of Helsinki. The human experimentation guidelines of the US Department of Health and Human Services were followed in the conduct of clinical research.

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response, consequently altering the clinical and histological course of HCV in this cohort of patients. Therefore, we investigated the specific $CD4^+$ proliferative response and cytokine profile against HCV and *S. mansoni* antigens in patients coinfected with HCV and *S. mansoni* and compared them with responses observed in patients infected with HCV or *S. mansoni* alone. The spectrum of these responses was correlated to the clinical and histological characteristics of these patients.

Patients and Methods

Patients. Eighty-five Egyptian patients (male:female ratio, 55:30; mean age, 38.0 ± 8.1 years) with proven HCV infection and/or schistosomiasis were enrolled. Patients were matched for age, duration of HCV infection, stage of liver disease at presentation, and HCV genotype (genotype 4). Patients were categorized into 3 groups (table 1). Group A included 30 patients infected with chronic HCV, and inclusion criteria were as follows: seropositivity for antibody to HCV (EIA 2; Abbott Laboratories), positive for HCV RNA by PCR, HCV genotype 4, elevated aminotransferase levels for ⩾6 months, liver biopsy sample showing evidence of chronic hepatitis, and no current or previous therapy with interferon (IFN) or ribavirin. Group B included 20 patients infected with schistosomiasis alone, and inclusion criteria were as follows: history of schistosomiasis, detection of *S. mansoni* ova in stool [26] or rectal biopsy sample, and seropositivity for schistosomal antibodies (indirect heamagglutination; Femouz laboratories). Twelve patients had active schistosomiasis (with viable *S. mansoni* ova in stool or rectal biopsy samples), whereas 8 had chronic infection (with a long history of schistosomiasis, repeated exposures, positive *S. mansoni* antibodies with either viable or nonviable ova in stool or rectal biopsy samples). Group C included 35 patients with chronic HCV and schistosomiasis coinfection, which were diagnosed by the above-mentioned criteria. All patients in group C acquired *S. mansoni* infection before HCV, and all had viable *S. mansoni* ova in stool or rectal biopsy samples. Ten healthy individuals, who were matched for age and sex, served as control subjects.

The patients enrolled in the study had no serological markers for the presence of hepatitis A, hepatitis B, hepatitis D, cytomegalovirus infection, Epstein-Barr virus infection, or other hepatic or intestinal parasites. Autoimmune alcohol- or drug-induced liver disease was ruled out. All patients were subjected to a physical examination, and a clinical history was obtained. The duration of the disease was defined as the interval between the probable time of acquisition of HCV infection (determined by the date of intravenous antischistosomal therapy and/or blood transfusion) and/or schistosomiasis (detected from the history of exposure and clinical presentation) and enrollment.

HCV RNA was detected by reverse-transcription polymerase chain reaction (RT-PCR; detection limit <2000 copies/mL), as described elsewhere [27]. HCV RNA was quantitated by using the Amplicor HCV Monitor kit (Roche Diagnostic Systems). HCV genotype [28] was determined with the line-probe assay (INNO-LiPA; Innogenetics).

Histological assessment. Liver biopsy samples were stained with hematoxylin-eosin and a connective tissue stain (chromotrope aniline blue). Liver biopsy samples were read in a blinded fashion adopting the grading and scoring system proposed by Ishak et al. [29], which includes grading of piecemeal necrosis, confluent necrosis, apoptosis, focal inflammation, and portal inflammation with a score of 0–18, and fibrosis was evaluated with a staging score of 0–6. Moreover, biopsy samples were assessed for morphologic features of schistosomiasis, such as *S. mansoni* ova, eosinophils, granuloma, pigment, and fibrosis of pipe stem type. Evidence for schistosomiasis was graded as follows: 0, no evidence for schistosomiasis; 1, poor evidence for schistosomiasis; 2, suggestive of schistosomiasis; and 3, strong evidence for schistosomiasis.

Table 1. Demographic and baseline characteristics of patients infected with hepatitis C virus (HCV) or *Schistosoma mansoni* alone and patients coinfected with HCV and *S. mansoni.*

Characteristic	Patients infected with		Patients coinfected with HCV and *S. mansoni*
	HCV alone	*S. mansoni* alone	
No.	30	20	35
Male:female ratio	21:9	12:9	22:12
Age, years	42.6 ± 8.1	38.5 ± 3.6	44.2 ± 6.7
Disease duration, years			
HCV	7.4 ± 4.1		6.5 ± 3.9
S. mansoni		10.4 ± 7.5	12.3 ± 6.4
ALT, U/mL	53.5 ± 31.1	23.5 ± 5.7[a]	68.2 ± 28.5[a]
AST, U/mL	58.5 ± 27.3	28.5 ± 5.7[a]	63.5 ± 30.8[a]
RNA titer, $\times 10^5$ copies/mL	14.5 ± 4.8[b]		28.8 ± 8.7[b]
Histological grading score[c]	5.9 ± 2.8	1.5 ± 0.1[a]	7.5 ± 3.1[a]
Histological staging score[c]	2.1 ± 1.1[d]	1 ± 0.1[a]	5.5 ± 0.2[a,d]

NOTE. Data are mean ± SD, unless otherwise indicated. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

[a] $P < .01$, patients infected with *S. mansoni* alone vs. coinfected patients.

[b] $P < .01$, patients infected with HCV alone vs. coinfected patients.

[c] Histological scoring is from Ishak et al. [29], including a necroinflammatory grading score of 0–18 and a fibrosis staging score of 0–6. Liver biopsy specimens also were assessed for morphologic features of schistosomiasis (0, no evidence for schistosomiasis; 1, poor evidence for schistosomiasis; 2, suggestive of schistosomiasis; 3, strong evidence for schistosomiasis).

[d] $P < .001$, patients infected with HCV alone vs. coinfected patients.

Antigens. Purified recombinant HCV proteins (core antigen, aa 2–120; nonstructural antigen 3 [NS3], aa 1192–1457; NS-4, aa 1569–1931; and NS5, aa 2054–2995), derived from the HCV-1 prototype sequence, were purchased from Chiron Mimotopes. All antigens were expressed as COOH-terminal fusion proteins with human superoxide dismutase (SOD) in yeast. Yeast and SOD were used as controls in each proliferation assay for nonspecific stimulation. Soluble egg antigen (SEA) and soluble adult worm antigen protein (SWAP) were a generous gift from Dr. K. Khalifa (Ain Shams University, Cairo) and were prepared as described elswhere [30, 31].

Proliferation assays. Peripheral blood mononuclear cells (PBMC) from patients and control subjects were isolated immediately after collection from fresh heparinized blood, using Ficoll-Hypaque density gradient centrifugation, were washed with PBS, and were resuspended in RPMI 1640 (Gibco) supplemented with 25 m*M* HEPES, 2 m*M* glutamine, 50 mg/mL gentamycin (Gibco), and 10% human AB serum (complete medium). PBMC (2×10^5/mL) were cultured in 200 μL of complete medium/well in triplicates in U-bottom 96-well plates (Costar) in the presence of HCV proteins (10 μg/mL), schistosomal antigens (15–50 μg/mL), and phytohemagglutinin (PHA; 1:200 final dilution; Murex Diagnostics) and tetanus toxoid (Wyeth Laboratories) as positive controls, SOD as

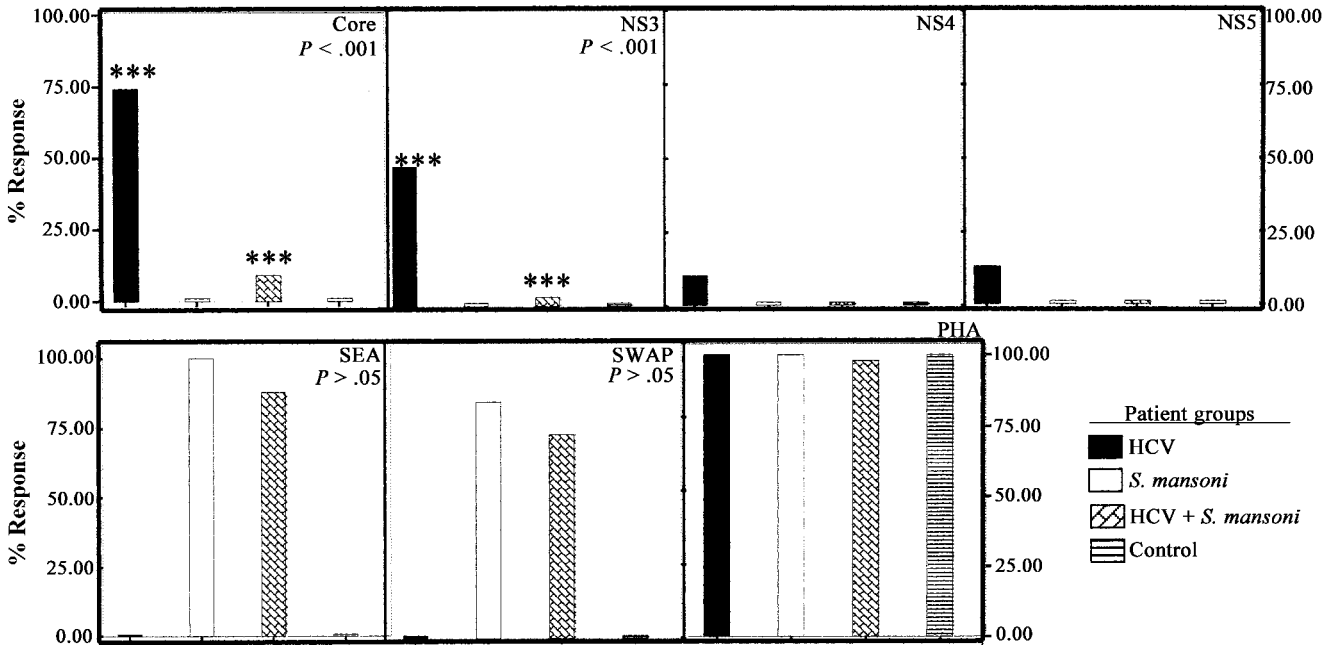

Figure 1. Comparison of overall $CD4^+$ cell response to hepatitis C virus (HCV) proteins (core antigen, nonstructural [NS] antigen 3, NS4, and NS5), *Schistosoma mansoni* antigens (soluble egg antigen [SEA] and soluble adult worm antigen protein [SWAP]), and control antigen (phytohemagglutinin [PHA]), expressed as the percentage of positive response in 3 patient groups (30 patients infected with chronic HCV alone, 20 patients infected with *S. mansoni* alone, and 35 patients coinfected with HCV and *S. mansoni*) and in 10 healthy control subjects. Although 73.3% of patients infected with HCV alone showed $CD4^+$ responses to at least 1 HCV antigen, only 8.3% of coinfected patients had a narrowly focused response to only HCV core antigen, despite responding to *S. mansoni* and control antigen (***$P = .0006$, patients infected with HCV alone vs. coinfected patients, Wilcoxon rank sum test). HCV core antigen was the most frequently recognized HCV antigen, followed by NS3, NS5, and NS4. Of 22 patients infected with HCV alone, 2 responded to all HCV proteins, 3 responded to 3 HCV antigens (core + 2 NS antigens), 5 responded to 2 antigens (core + 1 NS antigen), 8 responded to core antigen alone, and 4 responded to NS3 alone.

a negative control or no stimulation. Cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2.

All proliferation assays were performed after 5 days of culture with HCV antigens, schistosomal proteins, or controls. Proliferation assays were performed with the [^{3}H]-thymidine incorporation method. Cells were pulsed with 1 μCi [^{3}H]-thymidine per well (Amersham) for 18 h. Cells were harvested with an automatic cell harvester (Filter Mate Universal Harvester; Packard) on glass filter paper (Dunn), and [^{3}H]-thymidine incorporation was determined by liquid scintillation counting. Results were expressed as the stimulation index (SI; counts per minute [cpm] incorporated in response to antigen, divided by cpm incorporated in absence of antigen). An SI of 3 was considered to be positive, which represents 3 SD above the mean SI of healthy control subjects (data not shown). Since PBMC from coinfected patients showed poor or no proliferation in response to HCV antigens, proliferation assays also were performed in parallel, using the 5-bromo-2-uridine incorporation assay (ELISA detection kit; Boehringer Mannheim) for all patients and control subjects, at different time points. The results of both assays were comparable (data not shown).

$CD4^+$ and $CD8^+$ cell depletion. In some assays, proliferation assays and cytokine release were done before and after $CD4^+$ and $CD8^+$ depletion, to characterize the cell fractions that produced the response. PBMC (10×10^6) were suspended in 160 mL PBS supplemented with 0.5% bovine serum albumin and 2 m*M* EDTA. Cells were stained with 40 mL paramagnetic microbeads conjugated to a monoclonal mouse anti–human CD8 antibody (Micro Beads; Miltenyl Biotec) for 15 min at 4°C. Magnetic separation was performed by using a depletion column (Miltenyl Biotec), which was placed in a magnetic field of a suitable separator. Fluorescence-activated cell sorter analysis showed >95% $CD4^+$ after $CD8^+$ depletion. Separated cells then were tested in a proliferation assay for cytokine production.

Determination of cell phenotypes. Differentiation of $CD4^+$ and $CD8^+$ lymphocytes was performed by direct fluorescence, using a Coulter Epics II fluorescence-activated cell sorter with fluorescein isothiocyanate–conjugated monoclonal antibodies specific for $CD4^+$ and $CD8^+$ lymphocytes. T cell phenotype were determined as follows: cells ($>1 \times 10^5$) cultured with the respective antigen or mitogen were incubated for 30 min at 4°C with 2 μg/mL of the monoclonal antibodies in different combinations for double staining (Leu-4 and anti-CD3; Leu-3a and anti-$CD4^+$; and Leu-2a and anti-$CD8^+$; Becton Dickinson). Cells then were washed twice with cold PBS and were analyzed on a FACstar flow cytometer.

Cytokine measurements. Serum cytokines were quantitated by commercially available ELISA for IFN-γ (BioSource International) and for interleukin (IL)–4, IL-10, and tumor necrosis factor (TNF)–α (Boehringer Mannheim), according to the manufacturers' instructions. After 48 h of incubation and antigen-specific stimulation, 50 μL of cell-free supernatants were collected from all antigen-stimulated and control wells and then were stored at −85°C until assayed for their cytokine content.

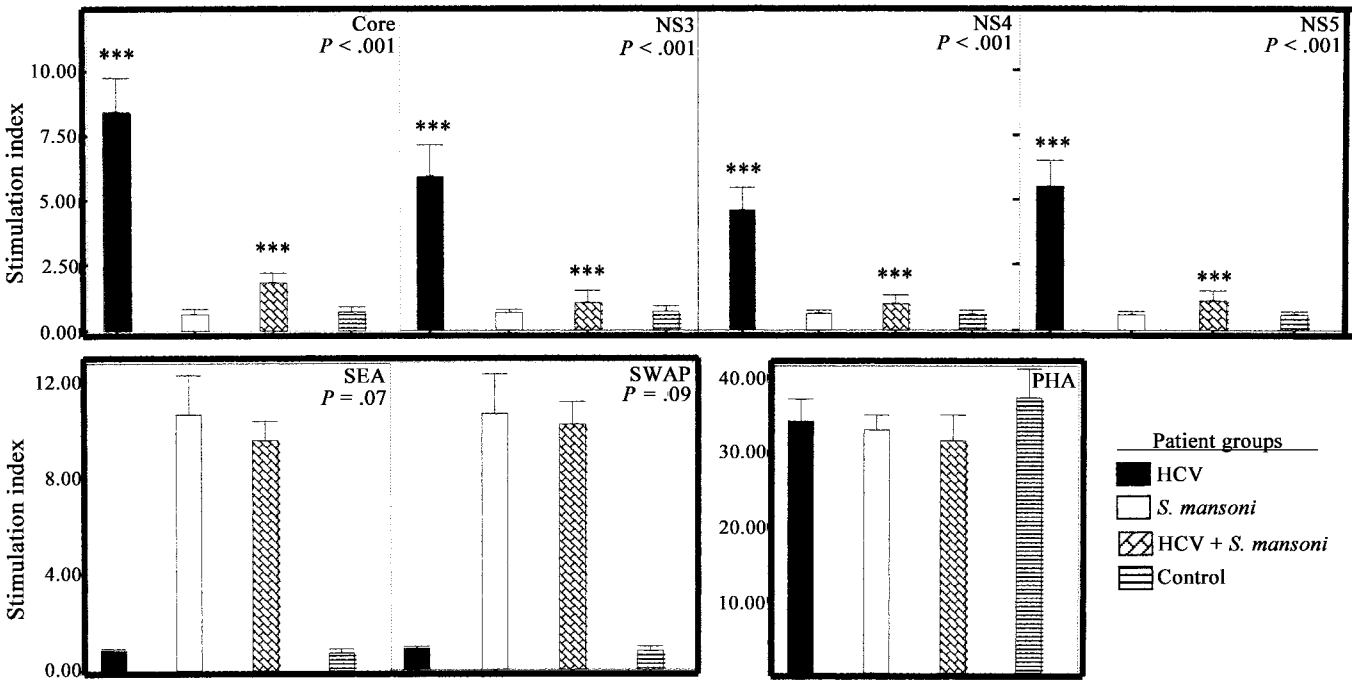

Figure 2. Strength of $CD4^+$ proliferative response to the hepatitis C virus (HCV) proteins (core antigen, nonstructural [NS] antigen 3, NS4, and NS5) and *Schistosoma mansoni* antigens (soluble egg antigen [SEA] and soluble adult worm antigen protein [SWAP]) relative to phytohemagglutinin (PHA). Results are expressed as the stimulation index (positive >3, which represents 3 SD above the mean stimulation index of normal control subjects) of 3 patient groups (30 patients infected with chronic HCV alone, 20 patients infected with *S. mansoni* alone, and 35 patients coinfected with HCV and *S. mansoni*) and 10 healthy control subjects. Patients infected with chronic HCV alone showed a more significant response to core antigen, NS3, NS4, and NS5, compared with that of coinfected patients (***$P < .001$, Student's t test; $P = .0006$, Wilcoxon rank sum test). There was no statistically significant difference in the strength of proliferative responses to *S. mansoni* antigens (SEA and SWAP) between patients infected with *S. mansoni* alone and coinfected patients ($P = .07$ and $P = .09$, respectively). There was no significant difference in the strength of proliferative response to PHA between patient groups and control subjects.

HLA typing. DNA extraction and DRB1, DRB3, DRB4, DRB5, DQA1, and DQB1 typing were performed by line-probe assay (INNO-LiPA; Innogenetics), according to the manufacturer's instructions. In brief, genomic DNA was extracted from PBMC of each patient and control subject, and biotin 5′ end–labeled primers for DRB and DQB were used in PCR amplification. The PCR products then were denatured before the addition of hybridization buffer and were incubated in genotyping strips for 30 min at different temperatures, which then were washed.

Statistical analysis. Results were expressed as mean ± SD and were analyzed by using paired and unpaired Student's t test, χ^2 test, nonparametric Mann-Whitney U test, Wilcoxon rank sum test, or Fisher's exact test, as appropriate. Correlation between different parameters was performed by using Pearson's or Spearman's rank test. $P \leq .05$ was considered to be significant. All statistical procedures were performed by using SPSS software, version 10 for Windows.

Results

The clinical, virological, and histological profile of all patients is shown in table 1. Patients coinfected with HCV and *S. mansoni* had higher HCV RNA titers and fibrosis scores, compared with patients infected with either HCV or *S. mansoni* alone, when matched for comparable disease duration and age. There was no significant difference in inflammatory scores between patients infected with HCV alone and patients coinfected with

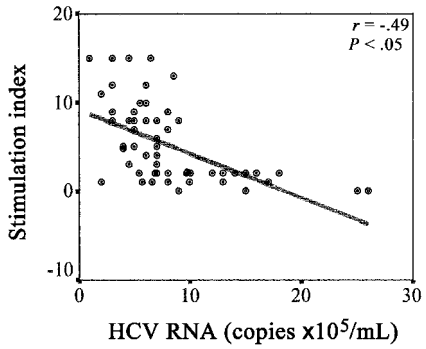

Figure 3. Relation of hepatitis C virus (HCV)–specific $CD4^+$ proliferative response to HCV RNA titers (virus load). The mean $CD4^+$ proliferative response to HCV core antigen (stimulation index) is plotted against the HCV RNA titers (copies $\times 10^5$/mL). The 2 parameters show a positive relationship ($r = -.49$; $P < .05$).

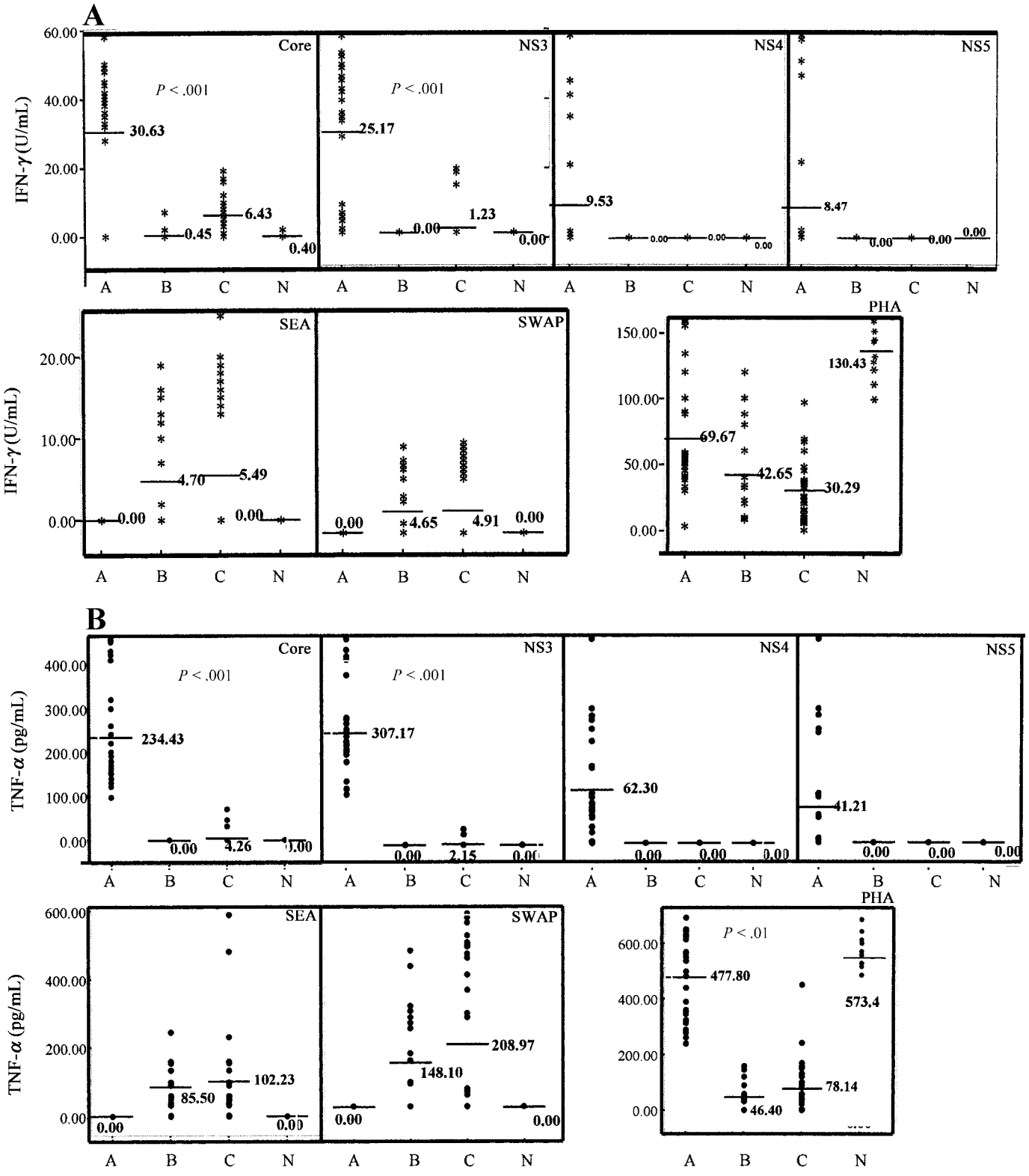

Figure 4. Cytokine production in response to hepatitis C virus (HCV) proteins (core antigen, nonstructural [NS] antigen 3, NS4, and NS5) and *Schistosoma mansoni* antigens (soluble egg antigen [SEA] and soluble adult worm antigen protein [SWAP]) relative to phytohemagglutinin (PHA). *A,* Interferon (IFN)–γ; *B,* tumor necrosis factor (TNF)–α; *C,* interleukin (IL)–4; *D,* IL-10. Results are expressed as mean ± SD. Sixteen patients infected with HCV alone showed significantly greater production of IFN-γ and TNF-α in response to HCV antigens, which is consistent with a type 1 $CD4^+$ T cell response, whereas 6 patients infected with HCV alone had IL-4 and IL-10 in addition to IFN-γ detectable in supernatants, which is consistent with a Th0 pattern. HCV stimulation produced IL-4 and IL-10 but no IFN-γ in 3 coinfected patients who recognized HCV core antigen and NS3 with a Th2 profile. A, 30 patients infected with chronic HCV; B, 20 patients infected with *S. mansoni;* C, 35 patients coinfected with HCV and *S. mansoni;* N, 10 healthy control subjects.

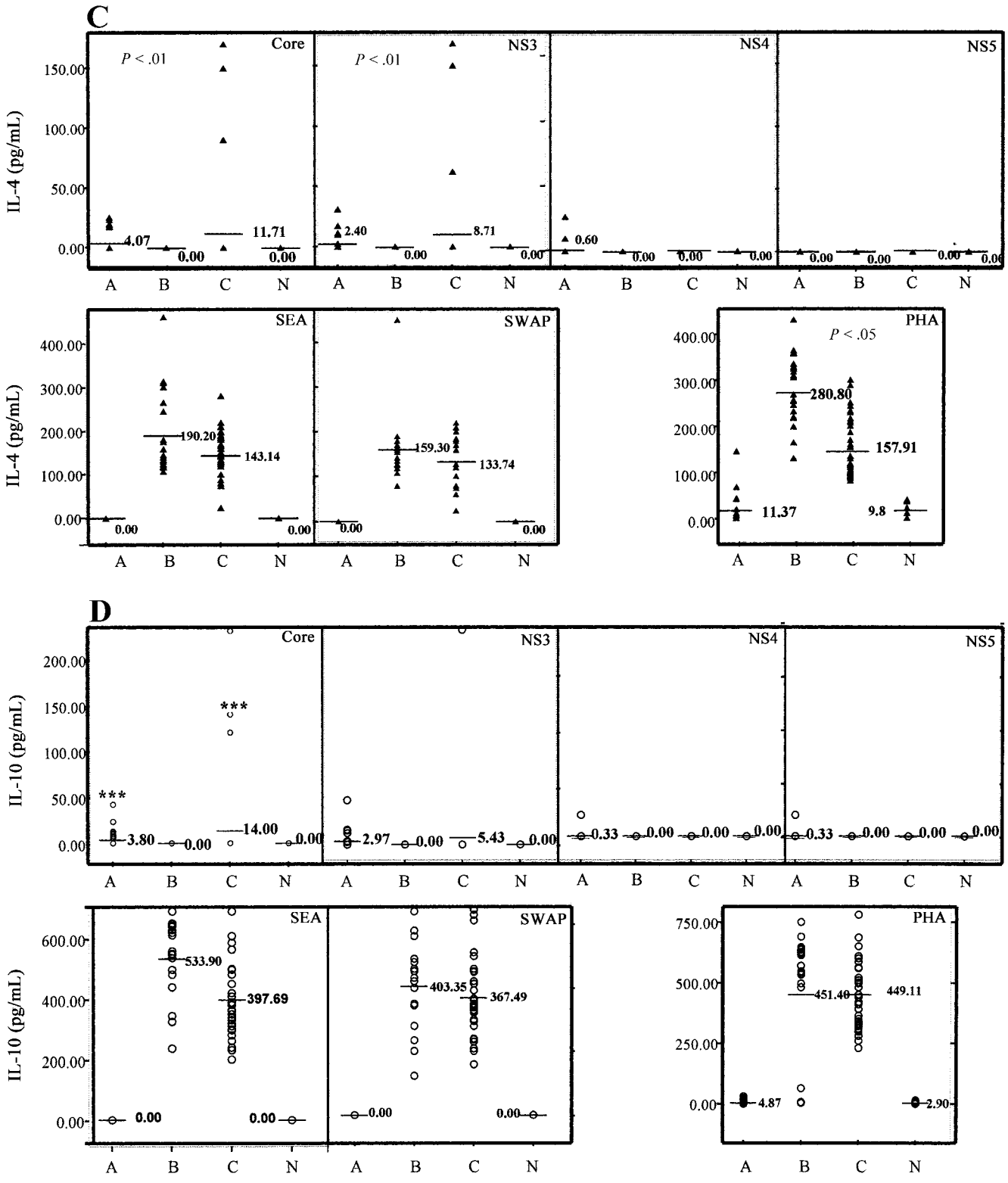

Figure 4. *(Continued.)*

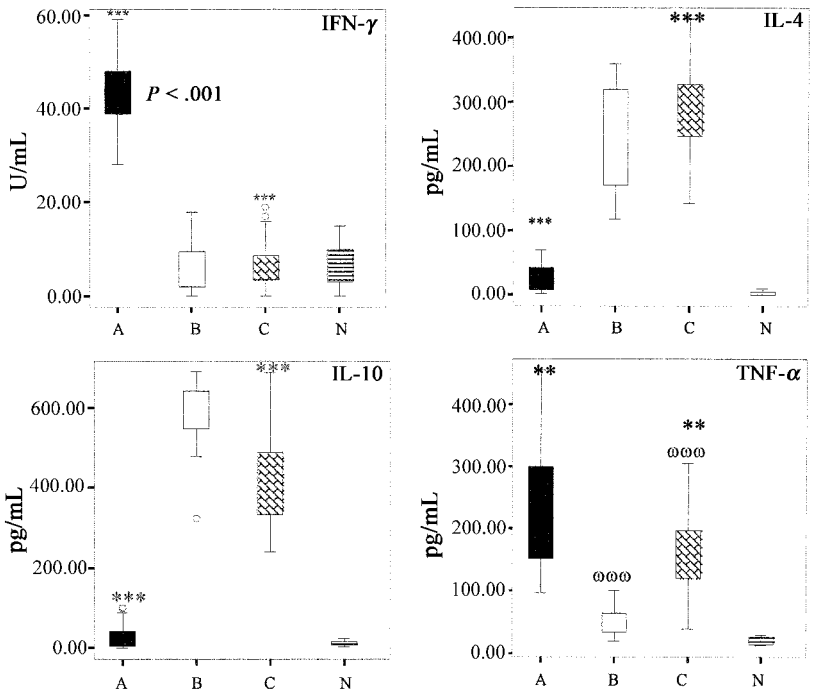

Figure 5. Serum cytokine levels of interferon (IFN)–γ, tumor necrosis factor (TNF)–α, interleukin (IL)–4, and IL-10 in the 3 patient groups and the control subject group: A, 30 patients infected with chronic hepatitis C virus (HCV); B, 20 patients infected with *Schistosoma mansoni;* C, 35 patients coinfected with HCV and *S. mansoni;* N, 10 healthy control subjects. Results are expressed as mean ± SD. *Boxes,* median values; *vertical lines,* ranges. $^{**}P < .01$; $^{***}P < .001$ between A and C; $^{\omega\omega\omega}P < .001$ between B and C.

HCV and *S. mansoni,* although inflammatory scores were significantly higher for patients infected with HCV alone and patients coinfected with HCV and *S. mansoni,* compared with those for patients infected with *S. mansoni* alone. There was no significant difference in HLA-DR phenotypes of the patients in the 3 groups. DRB1 0101, 0401, 1101, 0701, and 1104 were the most common HLA-DR molecules expressed in our patient population (data not shown).

We first characterized the overall magnitude of $CD4^+$ response in patients infected with HCV or *S. mansoni* alone and in patients coinfected with HCV and *S. mansoni* by measuring the level of PBMC proliferation in response to HCV proteins (core antigen, NS3, NS4, and NS5) or *S. mansoni* proteins (SEA and SWAP) relative to control antigens. The percentage of positive response to each antigen is shown in figure 1, and the strength of response to individual antigens is shown in figure 2. No patients infected with HCV alone responded to schistosomal antigens, and no patients infected with *S. mansoni* alone responded to any of the HCV antigens. Overall, 22 (73.3%) of 30 patients infected with HCV alone showed significant proliferative response to ⩾1 HCV antigen (SI, 3.4–13.8). Of these 22 patients, 16 responded to core antigen, 12 to NS3, 4 to NS5, and 3 to NS4. In striking contrast, only 3 (8.6%) patients coinfected with HCV and *S. mansoni* showed a weak, narrowly focused response to HCV core antigen (SI, 3.2–4.8; $P = .0006$, Wilcoxon rank sum test between patients infected with HCV and patients coinfected with HCV and *S. mansoni*), whereas 32 patients did not respond to any of the HCV proteins (figure 2). Despite the very poor proliferative response to HCV antigens, T cells from coinfected patients responded to PHA (figure 2) and/or tetanus toxoid (data not shown) without any statistically significant difference in the strength of proliferative responses to non-HCV antigens, when compared with responses in patients infected with HCV alone (figure 2). This suggests that infection with *S. mansoni* specifically inhibited the HCV-specific CD4 response in the coinfected patients. Comparison of coinfected patients who responded to recombinant HCV antigens with those who showed no response failed to show any significant differences in age, disease duration, HLA-DR phenotypes, or stage of liver disease. Moreover, this subset of coinfected patients mounted a vigorous proliferative response against SEA and SWAP that was not significantly different from the respones in patients infected with *S. mansoni* alone (SEA, $P = .07$; SWAP, $P = .09$; patients infected with *S. mansoni* alone and patients coinfected).

We then asked whether the vigor of the CD4 response could be related directly to the virus load or the degree of liver damage, since patients with advanced liver disease might have depressed cellular immunity. The association between $CD4^+$ proliferation assay results (SIs) against core antigen and HCV RNA titer is plotted in figure 3. Patients showing positive HCV-

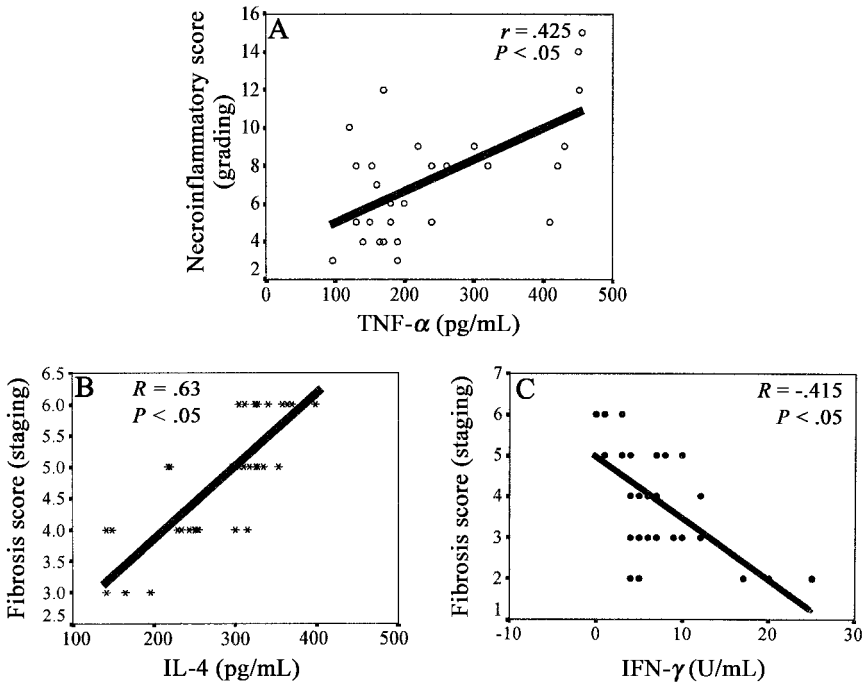

Figure 6. *A,* Relation of tumor necrosis factor (TNF)–α to necroinflammatory scores. The 2 parameters show a positive relationship in patients infected with hepatitis C virus alone ($r = .425$; $P < .05$), whereas no such significant correlation could be detected in patients infected with *Schistosoma mansoni* alone or in coinfected patients. *B,* Relation of interleukin (IL)–4 to fibrosis scores in all patients. The 2 parameters show a positive relationship ($r = .63$; $P < .05$). *C,* Relation of interferon (IFN)–γ to fibrosis scores in all patients. The 2 parameters show a positive relationship ($r = -.415$; $P < .05$).

specific responses had lower mean and median virus loads than those who did not respond to HCV antigens ($r = -.49$; $P < .05$). However, no statistically significant correlation was observed between the proliferative $CD4^+$ response and the histological necroinflammatory scores (grading) or fibrosis scores (data not shown). This suggests that the magnitude of the $CD4^+$ response is not a critical determinant of outcome in coinfection.

We then hypothesized that an alteration in the cytokine milieu exists with HCV and *S. mansoni* coinfection. Subsequently, cytokine production in response to HCV, *S. mansoni,* and nonspecific stimulation was analyzed in patients and control subjects. Figure 4 shows results of cytokine production in response to HCV, *S. mansoni,* and mitogenic stimulation. Among 16 patients infected with HCV alone, HCV stimulation led to the production of IFN-γ and TNF-α but not IL-4 or IL-10, which is consistent with a type 1 $CD4^+$ T cell response, whereas IL-4 and IL-10, in addition to IFN-γ, were detectable in supernatants derived from 6 patients (Th0 profile), but no Th2 pattern was found. In addition, all patients infected with HCV alone produced IFN-γ and TNF-α after mitogenic stimulation of PBMC. On the other hand, HCV stimulation produced IL-4 and IL-10, but IFN-γ (Th2 profile) was not produced in the 3 coinfected patients who recognized the HCV core antigen. Stimulation with SEA and SWAP produced high levels of IL-4 and IL-10 alone with a Th2 pattern in 16 of 20 patients infected with *S. mansoni* alone and in 26 of 35 coinfected patients. IFN-γ also was detected in supernatants from 4 of 20 patients infected with *S. mansoni* alone and from 9 of 35 coinfected patients. Therefore, only Th2 or Th0 patterns, but not the Th1 profile, of cytokine production were observed in coinfected patients after HCV (in 3 responding coinfected patients), *S. mansoni,* or nonspecific stimulation. In patients infected with HCV alone, the amount of IFN-γ produced after stimulation with core antigen and NS3 was significantly higher than that produced in the 3 coinfected patients who responded to the HCV core antigen ($P < .001$).

The levels of individual serum cytokines are shown in figure 5. Coinfected patients had high IL-4 and IL-10 levels, whereas patients infected with HCV alone had higher IFN-γ and TNF-α levels. For all studied patients, there was no significant correlation between TNF-α levels and the grading or staging scores. However, a significant correlation did exist between TNF-α and the necroinflammatory score when correlation was restricted to patients infected with HCV alone ($r = .425$; $P < .01$; figure 6*A*). On the other hand, there was a significant direct correlation between IL-4 levels and the fibrosis/cirrhosis score (staging) and an inverse correlation between IFN-γ levels and the fibrosis/cirrhosis score (figure 6*B* and 6*C*). However, no correlation was found between IFN-γ, IL-4, or IL-10 and necroinflammation (grading).

Discussion

In previous studies, we and others reported that patients coinfected with HCV and schistosomiasis have a more severe clinical course, higher HCV RNA titers, higher incidence of cirrhosis, hepatocellular carcinoma, poor response to IFN therapy, and higher mortality rates due to liver-related causes, compared with patients infected with HCV alone [23–25, 32]. Thus, we investigated the possibility that this difference in clinical outcome and course of HCV infection in coinfected patients might be related to the alteration in HCV-specific $CD4^+$ T cell response and cytokine profile caused by schistosomiasis. To test this premise, which has not been studied before, we assessed the HCV-specific $CD4^+$ T cell proliferative response and cytokine profile in the largest cohort, to date, of patients coinfected with HCV and *S. mansoni* versus patients infected with either HCV or *S. mansoni* alone.

Our results showed that significant differences in HCV-specific $CD4^+$ T cell proliferative response and cytokine profile could be detected between patients infected with HCV alone and coinfected patients. In general, coinfected patients were hyporesponsive to HCV antigen, despite their responsiveness to control antigens. They responded to schistosomal antigens and mitogen with a Th2/Th0 cytokine pattern, with elevated IL-4 and IL-10 levels similar to those detected in patients infected with *S. mansoni* alone, which has been described elsewhere [18–20, 33]. On the other hand, 73.3% of patients infected with chronic HCV alone were capable of mounting an HCV-specific $CD4^+$ proliferative response with a Th1/Th0 cytokine pattern, which is in accordance with the results of other studies [8, 34–36]. Our results showed a higher HCV-specific $CD4^+$ proliferative response in patients infected with HCV alone, compared with that reported elsewhere [8, 9, 10, 34]. This may be attributed to the relatively shorter duration of HCV infection in our patients.

Several lines of evidence suggest that the lack of HCV-specific $CD4^+$ T cell recognition detected in the coinfected patients is due to an influence of *S. mansoni* on the host immune system. It is unlikely that the HCV genotype is responsible for nonresponsiveness, since in our study all patients infected with HCV alone were infected with HCV genotype 4. Although proteins used in this study were derived from an HCV genotype 1a isolate, a high proportion of patients infected with HCV alone (73.3%), as well as 3 (8.6%) coinfected patients, had responses to ≥ 1 of the recombinant proteins used. Second, the lack of HCV $CD4^+$ response in coinfected patients was not due to a decline in responses, since, at entry, the patients were matched for age, duration of HCV infection, and stage of liver disease. Lack of recognition of viral antigens probably is not due to differences in HLA haplotypes, since all patients infected with HCV, who were with or without schistosomiasis, included in our study were from the same geographic area and displayed similar HLA-DR antigens. Therefore, patients coinfected with *S. mansoni* had the potential to present the immunodominant epitopes but were unable to mount a $CD4^+$ proliferative response.

In our study, the Th2 pattern was demonstrated to be dominant in schistosomiasis, which is in agreement with previous reports of human [20, 37] and murine models [18]. However, the present study is the first to investigate the impact of the *Schistosoma*-mediated predominance of a Th2 response and the lack of a Th1 response during the course of HCV infection. Although a predominant Th2 profile was shown to be beneficial in polyparasitism, in which mice infected with *S. mansoni* are capable of eliminating *Trichuris muris* infection more efficiently than are noninfected mice [38], it is assumed to be harmful in most viral infections. In our coinfected patients, HCV infection probably was acquired after *S. mansoni* infection was established, through parenteral antischistosomal therapy and/or blood transfusions. Thus, in the first encounter of HCV with the host immune system, it was confronted with a prevailing Th2 pattern. One may speculate that this Th2 profile induced by *S. mansoni* antagonized and down-regulated the antiviral activities of Th1 cytokines, resulting in increased viral replication and more-aggressive progression to fibrosis. Imbalance toward a Th2 profile at the time of acquisition of HCV infection or during the acute stage of the disease may favor the faster progression to chronicity in coinfected patients, which has been described elsewhere [23–25]. This finding is further corroborated by Tsai et al. [39], since their analysis of the cytokine profile of bulk cultures, as well as of $CD4^+$ T cell clones, from patients infected with HCV revealed that viral clearance is more likely in patients displaying a Th1 cytokine profile than in patients producing a Th2 cytokine profile.

In our study, coinfected patients had significantly high HCV RNA titers and an inverse relationship between virus load and $CD4^+$ T cell responses. This may be explained by the lack of viral control due to defective HCV-specific $CD4^+$ T cell response or abrogation of the HCV-specific Th1 response, which is antagonized by the *S. mansoni*–induced Th2 response. On the other hand, the high initial virus load in these coinfected patients may cause immune exhaustion [40], thereby aggravating HCV infection. This could not be confirmed in the current study, since we did not assess the cytotoxic responses, which we are currently assessing. However, Actor et al. [41] showed that mice infected with vaccinia virus alone rapidly cleared the virus, whereas viral clearance in animals coinfected with *S. mansoni* was delayed in the liver by as much as 3 weeks and in the spleen and lungs by several days. In another study [42], BALB/c mice infected 7 weeks earlier with *S. mansoni* and challenged with recombinant vaccinia virus vPE16, which expresses the human immunodeficiency virus envelope protein gp160, showed a marked delay in hepatic viral clearance, compared with that in mice infected with vPE16 alone. This increase in viral persistence was accompanied by reduced gp120-specific Th1-associated cytokine responses, as well as by impaired cytotoxic T lymphocyte activity against targets expressing epitopes

of the same antigen. These observations in the mouse model, further supports our findings, which suggest that helminth infection may influence immune responses to concurrent viral infections.

S. mansoni causes liver pathology through an immune-mediated mechanism, rather than through direct hepatic injury [33]. *S. mansoni* ova are trapped in the liver, evoking a highly skewed Th2 immune response profile with granuloma formation that progresses to periportal fibrosis. However, the ultrastructure and function of hepatocytes are minimally affected. Our study, as well as previous reports [23–25], showed that patients infected with schistosomiasis alone had very low inflammatory scores (<2), with no piecemeal necrosis. The high fibrosis scores of patients with concomitant infection may be immunologically mediated with predominance or up-regulation of the collagen-inducing cytokine IL-4 and the down-regulation of the collagen-suppressing cytokine IFN-γ, as was detected in our study and in other studies of schistosomiasis in mice [43, 44] or in humans [37]. However, it is important to consider that our study focused on the peripheral blood compartment, which may reflect only immune events that occur in the liver, because of compartmentalization of immune responses [9, 45].

In conclusion, our data show that patients coinfected with chronic HCV and schistosomiasis fail to mount significant HCV-specific $CD4^+$ T cell responses and show alteration in the cytokine milieu, along with more-severe liver disease. This also may have important implications for treatment, since it would imply that down-regulation of immune responses, at least early in disease, may have an adverse impact on disease progression.

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References

1. Seeff LB. Natural history of hepatitis C. Hepatology **1997**; 26(Suppl 1):S21–8.
2. Alter MJ. Epidemiology of hepatitis C. Hepatology **1997**; 26(Suppl 1):S62–5.
3. Dienstag JL. The natural history of chronic hepatitis C and what we should do about it. Gastroenterology **1997**; 112:651–5.
4. Cerny A, Chisari FV. Pathogenesis of chronic hepatitis C: immunological features of hepatic injury and viral persistence. Hepatology **1999**; 30: 595–601.
5. Rossini A, Ravaggi A, Agostinelli E, et al. Virological characterization and liver histology in HCV-positive subjects with normal and elevated ALT levels. Liver **1997**; 17:133–8.
6. Yuki N, Hayashi N, Moribe T, et al. Relation of disease activity during chronic hepatitis C infection to complexity of hypervariable region 1 quasispecies. Hepatology **1997**; 25:439–44.
7. Ray S, Wang Y, Layendecker O, Ticehurst J, Villano S, Thomas D. Acute hepatitis C virus structural gene sequences as predictors of persistent viremia: hypervariable region 1 as a decoy. J Virol **1999**; 73:2938–46.
8. Hoffmann RM, Diepolder HM, Zachoval R, et al. Mapping of immunodominant $CD4^+$ T lymphocyte epitopes of hepatitis C virus antigens and their relevance during the course of chronic infection. Hepatology **1995**; 21:632.
9. Minutello MA, Pileri P, Unutmaz D, et al. Compartmentalization of T lymphocytes to the site of disease: intrahepatic $CD4^+$ T cells specific for the protein NS4 of hepatitis C virus in patients with chronic hepatitis C. J Exp Med **1993**; 178:17–25.
10. Löhr HF, Schlaak J, Kollmannsperger S, Dienes HP, Meyer zum Büschenfelde KH, Gerken G. Liver-infiltrating and circulating $CD4^+$ T cells in chronic hepatitis C: immunodominant epitopes, HLA-restriction and functional significance. Liver **1996**; 16:174–182.
11. Wong DK, Dudley DD, Afdhal NH, et al. Liver-derived CTL in hepatitis C virus infection: breadth and specificity of responses in a cohort of persons with chronic infection. J Immunol **1998**; 160:1479–88.
12. Rehermann B, Chang KM, McHutchison JG, Kokka R, Houghton M, Chisari FV. Quantitative analysis of the peripheral blood cytotoxic T lymphocyte response in patients with chronic hepatitis C virus infection. J Clin Invest **1996**; 98:1432–40.
13. Nelson DR, Marousis CG, Davis GL, et al. The role of hepatitis C virus–specific cytotoxic T lymphocytes in chronic hepatitis. J Immunol **1997**; 158:1473–81.
14. Gerlach JT, Diepolder HM, Jung MC, et al. Recurrence of hepatitis C virus after loss of virus-specific $CD4^+$ T-cell response in acute hepatitis C. Gastroenterology **1999**; 117:993–41.
15. Warren K. Blood flukes (schistosomes) and liver flukes. In: McIntyre N, Benhamou JP, Bircher J, Rizzetto M, Rhodes J, eds. Oxford textbook of clinical hepatology. New York: Oxford University Press, **1991**:714–21.
16. El-Khoby T, Galal N, Fenwick A, et al. The epidemiology of schistosomiasis in Egypt: summary findings in nine governorates. Am J Trop Med Hyg **2000**; 62(Suppl 2):88–99.
17. Capron A, Dessaint JP. Immunologic aspects of schistosomiasis. Ann Rev Med **1992**; 43:209–18.
18. Sabin EA, Pearce EJ. Early IL-4 production by non-$CD4^+$ cells at the site of antigen deposition predicts the development of a T helper 2 cell response to *Schistosoma mansoni* eggs. J Immunol **1995**; 155:4844–55.
19. Estaquier J, Marguerite M, Sahuc F, Bessis N, Auriault C, Ameisen JC. Interleukin-10–mediated T cell apoptosis during the T helper type 2 cytokine response in murine *Schistosoma mansoni* infection. Eur Cytokine Netw **1997**; 8:153–60.
20. Malaquias LC, Falcao PL, Silveira AM, et al. Cytokine regulation of human immune response to *Schistosoma mansoni:* analysis of the role of IL-4, IL-5, and IL-10 on peripheral blood mononuclear cell responses. Scand J Imunol **1997**; 46:393–8.
21. Kamel MA, Miller FD, El-Masry AG, et al. The epidemiology of *Schistosoma mansoni,* hepatitis B and hepatitis C infection in Egypt. Ann Trop Med Parasitol **1994**; 88:501–9.
22. Bassily S, Hyams KC, El-Masry NA, Hassan NF, Watts DM. Hepatitis C virus infection and hepatosplenic schistosomiasis. Scand J Infect Dis **1992**; 4:687–8.
23. Kamal SM, Madwar MA, Bianchi L, et al. Clinical, virological, and histopathological features: long-term follow-up in patients with chronic hepatitis C coinfected with *Schistosoma mansoni.* Liver **2000**; 20:281–9.
24. Angelico M, Renganathan E, Gandin C, et al. Chronic liver disease in Alexandria governorate, Egypt: contribution of schistosomiasis and hepatitis virus infections. J Hepatol **1997**; 26:236–43.
25. Pereira LM, Melo MC, Saleh MG, et al. Hepatitis C virus infection in *Schistosomiasis mansoni* in Brazil. J Med Virol **1995**; 45:423–8.
26. Katz N, Chaves A, Pellegrino J. A simple device for quantitative stool thick-smear technique in *Schistosomiasis mansoni.* Rev Inst Med Trop Sao Paulo **1972**; 14:397–400.
27. Peters T, Mohr F, Scheiffele F, et al. Antibodies and viraemia in acute post-transfusion hepatitis C: a prospective study. J Med Virol **1994**; 42:420–7.
28. Simmonds P, Alberti A, Alter HJ, et al. A proposed system for the nomenclature of hepatitis C viral genotypes. Hepatology **1994**; 19:1321–4.
29. Ishak K, Baptista A, Bianchi L, et al. Histological grading and staging of chronic hepatitis. J Hepatol **1995**; 22:696–9.
30. Boctor FN, Nash TE, Cheever AW. Isolation of a polysaccharide antigen from *Schistosoma mansoni* eggs. J Immunol **1979**; 122:39–43.

31. James SL, Pearce EJ, Sher A. Induction of protective immunity against *Schistosoma mansoni* by non-living vaccine. I. Partial characterization of antigens recognized by antibodies from mice immunized with soluble schistosome extracts. J Immunol **1985**; 134:3432–8.
32. Kamal S, Madwar M, Peters T, Fawzy R, Rasenack J. Interferon therapy in patients with chronic hepatitis C and schistosomiasis. J Hepatol **2000**; 32:172–4.
33. Hoffmann K, Cheever A, Wynn T. IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. J Immunol **2000**; 164:6406–16.
34. Chang K, Thimme R, Melpolder J, et al. Differential CD4 and CD8 T-cell responsiveness in hepatitis C virus infection. Hepatology **2001**; 33:267–76.
35. Diepolder HM, Zachoval R, Hoffmann RM, Jung MC, Gerlach T, Pape GR. The role of hepatitis C virus specific $CD4^+$ T lymphocytes in acute and chronic hepatitis C. J Mol Med **1996**; 74:583–8.
36. Napoli J, Bishop GA, McGuinness PH, Painter DM, McCaughan GW. Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. Hepatology **1996**; 24:759–65.
37. Montenegro SM, Miranda P, Mahanty S, et al. Cytokine production in acute versus chronic human *Schistosomiasis mansoni:* the cross-regulatory role of interferon-γ and interleukin-10 in the responses of peripheral blood mononuclear cells and splenocytes to parasite antigens. J Infect Dis **1999**; 179:1502–14.
38. Curry AJ, Else KJ, Jones F, Bancroft A, Grencis RK, Dunn DW. Evidence that cytokine-mediated immune interactions induced by *Schistosoma mansoni* alter disease outcome in mice concurrently infected with *Trichuris muris.* J Exp Med **1995**; 181:769–74.
39. Tsai SL, Liaw YF, Chen MH, Huang CY, Kuo GC. Detection of type 2–like T-helper cells in hepatitis virus infection: implications for hepatitis C virus chronicity. Hepatology **1997**; 25:449–58.
40. Wodarz D, Klenerman P, Nowak MA. Dynamics of cytotoxic T-lymphocyte exhaustion. Proc R Soc Lond B Biol Sci **1998**; 265:191–203.
41. Actor JK, Shirai M, Kullberg MC, Buller RM, Sher A, Berzofsky JA. Helminth infection results in decreased virus-specific $CD8^+$ cytotoxic T-cell and Th1 cytokine responses as well as delayed virus clearance. Proc Natl Acad Sci USA **1993**; 90:948–52.
42. Actor JK, Marshall MA, Eltoum IA, Buller RM, Berzofsky JA, Sher A. Increased susceptibility of mice infected with *Schistosoma mansoni* to recombinant vaccinia virus: association of viral persistence with egg granuloma formation. Eur J Immunol **1994**; 24:3050–6.
43. Chiaramonte MG, Donaldson DD, Cheever AW, Wynn TA. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2–dominated inflammatory response. J Clin Invest **1999**; 104:777–85.
44. Farah IO, Mola PW, Kariuki TM, Nyindo M, Blanton RE, King CL. Repeated exposure induces periportal fibrosis in *Schistosoma mansoni*–infected baboons: role of TGF-β and IL-4. J Immunol **2000**; 164:5337–43.
45. Bertoletti A, D'Elios MM, Boni C, et al. Different cytokine profiles of intrahepatic T cells in chronic hepatitis B and hepatitis C virus infections. Gastroenterology **1997**; 112:193–9.