Effects of Interferon Treatment on the Antiviral T-Cell Response in Hepatitis C Virus Genotype 1b– and Genotype 2c–Infected Patients

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The viral genotype may influence the response to interferon (IFN) treatment in chronic hepatitis C virus (HCV) infection. To characterize potential mechanisms responsible for this effect, we assessed whether IFN modulation of HCV-specific T-cell responses differs in patients infected by different genotypes. The T-cell response to HCV core protein was sequentially analyzed before and during IFN treatment in two groups of patients chronically infected with HCV genotype 1b (eight patients) or 2c (eight patients). Overlapping 20-mer peptides corresponding to the amino acid sequence of the prevalent viral population identified in the serum of each patient were used for the analysis of the T-cell proliferative response to avoid possible problems caused by amino acid differences between infecting virus and HCV proteins used in vitro. Recombinant HCV core antigen was used in parallel. The level of viremia was monitored by competitive polymerase chain reaction (PCR). The T-cell response to HCV peptides and recombinant core protein detected throughout the follow-up was significantly more vigorous in genotype 2c– than in genotype 1b–infected patients. This difference was the result of a greater enhancement of the T-cell response caused by IFN treatment in genotype 2c– compared with genotype 1b–infected patients. The different IFN modulatory effect on T cells from genotype 1b– and genotype 2c–infected patients illustrates an aspect of the virus-host interaction, which may contribute toward the explanation of why different genotypes differ in responsiveness to IFN treatment. (HEPATOLOGY 1997;26:792-797.)

Interferon (IFN) treatment has a beneficial and long-lasting effect in approximately 20% of patients with chronic hepatitis C.1 Whether this effect is directly antiviral or is caused by the IFN immunoregulatory activity, leading to enhancement of major histocompatibility complex expression and more efficient antiviral immune response, is still unclear. Several factors including viral load, disease severity, age and duration of infection, and genotype of the infecting virus have been shown in different studies to have a predictive value for the outcome of the IFN treatment.2 Although definitive studies are still needed to define the influence of genotype, a higher rate of long-term response to IFN has been reported in genotypes 2 and 3 compared with genotype 1b–infected patients.2

To characterize the mechanisms by which different genotypes might influence responsiveness to IFN, we studied the effect of IFN treatment on the antiviral T-cell response in eight patients with chronic hepatitis C infected by genotype 1b and eight infected by genotype 2c. The study was focused on the T-cell response to hepatitis C virus (HCV) core antigen because of its high immunogenicity at the T-cell level. To limit possible problems arising from the use of proteins with amino acid sequences different from those of the infecting virus for T-cell analysis in vitro, the core region of the prevalent viral strains infecting each patient was sequenced, and different panels of synthetic peptides corresponding to the different sequences were used to stimulate individual T-cell responses.

Results show a different behavior of the HCV core-specific T-cell response in the two groups of patients; the response was significantly enhanced by IFN treatment in patients infected with genotype 2c, whereas a weaker effect was observed in patients infected with genotype 1b.

PATIENTS AND METHODS

Patients. Sixteen patients with chronic hepatitis C (12 males and 4 females; mean age, 49 years) of sporadic origin were selected for this study on the basis of the HCV genotype; 8 were infected by genotype 1b, and 8 by genotype 2c.

Diagnosis was based on biochemical and histological findings showing evidence of chronic active hepatitis, with different degrees of tissue damage and inflammation (Table 1). All patients were anti-HCV antibody–positive by second-generation enzyme-linked immunosorbent assay (ELISA II, Ortho Diagnostic Systems, Raritan, NJ), which detects antibodies to core, nonstructural 3, and nonstructural 4 proteins. They were all negative for hepatitis B surface antigen and for antibodies to hepatitis B surface antigen, hepatitis B core antigen, and human immunodeficiency virus. Other possible causes of chronic hepatitis (i.e., other viral infections, drugs, alcohol, autoimmunity, metabolic disorders) were excluded.

Abbreviations: IFN, interferon; HCV, hepatitis C virus; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cells.

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All patients received recombinant human IFN-α at a dose of 3 MU three times a week subcutaneously for 24 weeks. Treatment was continued at the same dosage for 24 additional weeks only in responder patients with normal alanine transaminase and negative HCV RNA.

HCV RNA was tested by nested polymerase chain reaction (PCR) at the beginning and every month during IFN treatment. HCV RNA was measured quantitatively by competitive reverse-transcriptase PCR before and after 6 months of IFN treatment.

The study was approved by the Ethics Committee of the University of Parma, and all patients gave informed consent before entering the study.

**Amplification of the Core Gene Sequence.** Two overlapping fragments covering the core region of the HCV genome were obtained by reverse transcription and amplification. The 3′ fragment of the C gene was reverse-transcribed with antisense primers HC6 (5′-GGCTGAGGCCGCAAGG-3′, nucleotides 573 to 557) or HC5 (5′-TAGTCTACGGCAGCCTCCAGAA-3′, nucleotides 491 to 469), then amplified by nested PCR using sense primer 4CH (5′-ATGGCCGTAGTATGAGTG-3′), specific proliferative responses. 4 MU three times a week subcutaneously for 24 weeks. Treatment was continued at the same dosage for 24 additional weeks only in responder patients with normal alanine transaminase and negative HCV RNA.

**Quantitation of HCV RNA.** The HCV RNA copy number was determined by a competitive PCR-differential hybridization assay. Briefly, known amounts of a competitive RNA template containing two point mutations (compared with the 5′-untranslated sequence) were added to RNA samples. After reverse transcription and nested PCR of the 5′-untranslated region, each sample was subjected to differential hybridization on microtiter plates, using probes specific for wild-type and competitor. The ratio between the optical density values obtained on each probe, plotted on a standard curve, allowed the estimation of HCV RNA copy number.

**HCV Synthetic Peptides.** Sixteen sets of 20-mer synthetic peptides, overlapping by 10 residues and spanning the whole sequence of the core antigen, were synthesized (Chiron Mimotopes, Clayton, Australia) according to the genomic sequences of the HCV core region of the prevalent viral strains infecting the 16 patients enrolled in our study (Fig. 1). An additional set of peptides based on genotype 1a sequence (HCV-1 isolate 3) was also available for the study.

**HCV Core Antigen Preparations.** Two recombinant preparations of HCV core antigen, one of genotype 1a (comprising the whole HCV-GCGTTAGTATGAGTG-3′, nucleotides 115 of the HCV core region) expressed in *E. coli,* and another, 1 core sequence, amino acids 1-191) expressed as a terminal fusion over the C region of the prevalent viral strains infecting the 16 patients enrolled in our study. An additional set of peptides based on genotype 1a sequence (HCV-1 isolate 3) was also available for the study.

**Isolation of Peripheral Blood Mononuclear Cells and T Cells.** Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation. T cells and non-T cells were separated by rosetting PBMC with 2-aminoethylisothiuronium bromide (Sigma Chemical Co., St. Louis, MO)—treated sheep erythrocytes. E-rosette-forming T cells were separated from the nonrosetting (non-T) fraction by Ficoll-Hypaque gradient. Purity of the T- and non-T-cell fractions was evaluated by direct immunofluorescence with anti-CD3 monoclonal antibodies after red cell osmotic lysis. The T-cell population contained more than 95% CD3+ cells, whereas the nonrosetting fraction contained less than 3% of CD3+ cells.

<table>
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<tr>
<th>Patient No.</th>
<th>Gender</th>
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<th>6 months IFN</th>
<th>Knodel Score*</th>
<th>T-Cell Response Enhancement</th>
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* Knodel score categories: 1, periportal and/or bridge necrosis; 2, lobular degeneration and necrosis; 3, portal inflammation; 4, fibrosis.
The results are expressed as the mean counts per minute of triplicate determinations. The stimulation index was calculated as the ratio between mean counts per minute obtained in the presence and absence of antigen.

Statistical Analysis. Stimulation indexes were compared by the Mann-Whitney U test. Frequencies of significant proliferative T-cell responses were compared by $\chi^2$ analysis.

RESULTS

Two patients infected with genotype 1b and four infected with genotype 2c showed biochemical and virological response to IFN during the first 6 months of treatment and cleared HCV RNA between 1 and 4 months from the start of IFN therapy. All other patients did not respond to IFN, and therapy was stopped after 6 months of treatment (Table 1).

Levels of viremia before treatment in patients infected by genotype 1b and 2 were comparable (Table 1), whereas the biochemical (mean pretreatment alanine transaminase values, genotype 1b = 96 U/L; genotype 2c = 56 U/L), and histological activity of the disease assessed according to the Knodell score (Table 1) was more severe in genotype 1b-infected patients.

Twenty-mer peptides overlapping by 10 amino acid residues and covering the whole HCV core region were synthesized according to the viral genomic sequences obtained from each patient by PCR amplification and molecular cloning. As shown in Fig. 1, short portions of the core sequence were not available in patients 2, 3, 5, and 6 infected by genotype 1b and in patients 1, 4, 7, and 8 infected by genotype 2c. PBMC stimulation was performed with peptides corresponding to the sequences of the prevalent infecting virus and with peptides derived from the HCV-1 strain where HCV sequences were missing. Proliferative responses were studied just before the start of IFN and at least twice between the second and the fifth month of therapy.

PBMC from 12 control HCV-uninfected subjects were tested with HCV proteins and peptides to define the levels observed in normal controls.

A peptide concentration of 2.5 $\mu$g/mL was used on the basis of the results of dose-response studies performed in seven responder patients, showing that this concentration was generally optimal for T-cell stimulation.

Proliferative T-cell responses to HCV core peptides expressed by IFN treatment can be influenced by the genotype of the infecting virus. All proliferation assays were performed in triplicate and $^3$H-thymidine (0.5 $\mu$Ci per well; specific activity, 2.0 Ci/mmol; Amersham International, Amersham, UK) was added 15 hours before harvest.
FIG. 2. T-cell response to HCV core peptides before and during IFN treatment in patients infected with genotype 1b and 2c. Synthetic peptides are indicated by the amino acid position from the NH2-terminus of the HCV core antigen. A proliferation experiment before treatment and two determinations during treatment (third and fifth month) are represented for each patient. To compare homogeneous groups of patients (all studied at the same time points), only these determinations were used for statistical analysis. Determinations at the first and second month of therapy (available only in a limited number of patients) were excluded from comparison. Groups were compared by χ² analysis.

1b–infected patients, but the difference between the two groups was not statistically significant. While T-cell responses detected during treatment (Figs. 2 and 3) in the eight patients infected with genotype 1b were slightly but not significantly higher than those detected before therapy, IFN caused a significant enhancement of the T-cell response expressed by patients infected with genotype 2c, as shown by increased stimulation index values and by the appearance of responses undetectable before treatment. A significant difference was observed by comparing pre- and during-treatment SI values (Mann-Whitney U test: \( P = .0001 \)), as well as the frequencies of significant T-cell responses (χ² analysis: \( P < .002 \)) (Fig. 3) before and during treatment.

As a result of the greater enhancement of the T-cell response caused by IFN treatment in genotype 2c– compared with genotype 1b–infected patients, a significant difference was observed between the two groups when all responses expressed at all time points were compared (χ² analysis: \( P < .00001 \)) (Fig. 2).

In line with these findings, the PBMC proliferative response to recombinant HCV core proteins analyzed throughout the study (before and during IFN treatment) was significantly more vigorous in genotype 2c– than in genotype 1b–infected patients (Mann-Whitney U test: \( P = .004 \)), although only genotype 1 proteins were available for the study, which should underestimate the T-cell reactivity in genotype \( \chi^2 \)–infected patients (not shown).

IFN caused an amplification of the T-cell response to HCV core peptides in all patients who became HCV RNA–negative, except one (patient 4 of Table 1). On the other hand, an amplification of the antiviral T-cell response was observed in five patients who did not show a virological response to IFN treatment (Table 1).

DISCUSSION

Several reports have suggested that the HCV genotype can influence the response to interferon treatment in chronic hepatitis C. However, little is known about the mechanisms responsible for this effect.

An efficient and vigorous T-cell response to HCV antigens seems to be critical to limit replication and spread of the virus within the infected host during the early phases of infection and to ultimately favor viral clearance. Given this crucial pathogenetic role played by the T-cell response, we assessed whether the type of the infecting genotype can exert a specific influence on the modulatory effect of IFN on antiviral T cells.

![Fig. 3. Frequencies of significant proliferative T-cell responses to HCV core peptides before and during IFN treatment in patients infected by (A) genotype 1b and (B) genotype 2c. Inserts represent mean values + SE of the frequencies of significant T-cell responses. Frequencies were compared by χ² analysis. (■) Frequencies of significant T-cell responses before treatment; and (□), frequencies of significant T-cell responses during treatment.](image-url)
Because the specific immune events induced by IFN are expected to occur early after initiation of therapy, the study was performed during the first 6 months of treatment. Two groups of patients infected by genotypes 1b and 2c, respectively (that were previously shown to differ in sensitivity to IFN therapy), were selected and their T-cell response to HCV core antigen was analyzed before and during IFN treatment. The two groups were similar for age, gender, epidemiology, and levels of viremia. Disease activity was more severe in genotype 1b–infected patients as shown by the transaminase levels and the histological activity index assessed before treatment. This reflects the reported prevalence of HCV genotype 2 among patients with normal or near-normal alanine transaminase levels and mild liver lesions.

To avoid interference of viral heterogeneity on the results of the study, short overlapping peptides based on the sequence of the prevalent viral population infecting each patient were synthesized and used to study the T-cell response. The results indicate that the T-cell response to HCV core antigen can be more strongly potentiated by IFN treatment in patients infected with genotype 2c than in those infected with genotype 1b.

All patients who cleared HCV RNA except one showed an enhancement of the HCV-specific T-cell reactivity; however, an increase of the cell-mediated immune response was also observed in some IFN nonresponder patients, suggesting that a vigorous and efficient HCV core-specific T-cell response is likely important, but not sufficient, for viral clearance to occur.

Our study was limited to an individual HCV protein and was performed by an experimental approach expected to preferentially give a quantitative measure of HLA class II restricted T-cell responses. Therefore, the lack of an absolute correlation between clinical and virological outcome and T-cell reactivity is not surprising in consideration of the fact that other factors, including the level of cytotoxic T-cell activity, the type of cytokine pattern, and the rate of virus variability, are believed to represent crucial determinants of the final evolution of infection.

Although a stimulation of the cell-mediated immune response by IFN treatment is an expected consequence of the known modulatory activity of the cytokine, more surprising is the finding of a different IFN effect on T-cell responses in genotype 1b– and genotype 2c–infected patients. The molecular basis of this observation is unknown at present, and we can only speculate about possible mechanisms responsible for this different T-cell susceptibility to the IFN immune modulatory effect.

This finding may be influenced at least in part by the different disease activity that is typically more severe in genotype 1b–infected patients. If HCV-specific T cells are more efficiently recruited into genotype 1b–infected livers because of a higher degree of inflammation, the low peripheral blood responsiveness in this group of patients may be related to the low frequency of circulating HCV-specific T cells, as a result of their sequestration within the infected liver. This interpretation, however, is apparently contradicted by the lack of improvement of the peripheral blood T-cell reactivity in one of the two genotype 1b–infected patients who showed a biochemical and virological response to IFN. This event should be associated with a decreased expression of HCV antigens within the liver, improvement of liver inflammation, and subsequent release of liver-infiltrating T cells into the peripheral blood compartment. On the other hand, an enhancement of the T-cell response was also observed in the absence of a biochemical and virological improvement, when liver inflammation and T-cell recruitment at the site of infection are expected to be unchanged. Therefore, different levels of compartmentalization of HCV-specific T cells within the liver of responder and nonresponder patients can, at best, only partially explain our findings.

An alternative interpretation is that different HCV genotypes may actually be able to exert a variable influence on the IFN immune modulatory effect. This implies that genotype-related differences of viral sequences can confer to different genotypes a variable capacity to interfere with the IFN modulatory effect.

A number of strategies adopted by different viruses to counteract the antiviral immune response have been previously reported. It has been shown that vaccinia virus and other poxviruses can encode soluble receptors for cytokines, including IFN-γ and IFN-α/β; Epstein-Barr virus can synthesize a homologue of interleukin-10, which can inhibit the synthesis of anti-viral Th1 cytokines. Other viruses can down-regulate the expression of HLA molecules or can inhibit processing and presentation of viral proteins.

Based on these examples, one could speculate that, by similar mechanisms, viral gene products synthesized by genotype 1b could mimic soluble cytokine receptors or other factors able to counteract the effect of host cytokines or other immune effector molecules more closely than genotype 2c–encoded amino acid sequences. If this were the case, one would expect that this viral interference on IFN immune modulation should be overcome by increasing concentrations of IFN; the observation that higher doses of IFN may be needed to achieve biochemical, histological, and virological effect in genotype 1b–infected patients might be in keeping with this interpretation.

Alternatively, administration of exogenous IFN would not significantly enhance the cell-mediated immune response in genotype 1b–infected patients as a consequence of a maximal, and not further inducible, activation of the endogenous IFN system by genotype 1b.

Furthermore, sequence variations between genotypes 1b and 2 in the 5′-untranslated region affecting the internal ribosomal entry site of the virus have recently been reported to provide genotype 2c with a translational advantage over genotype 1. This could lead to a more efficient synthesis of viral proteins and thus to a more efficient induction of the cell-mediated immune response, further amplified by IFN treatment.

Finally, the capacity of HCV core to interact with members of the tumor necrosis factor receptor family defines a mechanism that might explain the genotype-related effect of IFN on T-cell responses, as a possible result of a variable binding affinity of core sequences of different genotype to tumor necrosis factor receptors.

In conclusion, our results indicate that IFN treatment causes a greater enhancement of the peripheral blood T-cell response to HCV core antigen in patients infected with genotype 1b than in those infected with genotype 2c. Because the study was performed with synthetic peptides corresponding to the amino acid sequences of the infecting viruses, analysis was necessarily limited to an individual HCV protein. Therefore, we cannot exclude that other HCV antigens can play an important pathogenetic role in the stimulation
of the T-cell response in IFN-treated patients. The different effect in genotype 1 and genotype 2 infections may be related only indirectly to the infecting genotype and may be the result of different levels of compartmentalization of HCV-specific T cells within genotype 1– and genotype 2–infected livers. However, the alternative interpretation that a different sensitivity to the immunoregulatory effect of IFN is directly related to the type of infecting virus appears to be more likely. This could represent one of the pathogenic factors contributing to the different susceptibility of genotype 1b and genotype 2c to the curative effect of IFN treatment.

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