

Quantitative Analysis of the Peripheral Blood Cytotoxic T Lymphocyte Response in Patients with Chronic Hepatitis C Virus Infection

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Abstract

Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTL) are present in the peripheral blood and liver of chronically infected patients. The current study was performed to study the relationship between the strength of the CTL response, liver disease severity, and viral load. The results may be summarized as follows: first, using CTL precursor frequency (CTLpf) analysis to quantitate the peripheral blood CTL response, chronically infected patients were less strongly sensitized to a panel of well-defined HCV epitopes than they were to an epitope within the influenza matrix protein. Second, HCV-specific CTLpf did not correlate with disease activity or viral load in the majority of patients on a cross-sectional basis, although it did increase in three patients concomitant with sharp increases in liver disease. Finally, interferon therapy did not enhance the CTLpf against the HCV epitopes studied in these patients, indicating that its antiviral effect is independent of the CTL response. Since the HCV-specific CTLpf in the blood is actually quite low, the CTL may contribute to ongoing liver disease in these patients while being quantitatively inadequate to destroy all of the infected hepatocytes, thereby facilitating HCV persistence and contributing to chronic liver disease. (*J. Clin. Invest.* 1996. 98:1432-1440.) Key words: cytotoxic T lymphocyte • interferon • viral persistence • viral clearance • hepatitis C virus

Introduction

The hepatitis C virus (HCV)¹ is a hepatotropic RNA virus that causes acute and chronic liver disease and hepatocellular carcinoma (1-6).

It is generally believed that immune-mediated mechanisms, in particular HLA class I-restricted cytotoxic T lymphocytes (CTL), play an important role in viral clearance and disease pathogenesis in many viral infections (7, 8). In hepatitis C virus infection, however, HLA class I-restricted HCV-specific CD8+ CTL are detectable in the peripheral blood (9-14) and CTL clones have been isolated from the liver of chronically infected patients (15-17) and chimpanzees (18) despite persistent infection.

Thus, HCV can survive in the face of an intrahepatic and peripheral CTL response to its antigens. To investigate the basis for this observation, we quantitated the peripheral blood CTL response longitudinally in chronically infected patients treated with interferon and analyzed the relationship between the strength of the CTL response, liver disease activity, and viral load. To identify patients whose CTL responses to one or more of these peptides were strong enough to warrant CTL precursor frequency (CTLpf) analysis longitudinally by limiting dilution analysis, we screened HCV-infected patients before interferon therapy with a panel of 10 HCV-derived epitopes. We now report that the CTL precursor frequency for individual HCV epitopes is actually quite low in the peripheral blood of chronically infected patients as compared to the CTLpf for the influenza matrix epitope, and it is not enhanced by interferon therapy. Since in individual instances an increase in CTLpf preceded a rise in serum alanine aminotransferase (ALT) activity, the results suggest that the HCV-specific CTL response contributes to liver disease pathogenesis, but they are unable to clear the virus in patients with chronic hepatitis.

Methods

Patient population. 9 HLA-A2-positive patients with chronic hepatitis C (Table I) and 15 HLA-A2-positive healthy, uninfected subjects were studied. The diagnosis of HCV infection was based on standard clinical parameters and serological assays using the second-generation (c200/c22-3) Ortho HCV ELISA test system (Ortho Diagnostic Systems, Inc., Raritan, N.J.). All patients displayed elevated ALT activity and histological evidence of chronic active hepatitis before interferon therapy. Three (patients 2, 6, and 7) had liver cirrhosis. Treatment with interferon followed the protocols described in Table I. A decrease of transaminases below the upper limit of the normal range was achieved in five (patients 1, 4, 5, 7, and 9) during and immediately after cessation of therapy and serum HCV RNA was undetectable by branched chain DNA (bDNA) analysis in the same five patients and by nested polymerase chain reaction in two (patients 1 and 4) at the end of interferon treatment. Patient 1 showed a sustained reduction of serum ALT activity and remained HCV RNA negative by bDNA analysis for 6 mo after discontinuation of therapy, while Patient 4 displayed only a temporary biochemical and virologic response. All subjects studied were repeatedly negative for antibodies to HIV and HBV. The study protocol was approved by the Human Subjects Committee at the Scripps Clinic and Research Foundation (La Jolla, CA).

B. Rehermann and K.-M. Chang contributed equally to this study and should be considered co-first authors.

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1. Abbreviations used in this paper: ALT, alanine aminotransferase; bDNA, branched chain DNA; CTL, cytotoxic T lymphocytes; CRI, CTL response index; CTLpf, CTL precursor frequency; HCV (or HBV), hepatitis C (or B) virus.

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Table I. The HCV-infected Patients

| Patient | Age | Sex | HCV genotype | Anti-HCV | Before interferon therapy | | | End of interferon therapy | | | |
|---------|-----|-----|--------------|----------|---------------------------|---------|------------------|---|---------------|---------|------------------|
| | | | | | ALT [U/liter] | HCV-RNA | | Type of Interferon Therapy [§] | ALT [U/liter] | HCV-RNA | |
| | | | | | | bDNA* | PCR [‡] | | | bDNA* | PCR [‡] |
| 1 | 42 | F | 1a | positive | 97 | 110 | | 1 | 27 | < 3.5 | negative |
| 2 | 41 | M | 1a | positive | 306 | 220 | | 1 | 94 | 63.9 | |
| 3 | 37 | F | 1b | positive | 98 | 61 | | 3 | 133 | 159 | |
| 4 | 37 | M | 1a | positive | 223 | < 3.5 | 0 | 1 | 29 | < 3.5 | negative |
| 5 | 40 | M | 3a | positive | 83 | 5 | | 1 | 30 | < 3.5 | 2 |
| 6 | 51 | M | 1a | positive | 217 | 113 | | 4 | 159 | 75 | |
| 7 | 36 | M | 1b | positive | 140 | 20 | | 2 | 48 | < 3.5 | 3 |
| 8 | 39 | M | 1a | positive | 75 | 33 | | 3 | 69 | 39.3 | |
| 9 | 40 | M | 3a | positive | 86 | < 3.5 | 3 | 3 | 54 | < 3.5 | 3 |

*bDNA, branched chain DNA assay for HVC RNA quantitation in 100,000 genome equivalents/ml. [‡]Semiquantitative PCR with serial 10-fold dilution of cDNA was performed when HCV RNA was not detected by bDNA. Results indicate the negative logarithm (base 10) of the highest 10-fold dilution that was positive in this assay. [§]Type of interferon therapy: (1) 6 wk oral prednisone (60, 40 and 20 mg each for 2 wk) followed by 3 million U IFN alpha 2b (Intron A) (Schering Plough Corp., Kenilworth, NJ) subcutaneously thrice weekly for 6 mo; (2) IFN alpha 2a (Roferon; Hoffman-LaRoche), 6 million U subcutaneously thrice weekly for 6 mo; (3) 1–9 million U Monomethoxy-polyethylene glycol IFN alpha-2a (Hoffman-LaRoche) subcutaneously weekly for 6 mo; and (4) 10 million U IFN alpha 1 (Alferon; Interferon Sciences Inc., New Brunswick, NJ) subcutaneously thrice weekly for 6 mo.

HLA typing. HLA typing of PBMC from patients and from normal donors was performed by complement-dependent microcytotoxicity using HLA typing trays purchased from One Lambda (Canoga Park, CA).

Synthetic peptides. A panel of peptides representing 10 previously identified HLA-A2-restricted HCV CTL epitopes (12, 19, 20) and the influenza matrix peptide 58-66 (21) were selected for this study (Table II). Peptide sequences were derived from the nucleotide sequence of HCV-1 (22), which is genotype 1a. All peptides were synthesized with a free amine NH₂ terminus and a free acid COOH terminus by Chiron Mimotopes (Clayton, Australia). Lyophilized peptides were reconstituted at 20 mg/ml in DMSO (Mallinckrodt Inc., Paris, KY) and diluted to 1 mg/ml with RPMI 1640 medium (Gibco Laboratories, Grand Island, NY).

Stimulation of PBMC with synthetic peptides. PBMCs were separated on Ficoll-Histopaque density gradients (Sigma Chemical Co., St. Louis, MO), washed three times in HBSS (Gibco Laboratories), resuspended in RPMI 1640 (Gibco Laboratories) supplemented with

2 mM L-glutamine, 10 mM gentamycin, and 10% heat-inactivated human AB serum, and plated in a 96-well round bottom plate at 0.4 × 10⁶ cells/100 µl per well. Synthetic peptides were added at 10 µg/ml to eight replicate cultures. On day 7, the cultures were transferred to a 96-well flat-bottom plate and, on days 7 and 14, restimulated with 10 µg/ml peptide, 20 U/ml rIL-2 (Hoffmann-La Roche, Nutley, NJ), and 10⁵ irradiated (3,000 rad) autologous feeder cells. On days 3, 10, and 18, 100 µl of RPMI with 10% (vol/vol) human AB serum and rIL-2 at 10 U/ml final concentration was added to each well. Cultures were tested for peptide-specific cytolytic activity on day 21.

Cytotoxicity assay. For all experiments, JY cell targets (HLA-A2.1, B7, Cw7) were maintained in RPMI with 10% (vol/vol) heat-inactivated FCS (Gibco Laboratories). Target cells were incubated overnight with synthetic peptides at 10 µg/ml and then labeled with 100 µCi of ⁵¹Cr (Amersham Corp., Arlington Heights, IL) for 1 h and washed four times with HBSS. Cytolytic activity was determined in a standard 4-h ⁵¹Cr release assay using round-bottom 96-well plates containing 3,000 targets/well. Stimulated PBMCs from patients and

Table II. The Epitopes

| Peptide | Protein | Amino acid position | Amino acid sequence | Conservation by genotype (%) | | | Reference |
|----------------|------------------|---------------------|---------------------|------------------------------|-----|-----|-----------|
| | | | | 1a* | 1b* | 3a* | |
| 1 [‡] | Core | 35 | YLLPRRGPR | 100 | 77 | 0 | 12, 13 |
| 2 [‡] | Core | 131 | ADLMGYIPLV | 100 | 91 | 100 | 12–14 |
| 3 | E1 | 257 | QLRRHIDLLV | 100 | 0 | 0 | 14 |
| 4 [‡] | NS3 | 1073 | CINGVCWTV | 100 | 45 | 0 | 12, 17 |
| 5 | NS3 | 1169 | LLCPAGHAV | 67 | 0 | 0 | 12 |
| 6 [‡] | NS3 | 1406 | KLVALGINAV | 75 | 0 | 0 | 12 |
| 7 | NS4 | 1789 | SLMAFTAAV | 100 | 0 | 0 | 12 |
| 8 | NS4 | 1807 | LLFNILGGWV | 100 | 100 | 0 | 12, 13 |
| 9 | NS4 | 1851 | ILAGYGAGV | 100 | 91 | 100 | 13 |
| 10 | IS5 | 2727 | GLQDCTMLV | 89 | 0 | 0 | 13 |
| Flu epitope | Influenza-matrix | 58 | GILGFVFTLT | | | | 21 |

*Number of published (Genbank/EMBL/DDBJ) full length sequences analyzed: 1a (6), 1b (11), and 3a (2). [‡]Epitopes chosen for CTL precursor frequency analysis.

normal controls were tested at E/T ratios of 30:1–40:1. Percent cytotoxicity was determined from the formula: $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$. Maximum release was determined by lysis of targets by detergent (10% Triton X-100; Sigma Chemical Co.). In all instances, spontaneous release was < 25% of maximum release.

As previously described (23) the analysis of eight replicate cultures for each peptide allowed the strength of the CTL response to be expressed as the CTL response index (CRI). The CRI-P value is the sum of all specific cytotoxicities of the eight replicate cultures for a given peptide in a patient. The mean CRI-P values observed in the 15 normal controls are indicated at the bottom of Fig. 1. CRI-P values equal to or greater than the mean control value plus 3 SDs for each peptide were required for a CTL response to be considered positive. Positive responses are indicated in boldface type in Fig. 1 and the peptides chosen for limiting dilution analysis are underlined.

Limiting dilution analysis. Quantitative analysis of epitope-specific CTLpf was performed in nine HCV-infected patients and three uninfected healthy controls as previously described (24). To prepare antigen-presenting cells, autologous PBMCs were activated with

0.005% Pansorbin cells (Calbiochem Corp., La Jolla, CA), with 20 $\mu\text{g/ml}$ Immunobeads (Rabbit anti-human IgM; Irvine Scientific, Santa Ana, CA) and 20 ng/ml human rec IL-4 (PharMingen, San Diego, CA) at 2×10^6 cells/ml in RPMI 1640 supplemented with 2 mM L-glutamine, 10 mM gentamycin, and 5% heat-inactivated FCS. On days 4–6, they were washed in cold 0.9% NaCl, 1% BSA, resuspended at 10^7 cells/ml in cold citrate phosphate buffer, (0.13 M L-citric acid (J.T. Baker, Inc., Phillipsburg, NJ), 0.06 M sodium phosphate monobasic, pH 3 (Sigma Chemical Co.), 1% BSA, and 3 $\mu\text{g/ml}$ $\beta 2$ microglobulin for 2 min at 4°C, washed with 5 vol cold 0.15 M sodium phosphate monobasic, pH 7.5, containing 3 $\mu\text{g/ml}$ $\beta 2$ microglobulin (Scripps Laboratories, La Jolla, CA) and 10 $\mu\text{g/ml}$ peptide, and incubated with 50 $\mu\text{g/ml}$ peptide, 30 $\mu\text{g/ml}$ DNase (Sigma Chemical Co.), and 3 $\mu\text{g/ml}$ $\beta 2$ microglobulin for 4 h at 20°C. After irradiation (6,100 rad), 10^5 cells/well were plated in a 96-well round bottom plate and 400,000, 200,000, 100,000, and 50,000 autologous PBMCs were added in 24 replicates in 100 μl RPMI 1640 supplemented with 2 mM L-glutamine, 10 mM gentamycin, and 10% heat-inactivated human AB serum. If a high CTL precursor frequency was expected, 100,000, 50,000, 25,000, and 12,500 PBMCs were added to each well. Cultures

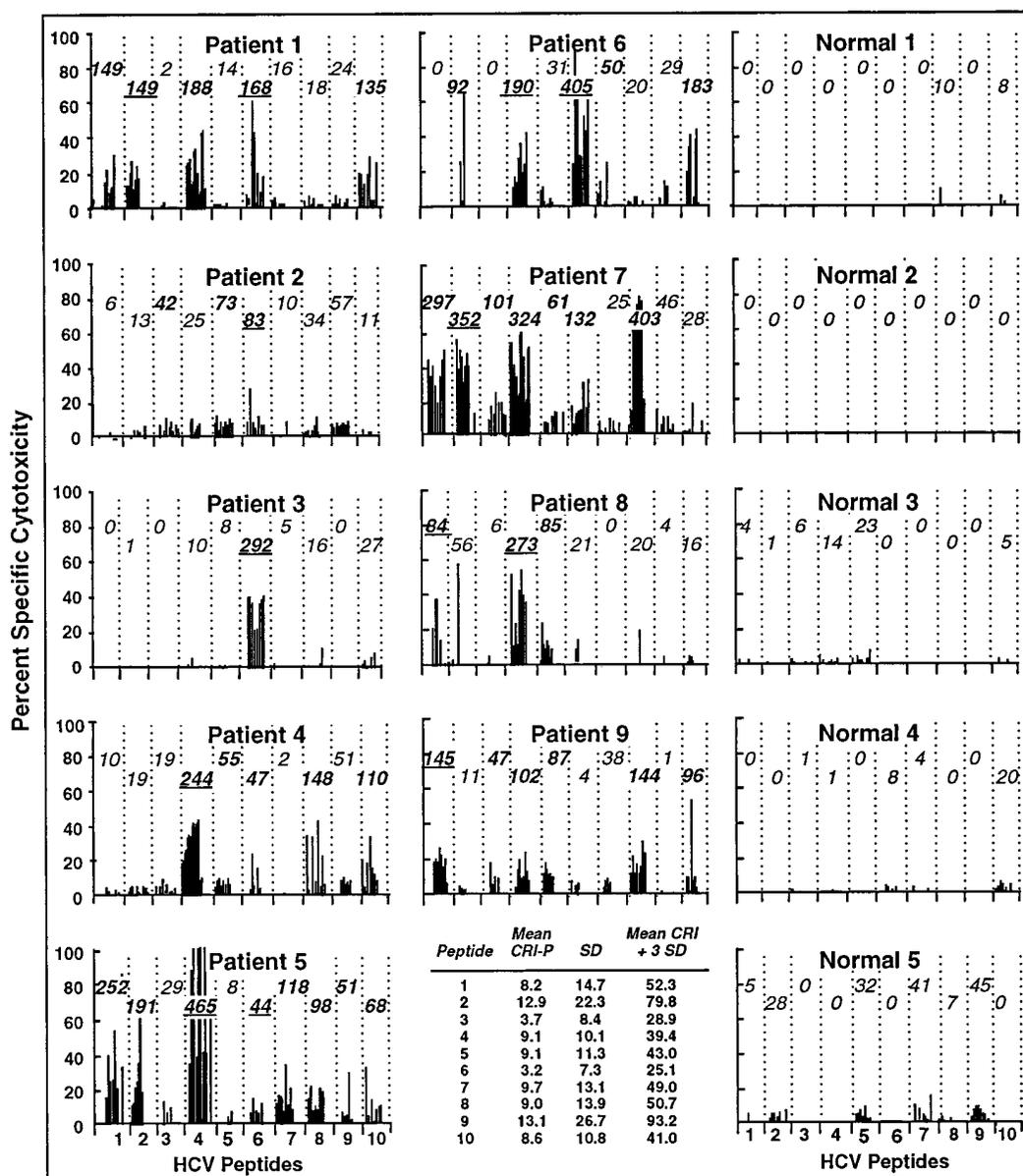


Figure 1. PBMC from 9 HCV-infected patients (patients 1–9) and 15 healthy, uninfected controls were stimulated with 10 $\mu\text{g/ml}$ of peptide for 3 wk as described in Methods and tested in a 4-h ^{51}Cr release assay against JY target cells prepulsed overnight with the same peptide. The bars represent the percent specific lysis in a 4-h ^{51}Cr release assay at an E/T ratio of 30:1–40:1 for all 9 patients studied and for 5 representative controls (Normal 1–Normal 5). The CRI-P is displayed above the bars. The CRI-P corresponding to the peptides chosen for limiting dilution analysis are underlined. The table at the bottom center displays the mean \pm 3 SDs of the CRI for each peptide in the 15 normal controls. This number is used as the operational cut off for a positive CTL response in this study.

were fed with 20 μ l rIL-7 (PharMingen) on day 7 and 20 μ l rIL-2 every 3 d to obtain a final concentration of 10 ng/ml rIL-7 and 10 U/ml IL-2. After restimulation with autologous, peptide-pulsed (20 μ g/ml), irradiated (3,000 rad) adherent cells on day 12, a split-well CTL-assay with 3000 targets/well as described above, was performed on day 17–19. Cultures were regarded as positive if the specific cytotoxicity was more than 2 SDs above the mean of the unspecific cytotoxicity of all cultures, and CTL precursor frequency was calculated using the maximum likelihood method (25). To follow changes in the HCV epitope-specific CTLpf in individual patients over time, frozen PBMCs from different bleed dates were thawed, cultured, and tested for specific cytotoxicity in the same experiment.

HCV RNA determination and HCV genotyping. RNA was extracted from 100 μ l of serum as previously described (26). Reverse transcription and nested polymerase chain reaction of the conserved 5' noncoding region was performed to establish the presence of HCV RNA (27). PCR products were analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining. HCV RNA was quantitated in serum using the bDNA nucleic acid hybridization assay Quantiplex™ HCV RNA Assay (Chiron Corp., Emeryville, CA) according to the manufacturer's instructions. Results were expressed as HCV genome equivalents per milliliter. Semiquantitative PCR was also performed using serial 10-fold dilutions of the cDNA. The negative logarithm of the highest 10-fold dilution of cDNA yielding a positive

Table III. HCV and Influenza CTLpf in Patients and Controls

| Subject | HCV peptide | HCV | Flu |
|-----------------|-------------|------|------|
| <i>CTLpf*</i> | | | |
| Patients | | | |
| 1 | 6 | 6.3 | 37.0 |
| 2 | 6 | 3.2 | 22.8 |
| 4 | 4 | 12.8 | 25.5 |
| Normal | | | |
| 6 | 4 | 1.8 | 10.7 |
| 7 | 4 | 0.3 | 74.0 |
| 8 | 2 | 0.5 | 76.4 |

*per 10⁶ PBMCs.

result was taken as the HCV RNA titer for each sample. HCV genotype in each patient was determined by the standard INNOLIPA assay (courtesy of Dr. Andrew Conrad, National Genetics Institute, Los Angeles, CA), using PCR-amplified viral nucleic acid and genotype-specific probes in the 5' noncoding region (28).

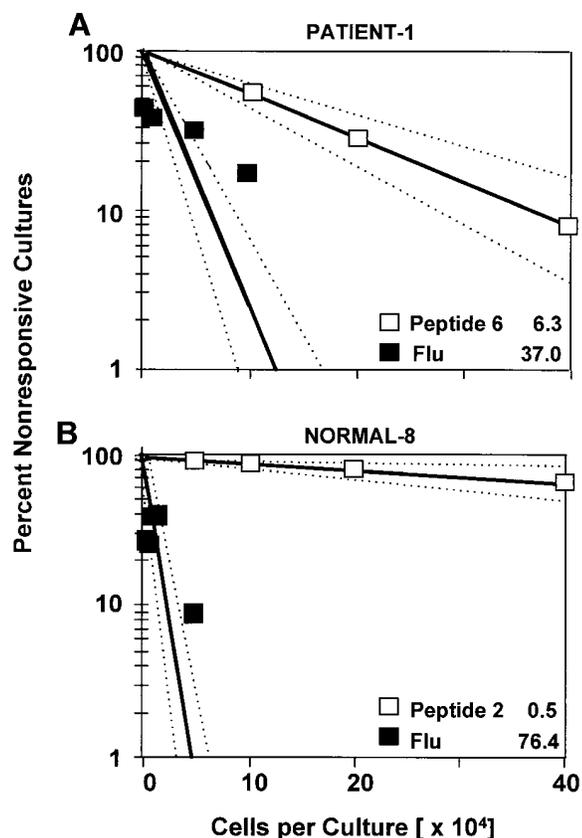


Figure 2. Precursor frequency of CTL specific for HCV-derived peptides (\square) and for the influenza matrix epitope M58-66 (\blacksquare) in an HCV-infected patient (A) and a healthy, uninfected control (B). An HCV-derived peptide that was efficiently recognized by semiquantitative CTL analysis (illustrated in Fig. 1) was chosen for limiting dilution analysis as described in Methods. The CTL precursor frequency is given as the number of specific CTL per 10⁶ PBMCs in the lower right hand corner of each panel. (A) Patient-1, peptide 6; (B) normal-8, peptide 2.

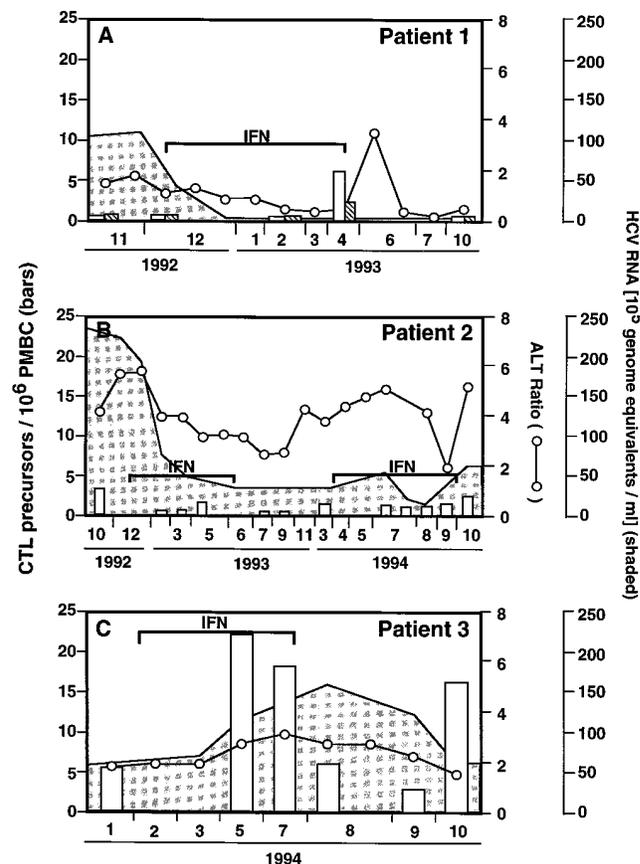


Figure 3. Serial analysis of the HCV epitope-specific CTL precursor frequency in patients 1 (A), 2 (B), and 3 (C). The open circles display serum ALT ratio (ALT activity divided by the upper limit of the normal range), the shaded area reflects the amount of HCV RNA (genome equivalents $\times 10^5$ /ml) detected by bDNA analysis in the peripheral blood. Frequency of CTL specific for peptide 2 (Core 131) (\boxtimes) and peptide 6 (NS3 1406) (\square). All limiting dilution analyses were done in the same experiment.

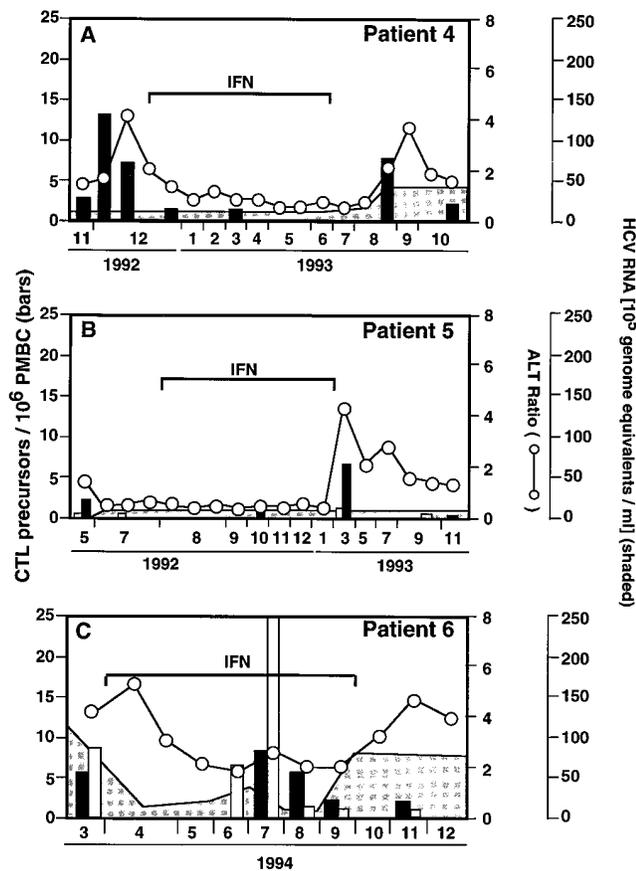


Figure 4. Serial analysis of the HCV epitope-specific CTL precursor frequency in patients 4 (A), 5 (B), and 6 (C). The open circles display serum ALT ratio (ALT activity divided by the upper limit of the normal range), the shaded area reflects the amount of HCV RNA (genome equivalents $\times 10^5$ /ml) detected by bDNA analysis in the peripheral blood. Frequency of CTL specific for peptide 4 (NS3 1073) (■) and peptide 6 (NS3 1406) (□). All limiting dilution analyses were done in the same experiment.

Results

Analysis of the CTL response to multiple HCV epitopes. To identify patients whose CTL responses to HCV-derived epitopes were strong enough to warrant CTL precursor frequency analysis longitudinally by limiting dilution analysis, we screened all patients before interferon therapy with a panel of 10 predefined HCV-derived peptides. Using a microwell peptide stimulation strategy with eight replicate cultures per peptide, we demonstrated that most of the patients with chronic hepatitis C displayed a characteristic multispecific CTL response (Fig. 1). A CRI-P of at least 3 SDs above the average CRI-P obtained in controls was observed in all patients. In total, 43 out of 90 (46%) possible CTL responses were positive in the patient group (Fig. 1) as opposed to 5 out of 150 (3%) possible CTL responses in the controls (not shown). Since we have recently described that the total CRI, which represents the sum of all CRI-P per patient, is inversely associated with viral load (29), and since interferon is thought to have antiviral as well as immunomodulatory effects, we were therefore interested in analyzing the relationship between CTL response, disease activity, and viral load longitudinally during interferon therapy in indi-

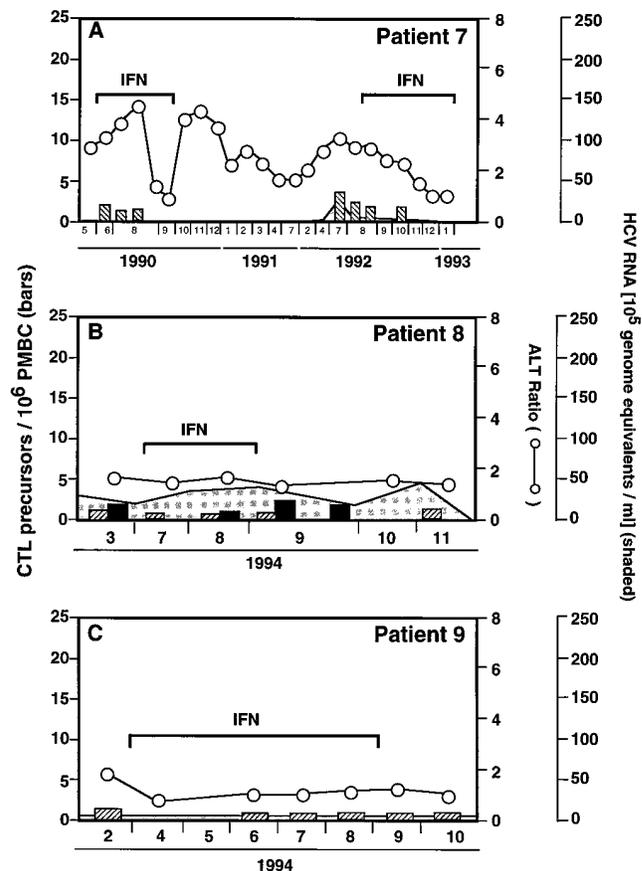


Figure 5. Precursor frequency of CTL specific for HCV-derived peptides 1 (Core 35) (▨), 2 (Core 131) (▩), and 4 (NS3 1073) (■) in patients 7 (A), 8 (B), and 9 (C). The open circles display serum ALT ratio (ALT activity divided by the upper limit of the normal range), the shaded area reflects the amount of HCV RNA (genome equivalents $\times 10^5$ /ml) detected by bDNA analysis in the peripheral blood.

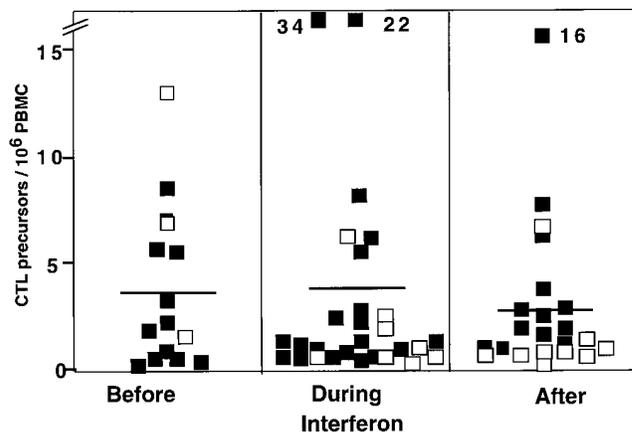


Figure 6. Precursor frequency of CTL specific for HCV-derived peptides 1 (Core 35), 2 (Core 131), 4 (NS3 1073), and 6 (NS3 1406) in all nine HCV-infected patients before, during, and after interferon therapy. Filled squares represent the CTL precursor frequency, given as the number of specific CTL per 10⁶ PBMCs, in patients with high viral load; i.e., HCV RNA was detectable by bDNA analysis. Open squares represent the CTL precursor frequency in patients with low viral load; i.e., HCV RNA was undetectable by bDNA analysis. The mean CTLpf for all of the peptides in each group is indicated by a horizontal line.

vidual patients. For each patient, one or two of the peptides that were best recognized (usually peptides 1 and 2 from the core protein and peptides 4 and 6 from the NS3 protein), displaying strong CTL response indices (underlined in Fig. 1), were chosen for CTLpf analysis (see below).

Relative strength of the CTL response to HCV and influenza virus matrix peptides in patients and controls. To compare the degree of sensitization to HCV and influenza virus (as a recall antigen), simultaneous limiting dilution analysis was performed to determine the CTLpf for HCV and influenza matrix peptides in three HCV patients and three normal controls. HCV- and influenza peptide-specific CTLpfs observed in a representative patient and a representative control (using the HCV peptide for which a high CTLpf was observed for each subject) are displayed in Fig. 2. Note that there are more influenza-specific CTL precursors than HCV-specific CTL precursors in the chronically infected patient and that essentially no HCV-specific CTL precursors were detectable in the uninfected control (i.e., < 1 in 2×10^6 PBMCs). The HCV- and influenza virus-specific CTLpf in all six subjects studied are summarized

in Table III. Importantly, all three patients displayed fewer HCV-specific CTL precursors for the HCV peptides they recognized efficiently in the screening assay (Fig. 1) than for the influenza recall antigen. Table III also illustrates that, despite the impressive degree of HCV peptide-inducible cytolytic activity illustrated in Fig. 1, the number of HCV-specific CTL precursors is actually quite low in these chronically infected patients, as we have previously reported (12).

Longitudinal analysis of HCV-specific CTL precursor frequency, disease activity, and viral load during interferon therapy. To examine the relationship between HCV-specific CTLpf and both disease activity and viral load, we studied the CTLpf for one or two epitopes per patient at 4–12 time points before, during, and after interferon therapy. For each patient, HCV-derived peptides that were recognized strongly in the screening assay (Fig. 1) were chosen for CTLpf analyses. For patients 2, 5, and 8, CTLpf for peptides that were less strongly recognized were also studied. As shown in Fig. 3, A and B and Fig. 4 C, three (patients 1, 2, and 6) responded to interferon with rapid and profound decreases in viral load (*shaded areas*).

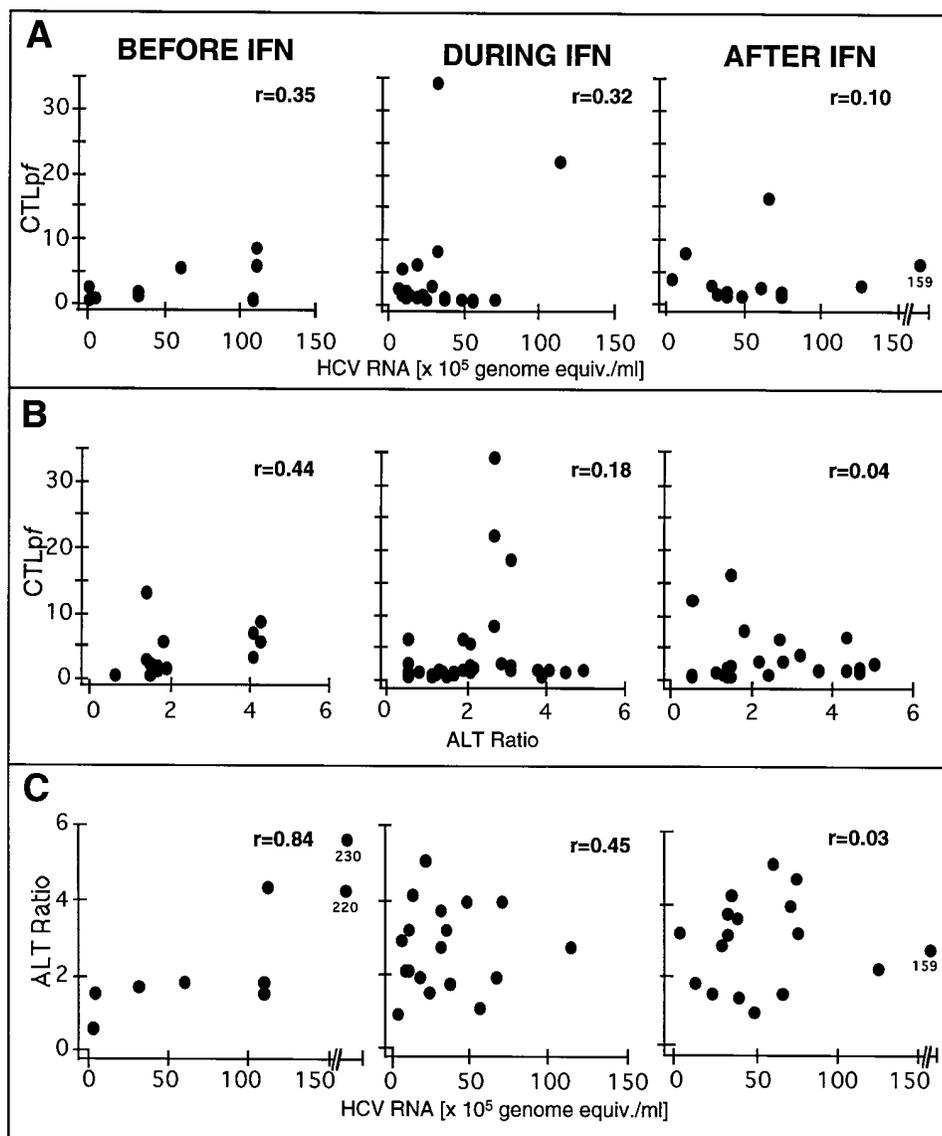


Figure 7. (A) Comparison of the frequency of CTL precursors (per 10^6 PBMCs) specific for peptides 1 (Core 35), 2 (Core 131), 4 (NS3 1073), and 6 (NS3 1406) with serum HCV RNA content determined by bDNA analysis before, during, and after interferon therapy. If HCV RNA was undetectable by bDNA analysis, but detectable by PCR, the data were not included in this figure but are shown in Fig. 8. r = correlation coefficient. (B) Comparison of the frequency of CTL precursors specific for the same epitopes with serum ALT activity (the ALT ratio represents ALT activity divided by the upper limit of normal). (C) Comparison of serum ALT ratio and HCV RNA content.

Importantly, the HCV-specific CTLpf (*bars*) did not change during this dynamic flux in viral load, indicating that CTL-independent mechanisms were probably responsible for the antiviral effect of interferon in these patients. In one atypical patient (*patient 3*) the HCV-specific CTLpf increased approximately fourfold during interferon therapy and fell back to baseline when treatment was stopped (Fig. 3 C). Nonetheless, serum HCV RNA levels rose paradoxically during this period, indicating that neither the CTL response nor interferon therapy was able to control the viral burden in this individual. Note that serum ALT activity did not change despite the fluctuations in viral load and CTLpf in this patient.

Five (patients 1, 2, 4, 5, and 6) displayed flares in serum ALT activity, defined as a greater than twofold increase of serum ALT activity during a period of 2 mo, either before initiation of therapy (Fig. 4 A) or after its withdrawal (Fig. 3, A and B, and 4, A–C). It is noteworthy that CTLpf activity appeared to increase just before or concomitant with the disease flares in four out of six instances in three of these five (patients 1, 4, and 5), suggesting that the CTL response may have played a role in these episodes. However, sharp increases in CTLpf were also observed in patients 3 (Fig. 3 C) and 6 (Fig. 4 C) without any associated changes in serum HCV RNA or ALT activity, and wide fluctuations in serum ALT activity observed in patient 7 were not associated with concomitant changes in CTLpf or viral load (Fig. 5 A). Patients 8 and 9 displayed minimal or no changes in HCV-specific CTLpf or serum HCV RNA levels over a similar observation period (Fig. 5, A and B).

HCV-specific CTL precursor frequency is not enhanced during interferon therapy and, on a cross-sectional basis, does not correlate with disease activity or viral load. The foregoing results demonstrate that interferon treatment did not enhance the peripheral blood HCV-specific CTL response in these patients. This was confirmed when all patients were analyzed cross-sectionally before, during, and after interferon therapy for HCV-specific CTLpf. As shown in Fig. 6, the mean HCV-specific CTLpf did not change significantly from pretreatment values during or after interferon treatment. Reflecting these observations, the HCV epitope-specific CTL precursor frequency did not correlate with either serum HCV RNA content as a measurement of viral load (Fig. 7 A) or the serum ALT activity as a measurement of disease activity (Fig. 7 B) before, during, or after interferon therapy. Finally, the serum HCV RNA content correlated with serum ALT activity in this group of patients before interferon treatment, although this correlation disappeared with interferon therapy (Fig. 7 C).

To determine if important correlations between viral load and CTLpf or ALT may be masked by the low sensitivity of the bDNA assay ($> 3.5 \times 10^5$ genome equivalents/ml), the bDNA-negative serum samples were also analyzed by semi-quantitative PCR. However, no linear correlation was found between HCV CTLpf and HCV RNA content even at the low levels detectable by this technique (Fig. 8 B).

Discussion

The current study was performed to examine the basis for HCV persistence in the face of a multispecific peripheral blood CTL response in patients with chronic hepatitis. The results can be summarized as follows: first, chronically infected patients display fewer HCV-specific CTL precursors in their pe-

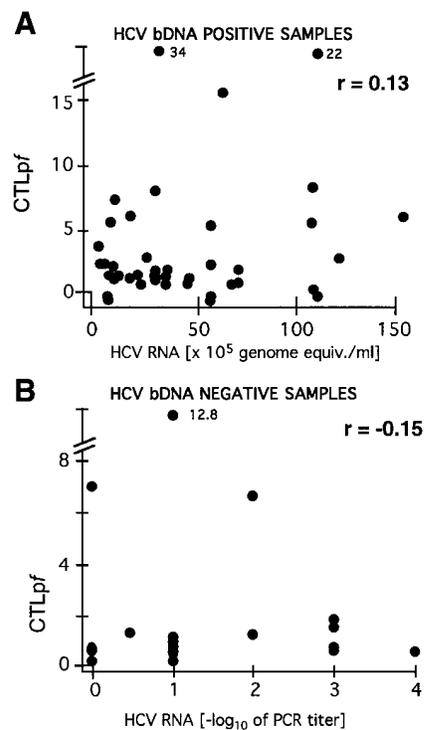


Figure 8. (A) Comparison of the frequency of CTL precursors (per 10^6 PBMCs) specific for peptides 1 (Core 35), 2 (Core 131), 4 (NS3 1073), and 6 (NS3 1406) with serum HCV RNA content determined by bDNA analysis. r = correlation coefficient. (B) Comparison of the frequency of CTL precursors (per 10^6 PBMCs) specific for the same peptides with serum HCV RNA content in bDNA-negative samples determined by semi-quantitative PCR before, during, and after interferon therapy. The negative logarithm (base 10) of the highest serum dilution still yielding a positive signal by PCR is given as the HCV RNA titer for each sample.

ripheral blood than influenza-specific CTL. Assuming that the CTL responses we monitored in this study are representative of the overall strength of the CTL response to both of these viruses, this suggests that the active CTL response to an ongoing HCV infection in these patients is not as strong as their recall response to a previous influenza infection. This may explain why HCV is commonly persistent while influenza is not. Second, although HCV-specific peripheral CTLpf increased before flares in liver disease activity in a few patients, this relationship was not absolute, apparently reflecting the indolent nature of the disease in most of the patients we studied or compartmentalization of the CTL in the liver. Third, the HCV-specific CTLpf did not correlate with viral load; in fact, the viral burden actually increased in the face of increasing CTLpf in one patient, emphasizing the ineffectiveness of the CTL response against single HCV epitopes in these patients. Finally, in some subjects interferon treatment was associated with a reduction in viral load without enhancing the CTL response or inducing liver disease, suggesting that HCV may be susceptible to control by noncytopathic antiviral mechanisms that can be induced by interferon but are not spontaneously activated in these chronically infected patients.

These results suggest that the chronically infected patients mount a CTL response to HCV that may be quantitatively insufficient for viral clearance, but is likely to kill some of the infected hepatocytes, thereby causing chronic hepatitis. The reason why most patients mount an apparently ineffective CTL response to HCV is unclear. Global immunosuppression can be eliminated based on the normal CTLpf these patients display to an unrelated viral antigen. Thymic deletion of HCV-specific T cells is unlikely since all of these patients were in-

ected as adults. CTL exhaustion due to antigen hyperstimulation is unlikely since the viral burden during HCV infection is not especially high. Conceivably, HCV might interfere with antigen processing or presentation by the hepatocyte, thereby diminishing its visibility to the immune system sufficiently to hamper development of an immune response; however, there is no direct evidence for this hypothesis in the literature at this time. Alternatively, it is possible that the low number of HCV-specific CTL precursors in the peripheral blood does not reflect conditions in the liver (15–17). If this is true, however, it implies that the cytolytic function of the intrahepatic HCV-specific CTL is not sufficient to kill or cure all of the infected cells in the liver even if the CTL response is vigorous.

Along these lines, based on evidence from a transgenic mouse model, we have recently suggested that hepatitis B virus (HBV)-specific CTL may control HBV infection not only by killing infected hepatocytes but also by inhibiting HBV replication by secreting certain antiviral cytokines when they recognize antigen (30, 31). If this or related potentially curative antiviral pathways are required for viral clearance to be complete in an organ with as many infectible cells as the liver, viruses that are either intrinsically resistant to noncytolytic control or that induce an immune response that does not produce the corresponding antiviral cytokines would have a survival advantage. The current data suggest that HCV may fall into one of these categories, and further studies should be done to pursue this hypothesis.

Finally, one must consider the possibility that HCV may have mutated and escaped the CTL response to the corresponding epitopes in the chronically infected patients we studied, and the response we measured is weak due to a lack of continuous stimulation by the original antigen. Furthermore, some patients did not display a CTL response to epitopes that should be encoded by the infecting virus as predicted by HCV genotyping. Others responded to HCV-1-derived peptides that were not identical with the predicted sequence encoded by their own viral genotypes. These very interesting observations are currently under investigation at the nucleotide sequence level, especially since emergence of a CTL escape variant of HCV has been reported in a chronically infected chimpanzee (18).

Collectively, these results and the published database suggest that the CTL response probably contributes to disease pathogenesis but is not vigorous enough to eradicate the virus during chronic HCV infection in most patients. The current study emphasizes that the peripheral CTL precursor frequency to individual HCV epitopes is very low in the chronically infected patients we studied. The reason for the failure to mount a stronger epitope-specific CTL response is unclear at this time. If the peripheral CTL response reflects the HCV-specific CTL_{pf} in the intrahepatic lymphomononuclear cell infiltrate, the total number of HCV-specific CTL in the chronically infected liver may simply be too low to clear the virus from all of the infected cells. It is premature at this time to conclude that HCV cannot be controlled by CTL under any circumstances, however, even though most of the patients studied were not able to clear the virus completely and only a few displayed sharp changes in liver disease activity. Quantitation of multi-specific CTL responses in patients who recover spontaneously from acute HCV infection is needed to better understand the role of the CTL response in HCV clearance and disease pathogenesis.

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