Analysis of Successful Immune Responses in Persons Infected with Hepatitis C Virus

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Abstract

Although hepatitis C virus (HCV) infection is very common, identification of patients during acute infection is rare. Consequently, little is known about the immune response during this critical stage of the disease. We analyzed the T lymphocyte response during and after acute resolving HCV infection in three persons, using interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) and human histocompatibility leukocyte antigen (HLA) peptide tetramer assays. Acute infection was associated with a broadly directed T helper and cytotoxic T lymphocyte (CTL) response, which persisted after resolution of clinical hepatitis and clearance of viremia. At the earliest time point studied, highly activated CTL populations were observed that temporarily failed to secrete IFN-γ, a “stunned” phenotype, from which they recovered as viremia declined. In long-term HCV-seropositive persons, CTL responses were more common in persons who had cleared viremia compared with those with persistent viremia, although the frequencies of HCV-specific CTLs were lower than those found in persons during and after resolution of acute HCV infection. These studies demonstrate a strong and persistent CTL response in resolving acute HCV infection, and provide rationale to explore immune augmentation as a therapeutic intervention in chronic HCV infection.

Key words: acute infection • cytotoxic T lymphocytes • T helper cells • tetramer staining • interferon-γ

Introduction

Hepatitis C virus (HCV) is a major public health problem, affecting an estimated 170 million people worldwide and more than 10% of the population in some countries (1). The virus persists in most cases, and can go on to cause hepatic inflammation, fibrosis, and ultimately death through liver failure or hepatocellular carcinoma. The mechanisms that cause both viral persistence and hepatic pathology are unknown. It is known that immune responses are present during chronic infection, and it has been suggested that these are present at too low a level to control or eliminate the virus yet sufficient to sustain low-grade liver inflammation (2–4). Analysis of these antiviral responses has often been hampered by the requirement for extensive in vitro culture of lymphocytes before detection, which renders quantification difficult, and also because studies have been largely confined to patients with established disease (2–10).

Although HCV infection is very common, it rarely presents acutely, as the disease is usually not accompanied by overt jaundice and patients rarely seek medical attention. Consequently, little is known about the specificity and kinetics of the immune response during this period. In other well-studied persistent viral infections, the immunological
Materials and Methods

Study Subjects and Samples. All subjects were HLA typed by PCR or conventional serology and screened for the presence of HCV antibodies by ELISA (Abbott Laboratories). Subject 1 was a 42-yr-old current intravenous drug user who had recently shared needles with a friend who was known to be HCV-seropositive and H1V-seropositive. He presented with an acute onset of malaise and jaundice. Initial laboratory investigations included alanine transaminase (ALT) 1073 U/liter, total bilirubin 8.6 mg/dl, anti-HCV- positive by ELISA, HCV RNA-positive, and H1V RNA-negative. Other causes of acute hepatitis, such as drug toxicity and other forms of viral hepatitis, were ruled out on history and serologic testing. A clinical diagnosis of acute HCV infection was made, and no specific pharmacologic treatment was advised. By 6 wk, his symptoms had resolved and his ALT had normalized. PCR for HCV RNA became negative at 4 wk and remained so during the 1 yr of follow-up. Subject 2 was a 67-yr-old woman who presented 5 wk after cosmetic surgery with symptoms of acute hepatitis. Her ALT was 703 U/liter, and she was seronegative for hepatitis A and B viruses but antibody- and PCR-positive for HCV. 4 wk later, she was HCV reverse transcription (RT)-PCR-negative, and ALT had normalized. She remained HCV RNA PCR-negative with normal ALT during 2 yr of follow-up. Subject 3 was a 32-yr-old female partner of an HCV-infected intravenous drug user. She presented to the hospital with signs of acute hepatitis and ALT of 882 U/liter. She was seronegative for hepatitis A and B and HIV, and positive for HCV antibodies and RNA. 10 wk later, she was HCV RNA PCR-negative and remained so for the duration of follow-up of 2 yr, during which time her ALT remained in the normal range. Long-term follow-up subjects who were HCV antibody- and HCV RNA-positive or -negative were recruited from Oxford (Table I). This group consisted of 36 HLA-A2+ subjects who had been HCV-seropositive for at least 2 yr before analysis (mean 16, range 2–30 yr). 19 subjects were persistently RNA-positive, and 17 subjects were persistently PCR-negative (at least 3 independent tests). PBMCs were obtained from patients by centrifugation over Lymphoprep (Nycomed Pharma SA), followed by washing in RPMI 1640 (Sigma Chemical Co.) and 10% FCS. PBMCs were frozen immediately and stored in liquid nitrogen until ELISPOT and tetramer analyses were performed. In most cases, analyses of frozen sequential samples by the different assays were performed simultaneously after thawing.

Synthesis of HCV-derived Peptides. Peptides corresponding to the amino acid (aa) sequences of the HCV-1 strain were synthesized as free acids by Cambridge Research Biochemicals or Chiron MImotopes using the Fmoc method. Peptides were 20 aa in length, overlapping adjacent peptides by 10 aa. Fine mapping was achieved using additional smaller peptides in free acid form, which were synthesized on an automated peptide synthesizer (model 432A, Applied Biosystems, Inc.). Peptides (9-aa length) for tetramer complexes were purchased from Reserach Genetics.

Identification of Virus-specific CTL Responses. Lympohocytes were expanded with a single round of antigen-specific stimulation, using a protocol modified from Lubaki et al. (29). Stimulator cells that presented all potential HCV antigens in the context of autologous HLA alleles were prepared by infecting 4 × 10^6 autologous EBV-transformed B lymphoblastoid cell lines (B-LCLs) with the vaccinia HCV recombinant viruses (see below) at a multiplicity of infection of 5–10 for 18 h. The vaccinia viruses were then inactivated by resuspending the stimulating cells in 5 ml of a 10 μg/ml psoralen solution (4-h-aminomethyl-4,5,8-trimethylpsoranyldrochloride; HR1 Associates) and then exposing the cells to long UV light (8-W bulb, 350–400-nm light; Fisher Scientific) for 5 min, followed by 100-Gy irradiation. PBMCs (4–6 × 10^6 cells) were stimulated with the stimulator B-LCL (2 × 10^4 cells infected with HCV-H 1–966 and 2 × 10^4 cells infected with HCV-H 827–3011 (30), a gift of Dr. C. Rice, Washington University School of Medicine, St. Louis, MO) and allogeneic irradiated (30-Gy) PBMCs (20 × 10^6) in 20 ml R10 medium (RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin) and incubated at 37°C in 5% CO_2. R10 medium containing rIL-2 (50 U/ml) was added on day 3. On day 14, clones were derived from bulk-expanded cells by subculturing in 96-well plates at limiting dilution (25, 10, 5, and 3 cells/well) with 2 × 10^4 allogeneic irradiated (30 Gy) PBMCs at each well in 100 μl R10 medium with the CD3-specific mAb 12F6 (0.1 μg/ml; a gift of Dr. J. Wong, Massachusetts General Hospital) and rIL-2 (100 U/ml). Developing cells were restimulated in 24-well plates with irradiated (30 Gy) allogeneic feeder cells (1 × 10^6 cells/well), 12F6 (0.1 μg/ml), and rIL-2 (100 U/ml) in R10 medium. Specificity of unselected clones was then identified by analysis of lytic activity against autologous B-LCLs infected with recombinant vaccinia vectors expressing aa 1–339 (9A), 1–966 (HCV-H), 339–906 (1H), 364–1619 (NNRd), 827–3011 (HCV-H), 1590–2050 (N54), 2055–2396 (N55A), 2396–3011 (N55B), and vMak2 (control vaccinia vector expressing the Esherichia coli β-galactosi- dase gene, a gift of Dr. M. Houghton, Chiron Corp., Emeryville, CA). HCV-specific clones (defined as clones having >20% spe-
Table I. Characteristics of HCV-seropositive Subjects

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Mean 47 76

*Liver histology before treatment: CAH, chronic active hepatitis; CAH/F, chronic active hepatitis with fibrosis.
‡Treatment: 1, IFN-α alone; 2, IFN-α and ribavarin.
§Minimal duration of HCV RT-PCR negative status at time of tetramer analysis.
iNo. of tetramers giving positive staining results/total no. of tetramers (not all patients were tested with the NS5B 2594 tetramer).
¶Patients with acute hepatitis C. ALT and PCR results are from the last time point of follow-up (>50 wk).
specific lysis and <10% background lysis) were maintained in long-term culture in T-25 flasks by restimulating 2–4 × 10^6 lymphocytes every 3–4 wk with 20 × 10^6 irradiated (30 Gy) allogeneic PBMC feeders, 0.1 µg/ml 12F6, and 50 U/ml rIL-2 in 20 ml R-10 medium. HLA restriction of individual clones was determined by using partially HLA-matched B-LCLs. The fine specificity of the clones was determined using peptides 20 aa in length, overlapping by 10 aa, and subsequently truncated peptides. Optimal epitopes were defined as the smallest peptide that sensitized target cells for maximal lysis in a cytotoxicity assay at the lowest peptide concentration. Cytotoxicity assays using 51Cr-labeled B-LCLs as targets were performed as described previously (2, 8). Longitudinal quantification of activity against peptide epitopes was then analyzed directly on PBMCs using optimal epitopes in an IFN-γ ELISPOT analysis as described below.

Quantification of T Cell Response Using IFN-γ ELISPOT Assay. Cryopreserved PBMCs were thawed and incubated at 37°C overnight in R-10 medium, 96-well nitrocellulose plates (Millipore) were coated with 2.5 µg/ml recombinant human anti-IFN-γ antibody (Endogen) in a carbonate/bicarbonate buffer (pH 9.6) overnight at 4°C. Autologous B-LCLs were infected with different recombinant HCV-vaccinia virus vectors overnight, washed, and 1 × 10^5 cells per well were used as antigen-presenting cells. PBMCs were added at 1 × 10^5, 0.5 × 10^5, and 0.25 × 10^5 cells per well in duplicates. For detection of peptide-specific CD8+ T cells, synthetic peptides (5 µg/ml) corresponding to defined optimal epitopes were added to PBMCs. For T helper cell assays, PBMCs were incubated with soluble protein antigens (10 µg/ml) in 96-well U-bottomed plates overnight and then transferred directly into the ELISPOT plate. The following protein antigens were used: HCV-1 C22-3 (core, aa 2-120), C33c (NS3, aa 1192-1457), C100 (NS4, aa 1569-1931), and N55 (aa 2054-2995) expressed as COOH-terminal fusion proteins with superoxide dismutase (SOD) in yeast or E. coli (provided by Chiron Corp.). Recombinant SOD was used as control antigen. The ELISPOT method using recombinant proteins was specific for CD4+ T lymphocytes as determined previously with other HCV blood donors using CD8- or CD4- lymphocyte-depleting antibodies. After incubation at 37°C for 20–24 h, the plates were washed, labeled with 0.25 mg/ml biotin-labeled anti-human IFN-γ (Endogen), and developed by incubating with streptavidin–alkaline phosphate (Bio-Rad) followed by incubating with BCIP/NBT (Bio-Rad) in Tris buffer (pH 9.5). The reaction was stopped by washing with tap water and allowed to dry before counting the spots at a magnification of 40, with a dissecting microscope. All wells with 10–150 spots were considered evaluable, and estimates of cell frequencies were obtained by linear regression analysis.

Tetrameric MHC Class I-Peptide Complexes. Tetrameric peptide-MHC class I complexes were made as described previously (31). In brief, recombinant human β2-microglobulin and the extracellular portion of the MHC class I heavy chain A*0201 containing the BirA recognition sequence in frame at its COOH terminus were expressed in E. coli as insoluble aggregates that formed inclusion bodies. Purified inclusion bodies were solubilized in urea and monomeric HLA class I complexes refolded around peptide by dilution of denaturing conditions. The following peptides were used: HCV N S3 1073-1081 (CINGVCWTV), HCV N S3 1406-1415 (KLVAGLNIV), N S4B 1807-1816 (LLFMLRDPQ), N S5B 2594-2602 (ALYDIVKT), EBV lytic protein BM LF1 (GLCLTVLAM) (33). After buffer exchange, a specific lysine residue in the heavy chain COOH-terminal tag was biotinylated with BirA enzyme (Avidity). Monomeric complexes were purified by gel filtration and anion exchange chromatography. Tetrameric arrays of biotinylated peptide-MHC class I complexes were formed by the addition of PE-labeled avidin (Extravidin; Sigma Chemical Co.) at a molar ratio of 4:1. For confirmation of specificity, tetramer staining was performed on HLA-A2-restricted HCV-specific CTL lines or clones derived from infected patients as described previously (5, 8, 34; data not shown). Staining of PBMCs from healthy HCV-seronegative individuals and of cloned CTLs diluted in PBMCs from healthy individuals established that the approximate limit of detection with tetramers was at 0.02% of CD8+ lymphocytes (data not shown). FACStm staining results of PBMCs were only considered specific when the tetramer-positive cells formed a cluster distinct from the tetramer-negative CD8+ lymphocytes. Staining of uninfected controls was routinely <0.02% of CD8+ lymphocytes. In some instances, results were confirmed by peptide pulsing of PBMCs and staining after 8 d, which led to a peptide-specific increase in numbers of tetramer-positive cells in HCV-seropositive but not control subjects (not shown).

Flow Cytometry. For tetramer staining, thawed PBMCs were washed three times in RPMI 1640 and 10% FCS and stained with tetrameric complexes and antibodies. The following mAbs were used: anti-CD8-TRICOLOR (CalTag Laboratories), anti-CD8-peridinin chlorophyll protein (PerCP; Becton Dickinson Immunocytometry Systems), anti-CD38-allophycocyanin (Becton Dickinson), anti-human MHC class II-FITC (HLA-DR, -DP, and -DQ; Dako), and anti-CC chemokine receptor (CCR)5-FITC (Pharmingen). 106 PBMCs were first incubated with tetrameric complexes for 20 min at 37°C, then the antibodies were added and incubated for an additional 20 min on ice. The cells were washed with PBS/2% FCS and fixed with 0.5% formalin/PBS. Samples were analyzed on a FACScan@libur™ flow cytometer, and the data were analyzed using CELQuest software (Becton Dickinson). For intracellular IFN-γ staining, thawed and washed PBMCs were incubated at 37°C for a total of 6 h in the presence of PMA (50 ng/ml) and ionomycin (500 ng/ml). Brefeldin A (10 µg/ml) was added for the last 4 h of incubation. After washing with PBS/2% FCS, the cells were stained with tetramers for 30 min at 37°C and again washed in PBS/2% FCS. The cells were fixed in 4% (wt/vol) paraformaldehyde/PBS for 10 min on ice and then permeabilized using PBS containing 2% FCS, 0.1% (wt/vol) sodium azide, and 0.1% (wt/vol) saponin (permeabilization buffer; 10 min on ice). Permeabilized cells were stained with anti-CD8-PerCP and anti-IFN-γ-FITC or FITC-conjugated isotype control antibody (Becton Dickinson). The stained cells were washed twice in permeabilization buffer and once in PBS/2% FCS, then fixed in 0.5% formalin/PBS, and analyzed by flow cytometry. For peptide-induced upregulation of CD69, thawed and washed PBMCs were incubated with 1 µM peptide for 4 h and then washed with PBS/2% FCS. The cells were stained with tetramers for 30 min at 37°C, followed by an incubation with FITC-conjugated anti-CD69 antibodies (Becton Dickinson) and PerCP-conjugated anti-CD8 antibodies for 30 min on ice. After washing with PBS/2% FCS, the cells were fixed and analyzed by flow cytometry.

**Results**

Analysis of the Breadth of the HCV-specific CD8+ Lymphocyte Response in a Subject with Resolving Acute HCV Infection. A 42-yr-old intravenous drug user presented to the hospital with acute HCV infection, characterized by ele-
vated ALT and HCV RNA positivity. ALT subsequently normalized, and HCV RNA became negative. Blood samples were available from this subject during and after acute illness such that the development of HCV-specific T cell responses during a successful elimination of the virus could be studied in detail. In an initial set of experiments, the CTL epitopes recognized were identified and characterized by in vitro expansion and cloning of PBMCs from a week 4 blood sample after stimulation with autologous B-LCLs infected with recombinant HCV-vaccinia viruses (see Materials and Methods). 120 clones were raised and screened for CTL recognition of autologous B-LCLs infected with recombinant HCV-vaccinia viruses. All HCV-specific CTL clones were further characterized using a panel of overlapping peptides representing the expressed gene products of HCV-1. A total of 80 HCV-specific CTL clones were generated and found to recognize one of 8 epitopes: 2 restricted by HLA-A2 (NS3 1073–1081 and NS5B 2594–2602), 1 by HLA-B37 (NS4 1966–1976), and 5 by HLA-A25 (NS2 832–841, NS4 1744–1754, NS4 1758–1766, NS4 2225–2233, and NS5A 2225–2233). This method was repeated for PBMCs from weeks 0, 10, and 24, yielding clones of the same specificities, but no additional epitopes were identified.

Except for the HLA-A2–restricted epitope NS3 1073, all defined epitopes recognized by subject 1 had not been described previously. Fig. 1 demonstrates the cytolytic activity of individual clones using target cells pulsed with the optimal epitopes. The newly identified HLA-A2–restricted NS3 2594 response targets a peptide conforming to the defined HLA-A2 motif, with a leucine at the COOH terminus and at position 2 (35). The A25–restricted responses all have a motif similar to that described in a conserved peptide from HIV p24 gag (36), which includes a tryptophan at the COOH terminus. Together, these data indicate that the CTL response in this subsequently controlled infection was simultaneously targeted at eight different epitopes.

Longitudinal Quantification of the CTL Response in Resolving Acute HCV Infection. The availability of longitudinal samples in subject 1 allowed for the detailed quantification of CTL responses over the course of resolving acute hepatitis. Comparison was made using two separate techniques. Initially, CTL responses to naturally processed viral proteins expressed by recombinant HCV-vaccinia viruses were determined using an IFN-γ ELISPOT assay. This assay confirmed the multispecificity of the response, and demonstrated that responses peaked between weeks 4 and 10, at a time when ALT levels had already normalized and no viral RNA was detectable in blood (Fig. 2 A). These results were confirmed using a peptide ELISPOT assay, in which PBMCs were stimulated with the individual optimal peptides as identified using cloned CTLs (Fig. 2 B). Both assays showed that the magnitude of the overall response was maximal at week 10 after onset of jaundice. Relative responses to individual proteins were similar with both ELISPOT assays. The HLA-A2–restricted response to NS4 1744 was maximal at the earliest time point tested (Fig. 2 B), although the overall response to NS4 (containing another two identified epitopes) peaked between weeks 5 and 10, similar to the other proteins (Fig. 2 A). Assuming that 10% of PBMCs are CD8+ lymphocytes, the total number of IFN-γ-producing cells was estimated to represent ~6% of CD8+ lymphocytes at the peak of the response. These results indicate that the magnitude of the HCV-specific IFN-γ-producing CTLs in resolving acute hepatitis continued to increase at a time when the ALT was decreasing and the HCV RNA had become negative.

Phenotypic Characterization of the Antiviral CD8+ Lymphocyte Response. The above studies provide a functional analysis of the CTL response in terms of IFN-γ production, but do not assess for the presence of CTLs that may be unable to mediate this specific effector function. The use of peptide–HLA tetramers to directly visualize antigen-specific CD8 cells by flow cytometry allows a more precise quantification of the full complement of antigen-specific cells. The HLA-A2–restricted CTL response was studied...
in subject 1 using such tetrameric complexes to accurately define the dynamics and phenotype of these populations. Fluorescently labeled tetramers specific for four HLA-A2-restricted epitopes (NS3 1073–1081, NS3 1406–1415, NS4B 1807–1816, and NS5B 2594–2602) were used to stain antigen-specific CD8+ lymphocytes in PBMCs (Fig. 3 A). PBMCs were also stained for the presence of the activation markers CD38, MHC class II, and CD69, as well as the chemokine receptor CCR5. A very strong CD8+ lymphocyte response was observed directed against the epitope NS5B 2594, comprising 7.40% of circulating CD8+ lymphocytes at the first time point when the patient was jaundiced (Fig. 3, A and B). These high levels of tetramer-positive cells, which expressed the activation markers CD38 and HLA class II, occurred at the time of maximal ALT elevation, reflecting destruction of hepatocytes (Fig. 3, B–D). The initial NS5B 2594–specific CD8+ response was also associated with the expression of high levels of the chemokine receptor CCR5, which is mainly found on Tc1 cells (37; Fig. 3 E). The frequency and activation status of NS5B 2594–specific CD8+ lymphocytes decreased parallel to the rapid drop of ALT. Although the NS5B 2594–specific CD8+ response persisted at a level of ~2% of CD8 cells, these cells no longer expressed elevated levels of CD38 and HLA class II, as had been observed during the peak of the response. The CD8+ response

Figure 2. Antigen-specific IFN-γ production of HCV-specific T lymphocytes during resolution of acute HCV infection. PBMCs from subject 1 were tested for HCV-specific IFN-γ release in an ELISPOT assay at the time points shown after presentation with acute HCV infection. (A) Results from IFN-γ ELISPOT assays using autologous B-LCLs infected with the designated recombinant HCV-vaccinia vectors as stimulator cells. (B) Results from IFN-γ ELISPOT assays using designated HCV peptides as stimulators. The y-axis shows numbers of HCV-specific PBMCs in 10^6 PBMCs producing IFN-γ.
against the A2-restricted epitope NS3 1073 was subdominant at the time of initial analysis, with 1.1% of CD8 cells reacting with this tetrameric complex. It also peaked later than that against N55B 2594 (maximum at week 10 = 2.35% of CD8+ lymphocytes; Fig. 2 A) and remained roughly at this level throughout follow-up. These cells demonstrated lower levels of CD38 expression and only slightly elevated HLA class II expression compared with total CD8+ lymphocytes, and peaked at a time when ALT levels had already normalized (Fig. 3). No lymphocytes stained with tetramers for the two HLA-A2–restricted HCV epitopes NS3 1406 and NS4B 1807, consistent with the lack of detection of these responses in the cloning assays. It should be noted that acute hepatitis was also associated to a lesser extent with increased expression of CD38, HLA class II, and CCR5 on total CD8+ lymphocytes (Fig. 3, C–E). These tetramer-negative CD38+HLA class II+CCR5+ cells detected during clinical illness are likely to include HCV-specific T cells recognizing epitopes other than the HLA-A2–restricted one, e.g., those restricted by HLA-A25, for which no tetramers were available. In addition, these cells may also represent activation of bystander (non–HCV-specific) CD8+ lymphocytes. This possibility is suggested by the detection of EBV–specific cells expressing elevated levels of CD38 and HLA class II at early time points (Fig. 3, C and D). Expression of CD69 was not elevated on tetramer-positive cells compared with total CD8+ lymphocytes at any time point examined (data not shown).

**Figure 4.** Functional analysis of HCV–specific PBMCs. (A) Intracellular IFN-γ staining. PBMCs from subject 1 were stimulated with PMA and ionomycin for 6 h or left unstimulated and subsequently stained with PE-labeled N55B 2594 tetramers, PerCP-conjugated anti–CD8, and FITC-conjugated anti–IFN-γ or isotype control antibodies. Results from the gated CD8+ lymphocytes are shown for different time points. The percentage of tetramer-positive CD8+ lymphocytes that express IFN-γ is shown in each panel. (B) Peptide-induced upregulation of CD69. PBMCs were incubated with NS5B 2594 peptide (1 μM) for 4 h and stained with PE-labeled NS5B 2594 tetramers, FITC-conjugated CD69, and PerCP-conjugated CD8 antibodies. Results from the gated CD8+ lymphocytes are shown for different time points. The percentages of tetramer-positive and tetramer-negative CD8+ lymphocytes expressing CD69 are shown in each panel. (C) Cytolytic capacity of PBMCs from the first time point (week 0) was tested after 9 d of in vitro culture. PBMCs were preincubated with NS5B 2594 peptide (10 μM) for 1 h, washed, and incubated for 3 d, after which 10% (vol/vol) IL-2 containing Lymphocult-T (Biotest AG) was added. T2 cells (HLA-A2++) were preincubated with 1 μM cognate peptide (●) or no peptide (○) and used as targets in a 6-h 51Cr-release assay at E/T ratios shown.
the cell surface, both of which are downregulated after antigen exposure (39–42). Interestingly, at the first time point evaluated, a percentage of NS5B 2594–specific cells showed a reduced intensity of anti-CD8 antibody and tetramer staining compared with later time points, a feature that was not seen on NS3 2073– or EBV–specific lymphocytes (Fig. 5). These latter cells also showed lower levels of CD38 and HLA class II expression consistent with a lower level of activation. To investigate the proliferative and cytolytic potential of these cells, PBMCs from week 0 were placed in culture for 9 d with peptide and IL-2. After this period, no expansion of the tetramer-positive population was seen (total 5% of CD8⁺ lymphocytes, data not shown), nor was CD69 upregulated. However, these cells showed a very marked lytic capacity (Fig. 4C). These results indicate that at the time of acute illness, the NS5B 2594–specific cells were not able to produce IFN-γ or respond to peptide in culture, possibly due to recent encounter of antigen in vivo; however, after cultivation they showed strong lytic capacity. Due to a limited number of cells, we could not test if the NS5B 2594–specific cells also exhibited lytic activity when tested directly after isolation.

A niltriviral T Helper Response in Acute Resolving HCV Infection. In subject 1, we were also able to study the antiviral T helper response during the acute phase of the disease (Fig. 6). IFN-γ ELISPOT assays were performed on PBMCs, using recombinant proteins derived from HCV as target antigens. Unlike IFN-γ–secreting CTL responses, antigen-specific T helper responses were maximal at the first time point and remained high up to week 10, after which the responses decreased 5-10-fold but still remained readily detectable. The response was multispecific at all times, a feature previously associated with clearance after acute infection (23, 24, 43), and was calculated to comprise ~3% of CD8⁺ lymphocytes at the time of maximal response. These results indicate that the peak magnitude of the detectable IFN-γ T helper cell response preceded the peak magnitude of the IFN-γ CTL response.

Analysis of HCV–specific T Lymphocyte Responses in Patients with Recent Acute HCV. Two additional subjects were identified with acute HCV infection, in whom blood for immunologic analyses was first available a few weeks or months after documented symptomatic illness. These two HLA-A2⁺ subjects had, as did subject 1, an acute self-limiting illness, with jaundice and elevation of ALT after exposure to HCV. Both subjects became HCV PCR–negative before blood was taken for T cell analysis. In subject 2, the first sample was obtained at week 4, and in subject 3, the first sample was available at week 36. Using tetramer staining of PBMCs, antiviral CD8⁺ responses were detected against two (subject 2) or three (subject 3) of the three tested HLA-A2–restricted epitopes, respectively. Responses to the HLA-A2–restricted epitope NS5B 2594 could not be determined because at the time of analysis this epitope had not yet been characterized and therefore no tetramers were available. In both subjects, switches in the dominance of HCV–specific T cell responses occurred over time, even weeks after the original illness and after clearance of HCV from the blood by PCR and normalization of ALT (Fig. 7). As in subject 1, the tetramer-positive populations in subjects 2 and 3 persisted well beyond the clearance of viremia. Interestingly, expansions of HCV–specific lymphocytes after resolution of acute illness, despite their magnitude (in subject 2 especially), did occur without elevation of ALT, and the tetramer-positive cells showed low expression of CD38 and HLA class II (~20% for CD38 and <7% for HLA class II in both cases, not shown), similar to what was seen at later stages in subject 1. Potential responses to other peptides could not be tested because of the lack of available cells for additional tetramer or ELISPOT analysis. However, sufficient PBMCs from subject 3 were available at week 93. We were able to demonstrate IFN-γ...
secretion in PMA-stimulated tetramer-positive CD8\(^+\) lymphocytes as well as in peptide IFN-γ ELISPOT assays at this time point (peptides NS3 1073 and NS3 1406; data not shown). In addition, a multispecific T helper response was detected using recombinant proteins in an IFN-γ ELISPOT assay, with responses to NS3, NS4, and NS5 (not shown). This indicates that the secretory capacity of the antiviral CTLs as well as T helper cells was preserved in this subject, similar to the later time points of subject 1. These results suggest that patients with resolving acute HCV infection generate strong and multispecific T helper cell as well as CTL responses that are maintained after resolution of viremia and normalization of ALT.

A analysis of HCV-specific T lymphocytes in Long-Term HCV-seropositive Persons. 36 HLA-A2\(^+\) persons who had been HCV-seropositive for several years (see Materials and Methods) were also screened using HLA-A2 tetramers. These subjects were divided into two groups, depending on the continued presence or not of virus in blood, as detected by PCR. In those who remained PCR-positive, irrespective of ALT or treatment history, the percentage of HCV-specific CD8\(^+\) lymphocytes in peripheral blood was at or below the limit of detection by tetramers (Fig. 8). This is consistent with previous studies showing that CTL precursor frequencies in chronic hepatitis C infection are relatively low (4, 5). In those patients where HCV-specific cells

**Figure 7.** Tetramer analysis of PBMCs from two subjects after resolution of acute hepatitis infection. Results of tetramer stainings of PBMCs are illustrated as in Fig. 2. Subjects 2 and 3 had developed acute disease, but the first blood samples available were from time points when ALT levels had already normalized and HCV RT-PCR was negative. The expression on tetramer-positive CD8\(^+\) lymphocytes of CD38 was <20% and <7% for HLA class II at all stages (not shown). The N55B 2594 tetramer was not available at the time these specimens were analyzed.

**Figure 8.** Comparison of HCV-specific CD8\(^+\) lymphocyte responses in subjects with acute HCV infection and in long-term HCV-seropositive subjects. Tetramer staining was performed on PBMCs from subjects 1, 2, and 3 with documented primary acute HCV infection. Peak responses detected during the first 20 mo after onset of disease are shown for each CTL epitope. 36 subjects who were long-term HCV-seropositive and either RNA PCR-positive or -negative were also analyzed. As a comparison, HLA-A2-restricted EBV responses are shown for all patient groups. Results are shown as the percentage of tetramer-positive cells among total CD8\(^+\) lymphocytes. Filled symbols, subjects who were or became HCV RNA PCR-negative during follow-up; open symbols, subjects who remained HCV RNA PCR-positive.
could be detected (n = 9/19), the average percentage of tetramer-positive CD8⁺ lymphocytes was 0.07 (Fig. 8). In persons who were persistently antibody-positive but PCR-negative, either spontaneously or after treatment, similarly low levels of tetramer-positive CD8⁺ lymphocytes were seen (average 0.09%) compared with those observed during or after acute infection, although in two cases, levels >0.2% were seen. In one subject tested on two occasions >1 yr apart, this population was maintained (not shown).

Compared with the patients who remained PCR-positive, there was overall a greater proportion of positive tetramer responses observed in persons who cleared their RNA (18/59 vs. 11/65 tests; P = 0.07 by Fisher’s exact test). There was also an excess of responses seen to the NS5B 2594 epitope (4/8 vs. 0/10 persons; P = 0.02 by Fisher’s exact test). In the PCR-positive group, only one subject (1/19) showed a response against more than one tested epitope, whereas in the PCR-negative group 50% of the responding persons had CTLs specific for more than one tested epitope (P = 0.04 by Fisher’s exact test). By comparison, the percentage for EBV-specific cells in those patients who had detectable EBV-specific CD8⁺ T lymphocytes (n = 22/36) was 0.32, thus higher in most patients than the responses seen against HCV (P = 0.047 by Wilcoxon signed rank test).

In both PCR-positive and -negative persons, CD38 expression on HCV and EBV tetramer-positive CD8⁺ lymphocytes was always <15%, and HLA class II expression was <7% (data not shown). These results indicate that CTL responses were more common in PCR-negative subjects than in PCR-positive subjects, but these responses were weak compared with patients who had recently resolved acute HCV infection and did not express activation markers.

**Discussion**

There are two clearly defined outcomes after acute HCV infection—on the one hand, clearance of the virus below the level of detection by PCR, and on the other, viral persistence associated with varying degrees of liver damage. It is thought that the cellular immune response contributes to control of the virus in the first instance, and potentially to liver inflammation in chronic disease, but the details of these associations have been difficult to disentangle. There are at least four fundamental problems in doing so: the virus is highly variable, available immunologic assays have lacked sensitivity, people typically present only years after infection, and antiviral lymphocyte populations may be sequestered in the liver (10, 44). Despite these problems, it is important to understand the immune responses in early HCV infection, as the events occurring during this period may be crucial to the eventual outcome of this infection (11, 43, 45, 46).

Fluctuations of number and function of T cells in acute disease have been evaluated by several groups. A very broad antiviral T helper as well as CD8⁺ T lymphocyte response was detected in subject 1 from the earliest time points. Using tetramers, maximal antiviral CD8⁺ lymphocyte frequency against one of the epitopes (NS5B 2594) was determined to be 7.4% at the peak of clinical illness when virus was still detectable (Fig. 3). ELISPOT data suggest that the total frequency of antiviral CTLs at early stages was even higher because the IFN-γ responses found against other epitopes (for which no tetramers were available) were as high as the response to NS5B 2594 (Fig. 2 B). This level of expansion of CTLs during acute disease is of the same order of magnitude as that seen during resolution of acute mononucleosis syndrome caused by EBV, and higher than the levels found in chronic HCV infection (33, 47).

A total of eight different epitopes were targeted simultaneously in subject 1 that had been characterized in the greatest detail, and were presented by three different class I alleles. In addition, these responses persisted for the duration of follow-up, despite clearance of viremia and resolution of hepatitis. The breadth of the response was thus greater than that typically observed in chronic infection (8). The quantitative data in the present study thus add significantly to earlier studies of experimental acute HCV infection in chimpanzees, which showed variable recognition of up to nine different epitopes in a single animal with resolving acute infection (25). Although the techniques used in that study did not allow quantification of responses, persistence of responses for up to 1.5 yr after infection was also documented.

The availability of early samples as well as tetramers and IFN-γ ELISPOT assays allowed for the detection of a dominant initial immune response in subject 1 that would not have been appreciated with the more limited assays that have typically been used to study HCV infection in humans and animals. Tetramer analysis revealed that the NS5B 2594-specific CTLs expressed high levels of CD38 and HLA class II, representing an activated phenotype. These cells also had reduced expression of TCR (as judged by tetramer binding and anti-CD8 staining), suggesting recent exposure to cognate antigen in vivo (39–41). Interestingly, at this stage, the cells showed limited capacity to secrete IFN-γ in vitro (in ELISPOT assays and after PMA stimulation) and to upregulate CD69 after exposure to peptide. The phenotype of these cells was unexpected, particularly as at later time points the cells adopted a more conventional memory phenotype (CD38low and HLA class IIlow, with capacity to secrete IFN-γ and upregulate CD69 upon peptide stimulation).

The finding of subpopulations of CTLs with impaired ability to secrete IFN-γ in acute HCV infection has important implications for HCV pathogenesis. We propose three possible explanations for this phenomenon. First, the dominant NS5B 2594-specific cell population at week 0 may represent a subset of cells without IFN-γ secreting capacity. Despite this, NS5B 2594-specific CTLs detected at later time points were capable of producing IFN-γ. They may have changed their cytokine secretion profile over time, or alternatively, represent a different population of cells with the same peptide specificity. Due to limitations in cell numbers, we were unable to test exhaustively for dif-
differential cytokine secretion at the first time point, but they may theoretically have secreted IL-4 or IL-10 as has been previously observed in HCV-specific CTL clones (2), or only IL-2 but no IFN-γ, a cytokine pattern found in naive and a subset of memory CD4+ lymphocytes (48). Second, exposure to natural altered peptide ligands (APLs) emerging as the virus is suppressed may have altered the phenotype of these cells temporarily (49). Finally, the cells might have undergone a process of antigen-induced partial deactivation or exhaustion (22, 26, 38, 50). This may be particularly facilitated by exposure to high concentrations of antigen in the liver, without costimulation; it is possible that CTLs detected in blood could theoretically represent those cells leaving the liver after encountering antigen there, although it has recently been shown that the majority of activated CD8+ T cells entering the liver undergo apoptosis within the liver environment (51). Although not completely exhausted, the NS5B 2594–specific cells showed a temporarily dysfunctional phenotype, which we would describe as “stunned.” Their recovery may be due to the efficient control of virus by the evolving broadly directed CTL response, or through other mechanisms such as interaction with antigen-specific CD4+ lymphocytes (22).

This study also illustrates the rapid emergence of T helper responses in concert with CTL responses in early disease. These IFN-γ-producing T helper responses were of broad specificity, and were of greatest magnitude at the earliest time point sampled, in contrast to the CD8 IFN-γ responses. It is very likely these cells contributed to the resolution of disease, as suggested previously (23, 24, 43, 52). The future development of HLA class II tetramers will be important in dissecting the functional properties of these cells and determining the relationship between the magnitude of antigen-specific CD4 responses and the ability to proliferate and secrete cytokines.

Persistence and Fluctuation in CTL Responses after Acute Disease. A unique feature of this study was the fluctuation of populations of CTLs with different antigenic specificities at a time when plasma viremia had been cleared below the detection limit in blood and there was no further evidence of biochemical hepatitis (Fig. 7). These expansions of antiviral CTLs months after infection may be caused by bystander activation by other immunogens, or more likely may represent continued presentation of HCV antigens to CTLs in lymphoid organs or the liver. There is evidence for very low level presence of HCV RNA in the liver even in some patients who clear the virus from blood, and also in extrahepatic sites (53, 54).

The phenotype of CTLs in this phase after acute infection was also of interest. These populations of tetramer-positive cells, which could reach frequencies of up to 4% of CD8+ lymphocytes, did not show an activated phenotype, which could explain why no concomitant rise in ALT was observed. Alternatively, antigenic load was too low in these subjects to cause detectable liver damage at this stage. Low expression of CD38 and HLA class II on antiviral cells is similarly found in patients who have successfully cleared hepatitis B virus (13) or controlled acute EBV infection (33). It is unlike HIV-infected patients, in whom CD38 and HLA class II expression remains high on lymphocytes in the chronic phase of uncontrolled infection but decreases after successful antiretroviral treatment (47, 55–57).

In our group of persistently HCV antibody–positive subjects, where initial exposure to HCV had taken place several years previously, the levels of tetramer-positive CD8+ lymphocytes were generally low and the few cells that were detectable expressed low levels of CD38 and HLA class II, even in those in whom virus persisted. Several potential confounding factors may influence the magnitude of these responses, including viral variation leading to escape from immune recognition, reactivity to epitopes for which no tetramers were available, or sequestration of activated effector cells in the liver, which could explain why the few cells detected in the periphery expressed no activation markers. The finding of low to undetectable levels of tetramer-positive cells in chronic infection was consistent with additional studies using two HLA-B7 and two HLA-B8 tetramers containing defined HCV epitopes (Lechner, F., and P. Kleinerman, unpublished results). Likewise, the HLA-A2, -B7, and -B8 tetramers failed to detect HCV-specific CD8+ lymphocytes derived from explanted livers in situations where PBMcs were negative (58). Our results are consistent with other studies of chronic infection, in that even in situations where CTLs have been found by tetramers in the nonacute situation, the levels have been in general low (typically <0.1% of CD8+ cells) [10]. If the levels of antiviral CTLs are truly low, particularly in the presence of virus, the mechanisms leading to low CTL responses with a nonactivated phenotype require further study. Apart from antigenic variation leading to epitope loss or antagonism (59–61), or loss of CD4+ T helper responses (23, 24, 43), some long-term immunomodulatory effect of the virus may also be involved in chronic infection, which for example directly interferes with lymphocyte activation or antigen presentation.

Implications for HCV Pathogenesis. The data presented here indicate that strong and broadly directed CTL responses can be observed in persons with acute hepatitis who go on to successfully control HCV infection. These findings, coupled with the demonstration of a greater frequency of detection of CTL responses in long-term seropositive persons who remain PCR-negative, provide a rationale to explore immunotherapy as an adjunctive therapy in persons with chronic progressive HCV infection. In this regard, it will be important to perform similar detailed longitudinal studies in persons who develop acute HCV hepatitis and do not go on to clear infection but sustain viral replication.

It has been argued on the basis of animal models of HCV and lymphocytic choriomeningitis virus infection that the breadth of the CTL response may be important in maintaining viral control (25, 62, 63). This study also demonstrates a broadly directed CTL response in resolving infection, and emphasizes the fact that function as well as number must be taken into account in drawing conclusions about pathogenesis or protective roles of these cells.
We speculate that there is a short period of time when CTLs, acting in concert with CD4+ lymphocytes and possibly NK cells, are effective in antiviral control—beyond this, if virus is cleared, they may initially maintain a conventional phenotype, but if virus persists their numbers and effector function may decline (22). CTL activity is always a two-edged sword, and it is likely that CTLs contribute to hepatic inflammation and damage during acute disease when they have appropriate number, phenotypic markers, and lytic function. How they function or fail to function in chronic disease remains an open question that demands further study.

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