

# Determinants of Viral Clearance and Persistence during Acute Hepatitis C Virus Infection

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## Abstract

The virological and immunological features of hepatitis C virus (HCV) infection were studied weekly for 6 months after accidental needlestick exposure in five health care workers, four of whom developed acute hepatitis that progressed to chronicity while one subject cleared the virus. In all subjects, viremia was first detectable within 1–2 weeks of inoculation, 1 month or more before the appearance of virus-specific T cells. The subject who cleared the virus experienced a prolonged episode of acute hepatitis that coincided with a CD38<sup>+</sup> IFN- $\gamma$ <sup>-</sup> CD8<sup>+</sup> T cell response to HCV and a small reduction in viremia. Subsequently, a strong CD4<sup>+</sup> T cell response emerged and the CD8<sup>+</sup> T cells became CD38<sup>-</sup> and started producing IFN- $\gamma$  in response to HCV, coinciding with a rapid 100,000-fold decrease in viremia that occurred without a corresponding surge of disease activity. Chronic infection developed in two subjects who failed to produce a significant T cell response and in two other subjects who initially mounted strong CD4<sup>+</sup> T cell responses that ultimately waned. In all subjects, viremia was higher at the peak of acute hepatitis than it was when the disease began, and the disease improved during the viremia. These results provide the first insight into the host–virus relationship in humans during the incubation phase of acute HCV infection, and they provide the only insight to date into the virological and immunological characteristics of clinically asymptomatic acute HCV infection, the commonest manifestation of this disease. In addition, the results suggest that the vigor and quality of the antiviral T cell response determines the outcome of acute HCV infection, that the ability of HCV to outpace the T cell response may contribute to its tendency to persist; that the onset of hepatitis coincides with the onset of the CD8<sup>+</sup> T cell response, that disease pathogenesis and viral clearance are mediated by different CD8<sup>+</sup> T cell populations that control HCV by both cytolytic and noncytolytic mechanisms, and that there are different pathways to viral persistence in asymptomatic and symptomatic acute HCV infection.

Key words: hepatitis C • acute infection • immune response • infectious immunity–virus • cytokines

## Introduction

Hepatitis C virus (HCV)\* is a parenterally transmitted hepatotropic RNA virus that causes acute and chronic hepatitis and hepatocellular carcinoma (1). The mechanisms that de-

termine the outcome of HCV infection are not well understood, although it is widely assumed that cellular immune responses play an important role (2–5). Indeed, viral clearance is usually associated with a multispecific CD4<sup>+</sup> and CD8<sup>+</sup> T cell response to the virus (6–10) that usually persists after viral clearance, while it is not readily detectable in chronically infected subjects or in subjects who initially control the infection but in whom viremia subsequently recurs (6, 8, 11–26). It is unclear, however, whether chronically infected subjects never respond to the virus or whether they mount an early, vigorous T cell response that isn't maintained.

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\*Abbreviations used in this paper: GE, genome equivalents; HCV, hepatitis C virus; HCW, health care worker; sALT, serum alanine aminotransferase; SI, stimulation index.

The kinetics, vigor, and quality of the immune response to HCV during the first few days and weeks after infection probably contribute very importantly to the outcome of infection. The role of CD8<sup>+</sup> T cells during acute HCV infection has been most clearly demonstrated in experimentally infected chimpanzees where an early, multispecific intrahepatic CD8<sup>+</sup> T cell response to HCV was detected in 2/2 chimpanzees that cleared the virus but only in 1/4 chimpanzees that became chronically infected. In contrast, 3/4 chimpanzees that became chronically infected displayed a more narrowly focused and delayed response to the virus (27). In a separate study, the development of persistent infection in another chimpanzee was associated with the emergence of a CTL escape variant (28).

In view of these observations, the early T cell response to HCV is likely to play a critical role in the outcome of infection in humans. Because of the lengthy clinically silent incubation period of HCV, however, the immune response has never been studied before the onset of clinical symptoms of acute hepatitis (6, 7, 9, 10, 14); nor has it been studied at all in acutely infected subjects who don't become symptomatic, the commonest course of HCV infection (29). Therefore, in this study we analyzed the early viral and immune events immediately after needlestick exposure to HCV-positive blood and throughout the course of both clinically asymptomatic and symptomatic acute HCV infection. The results demonstrate the existence of a dynamic interplay between the virus and host in the early phase of infection, and they identify multiple virological and immunological parameters that are likely determinants of viral clearance and persistence.

## Materials and Methods

**Subjects.** 93 health care workers (HCWs) who were exposed to HCV after accidental penetrating needle stick exposure to HCV-positive blood were enlisted into this study. The demographic characteristics of the HCWs and the design of the study are described in detail (unpublished data). At baseline and at 1–4 weekly intervals after exposure, blood samples were drawn for serological, virological, and immunological analysis and the

medical history of each subject was recorded. A summary of the five HCWs who became infected is provided in Table I. The study protocol was approved by the Institutional Review Boards at all institutions.

**PBMCs.** At enrollment and at each study visit, ~50 ml of EDTA anticoagulated blood was obtained and either transported by overnight courier to the Scripps Research Institute for isolation of the next day, or PBMCs were immediately isolated at the Institute of Human Virology where they were cryopreserved for subsequent shipment and analysis in La Jolla. PBMCs were isolated on Ficoll-Histopaque density gradients (Sigma-Aldrich), washed three times in HBSS (GIBCO BRL), and they were either studied immediately or they were cryopreserved in media containing 80% FCS (GIBCO BRL), 10% DMSO (Sigma-Aldrich), and 10% RPMI 1640 (GIBCO BRL). The results of functional assays performed with fresh and cryopreserved PBMCs from the same bleed date were comparable.

**Recombinant HCV Proteins.** All recombinant HCV proteins (genotype 1a) and control superoxide dismutase (SOD) protein were provided by Michael Houghton (Chiron Corporation, Emeryville, CA). These proteins, designated HCV core (c22, amino acids 2–120), NS3 (c33, amino acids 1192–1457), NS4 (c100, amino acids 1569–1931), and NS5 (NS5 amino acids 2054–2995), were expressed as COOH-terminal fusion proteins with human SOD in yeast or *Escherichia coli*, as described previously (11, 13).

**Synthetic Peptides and HLA A2 Tetramers.** HCV-derived peptides (genotype 1a) previously shown to be HLA-A2-restricted HCV epitopes were synthesized with a free amine NH<sub>2</sub> terminus and a free acid COOH terminus by standard Fmoc chemistry by Research Genetics. The amino acid sequences of the five HLA-A2-restricted CTL epitopes and the FLU epitope are shown in Table II. The choice of these epitopes was based on their immunogenicity, HLA binding affinity and immunogenicity in infected subjects as reported previously (9, 20, 22, 30, 31). These peptides were initially dissolved in 100% DMSO (Sigma-Aldrich) at 20 mg/ml and further diluted to 1 mg/ml with RPMI 1640 before used as described previously (22). HLA-A2 tetramers corresponding to the HCV peptides were provided by the National Tetramer Core Facility at Emory University (Atlanta, GA). The specificity of the tetramers was determined by using peptide-specific CTL lines generated by repetitive stimulation of PBMCs from HLA-A2 positive chronically HCV-infected subjects with the corresponding peptides (data not shown).

**Table I.** Clinical and Serological Features of the Five Subjects Studied

| Subj. | Age | Sex | HLA class I |             | HLA class II |          | Genotype | HCV RNA of source               | Peak                            |       |      |          |             |  |
|-------|-----|-----|-------------|-------------|--------------|----------|----------|---------------------------------|---------------------------------|-------|------|----------|-------------|--|
|       |     |     | HLA-A2      | DRB1        | DQB1         | Genotype |          |                                 | HCV RNA                         | sALT  | Bili | Symptoms | Outcome     |  |
|       |     |     |             |             |              |          |          | <i>GE</i> × 10 <sup>6</sup> /ml | <i>GE</i> × 10 <sup>6</sup> /ml |       |      |          |             |  |
| 1     | 46  | F   | yes         | *0401 *1001 | *0303 *0501  | 1b       |          | 0.2                             | 59.1                            | 1,386 | 1.1  | no       | clearance   |  |
| 2     | 29  | M   | yes         | *1301 *1401 | *0503 *0603  | 1b       |          | 2.9                             | 48.3                            | 1,809 | 1.1  | no       | persistence |  |
| 3     | 32  | F   | no          | *0901 *1101 | *0202 *0602  | 1a       |          | 7.4                             | 0.7                             | 299   | 1.2  | no       | persistence |  |
| 4     | 56  | F   | no          | *0102 *0301 | *0201 *0501  | 1a       |          | 1.2                             | 15.4                            | 876   | 12.4 | yes      | persistence |  |
| 5     | 27  | M   | no          | *0401 *1501 | *0301 *0602  | 1a       |          | 12.4                            | 0.4                             | 1,883 | 8.3  | yes      | persistence |  |

**Table II.** *HLA-A2 Peptides used in this Study*

| Virus | Peptide No. | AA position | AA sequence |
|-------|-------------|-------------|-------------|
| HCV   | 1           | CORE 132    | DLMGYIPLV   |
|       | 2           | E2/NSA1 728 | FLLADARV    |
|       | 3           | NS3 1073    | CINGVCWTV   |
|       | 4           | NS3 1406    | KLVALGINAV  |
|       | 5           | NS5B 2594   | ALYDVVTKL   |
|       | 6           | FLU         | GILGFVFTL   |

**Antibodies.** Anti-CD8 FITC, anti-CD8-PE, anti-CD38-FITC, anti-IFN- $\gamma$ -PE, isotype FITC, isotype APC, and isotype PE antibodies were obtained from BD PharMingen. All antibodies were used for immunostaining and FACS<sup>®</sup> analysis according to the manufacturer's instruction.

**HCV RNA.** Detection HCV RNA was detected using the reverse transcription (RT)-PCR with primers targeting the 5' noncoding region of the HCV genome (HCV-Monitor Amplification kit; Roche Diagnostics Version 2.0) following the manufacturer's instructions. The quantity of HCV RNA in each specimen was expressed in HCV RNA genome equivalents (GE) per milliliter of serum. In Subject 1, the assay was performed in triplicate for each time point between weeks 2–15. The lower limit of detection by this method is 100 GE/ml.

**Sequence Analysis of CTL Epitopes.** After serum RNA extraction, RT-PCR, and cDNA cloning, the predominant clonotype was determined by using combined analysis of heteroduplex mobility shift and single-stranded conformational polymorphism based on hypervariable region 1 (32). The sequences of the epitopes of interest were determined bidirectionally from cloned cDNAs representing the predominant clonotype for each serum specimen.

**HCV Antibody Measurements.** HCV antibody testing was performed with the HCV EIA-2 (Abbott Diagnostics).

**HCV-Specific Proliferative T Cell Response.** PBMCs were re-suspended in complete media with 10% human AB serum (ABS;  $10^6$  cells per milliliter), plated at  $2 \times 10^5$  cells per 200  $\mu$ l/well, and stimulated with 10  $\mu$ g/ml of recombinant HCV and control proteins in five replicate wells of 96-well U-bottomed plates. [<sup>3</sup>H]thymidine (1  $\mu$ Ci) (Dupont NEN) was added to each well on day 6. Cultures were harvested on day 7 after 16 h of [<sup>3</sup>H]thymidine incorporation. The degree of cellular proliferation was expressed as stimulation index (SI) in which the average [<sup>3</sup>H]thymidine uptake (in cpm) in replicate wells stimulated with each HCV protein was divided by the average [<sup>3</sup>H]thymidine uptake in replicate wells stimulated with the control protein. The positive cutoff value was determined as 2.1 which was 2 SD above the mean SI for the seronegative controls. The sum of all positive stimulation indices was calculated to estimate the relative strength of the overall CD4<sup>+</sup> T cell response and the result was expressed as the "sum of all SI" for each subject.

To determine the contribution of CD4<sup>+</sup> and CD8<sup>+</sup> T cells to the proliferative response, CD4<sup>+</sup> and CD8<sup>+</sup> T cells in PBMCs were positively selected using CD4 and CD8 ferrous Miltenyi microbeads, positive separation column MS and MidiMACS<sup>™</sup> magnet (Miltenyi Biotec) according to manufacturer's instructions. The cells eluted from the column were >99% positive for CD4<sup>+</sup> and CD8<sup>+</sup> cells. Cells ( $2 \times 10^5$  cells per well) were plated with autologous irradiated PBMCs ( $10^5$  cells per well) as antigen present-

ing cells and stimulated with HCV antigens in five replicates as described previously. Concurrently, to assess the overall proliferative T cell responsiveness in each subject, PBMCs were plated at  $2 \times 10^5$  cells per 200  $\mu$ l/well, stimulated with and without PHA at 2  $\mu$ g/ml in three replicates, and harvested after 3 d of stimulation.

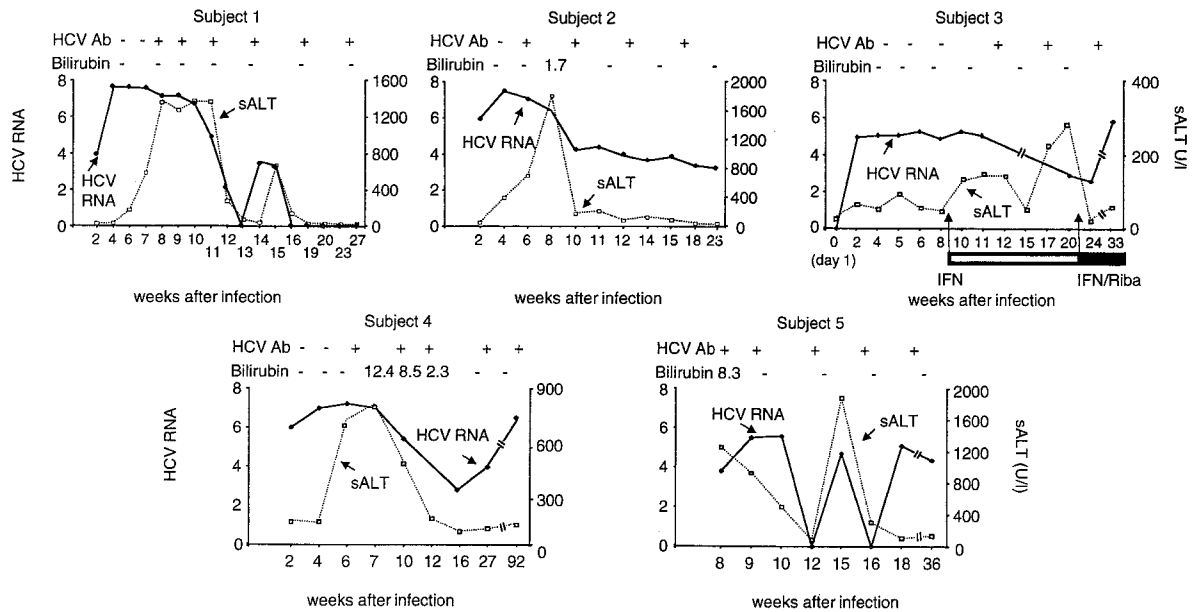
**HCV-specific CD8<sup>+</sup> T Cell Enumeration.** Quantification of HCV-specific CD8<sup>+</sup> cells was performed in HLA-A2-positive subjects by using HLA-A2 tetramers containing the peptides shown in Table II. PBMCs were incubated for 30 min with 1  $\mu$ g of the APC-labeled tetramers in a 96 well v-bottomed plate. Cells were washed in PBS containing 1% FCS and incubated with anti-CD8 and, in some experiments, also anti-CD38 for 30 min. Cells were again washed three times by consecutive resuspension in 250  $\mu$ l (total vol/well) of staining buffer, and then resuspended in 200  $\mu$ l PBS with 2% paraformaldehyde before FACS<sup>®</sup> analysis.

**Intracellular IFN- $\gamma$  Staining.** These procedures were performed essentially as described previously (12). Briefly, PBMCs ( $2 \times 10^6$  cells per milliliter) were stimulated with the peptides (10  $\mu$ g/ml) shown in Table II plus two additional peptides described in the results in duplicate wells in the presence of 50 U/ml human rIL-2 (Hoffmann-La Roche, Inc.) and 1  $\mu$ l/ml Brefeldin A (BD PharMingen). After 5 h of incubation (37°C, 5% CO<sub>2</sub>), the cells from each well were stained with antibodies to CD8 and IFN- $\gamma$  (BD PharMingen). Duplicate wells without peptide were also included to determine the background level of IFN- $\gamma$  production. In addition, one well without peptide was included for isotype antibody staining, and another well was stimulated with 10 ng/ml PMA (Sigma-Aldrich) and 200 ng/ml ionomycin to serve as a positive control for IFN- $\gamma$  staining. FACS<sup>®</sup> analysis was performed at the Scripps Research Institute Flow Cytometry Core Facility using FACSort<sup>™</sup> and FACScan<sup>™</sup> flow cytometers and analyzed with CELLQuest<sup>™</sup> software (Becton Dickinson). The frequency of cytokine-positive CD8<sup>+</sup> T cells was defined as the difference between the frequency detected in peptide-stimulated and unstimulated cells in the PBMCs.

**HLA Typing.** Class I (HLA) typing of PBMCs was performed by complement microcytotoxicity, using HLA typing trays (One Lambda). Class II typing was performed by PCR using sequence specific primers at the Immunogenetics and Transplantation Laboratory of the UCSD Medical Center, San Diego, CA (33).

## Results

**Course of Acute HCV Infection.** 93 HCWs who were exposed to HCV-positive blood by accidental penetrating needlestick injury were entered into this study. The five subjects who became acutely infected form the basis of this report. The salient characteristics of these individuals are summarized in Table I and Fig. 1. Subjects 1 and 2 were infected by genotype 1b while the others were infected by genotype 1a. All five subjects displayed serum ALT elevations of varying severity but only Subjects 4 and 5 became symptomatic with elevated serum bilirubin levels and jaundice. Thus, Subjects 1–3 were clinically unaware of their hepatitis, thereby permitting us to study asymptomatic acute HCV infection. Subjects 1 and 2 were HLA-A2-positive, thus allowing quantitative analysis of their CD8<sup>+</sup> T cell responses to HCV directly ex vivo with HLA-A2 tetramers containing five well-defined CTL epitopes derived from the viral core, E2, NS3, and NS5b proteins (see below).



**Figure 1.** Courses of acute HCV infection in five subjects after accidental needlestick exposure to HCV-positive blood. HCV RNA values were expressed as log GE per milliliter of serum. sALT activity was expressed in U per liter. Total bilirubin levels are expressed as mg/dl and <1.2 mg/dl are identified as negative (-). HCV-specific antibody responses reflect the results of an HCV EIA-2 assay as described in Materials and Methods.

Subjects 1–4 were viremic by 2 wk after exposure, whereas Subject 5, first evaluated 8 wk after exposure, was already viremic at that time. In all subjects, except patient 5 who was first seen after the onset of symptoms, high titers of HCV RNA initially occurred in association with completely normal serum ALT levels, indicating the noncytotoxic nature of HCV. All subjects developed acute viral hepatitis, detectable as elevated serum ALT activity. Slight serum ALT elevations were detectable as early as day 14 in some subjects, but they didn't reach peak levels or plateau until 7–8 wk after inoculation or later. HCV antibody conversion occurred 7–12 wk after exposure in all five subjects, corresponding fairly well with the onset of serum ALT activity and preceding significant decreases in viremia (Fig. 1). Only subject 1 spontaneously cleared the infection, while subjects 2–5 remained HCV RNA positive for 6 or more mo after infection. Subject 3 was treated acutely with IFN 8 wk after infection and ribavirin was added to the regimen at week 20 after infection. Nonetheless, this subject became chronically infected.

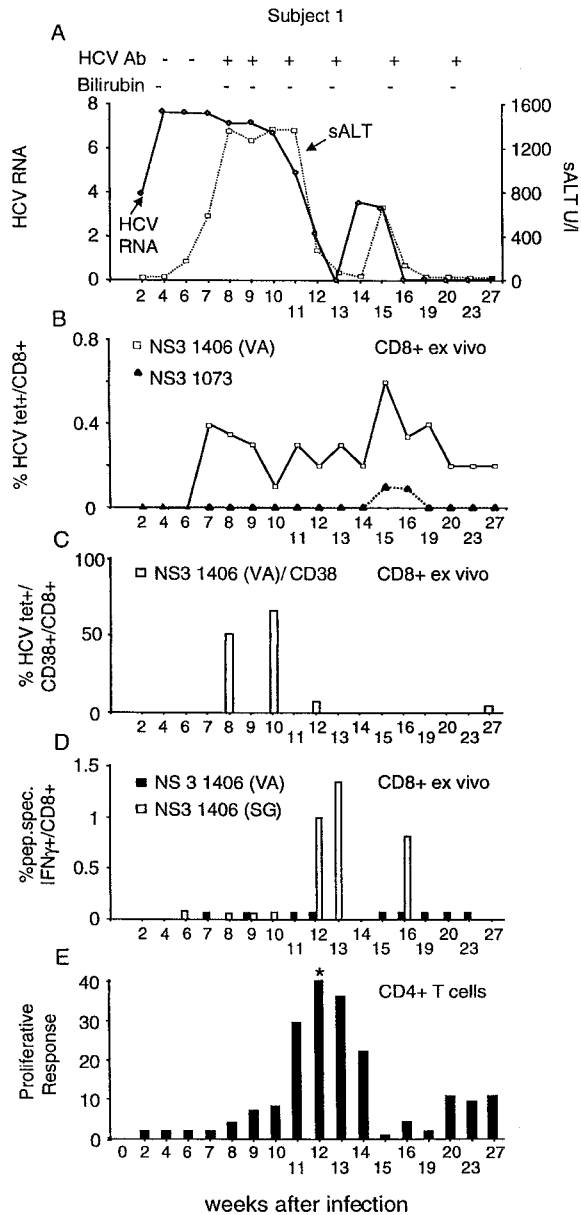
*Host-Virus Interactions in Asymptomatic Acute Viral Hepatitis.* The HCV-specific T cell responses produced by asymptomatic subjects 1–3 are shown in Figs. 2 and 3 and Table III. The CD4<sup>+</sup> proliferative T cell response was monitored in all three subjects. Because Subjects 1 and 2 were HLA-A2-positive, we were also able to determine the number of HCV-specific CD8<sup>+</sup> T cells in their blood. Because these subjects were asymptomatic, the following results represent the first description of the virological and immunological characteristics of the most common form of acute HCV infection in humans.

*The Immune Response to HCV in a Subject who Clears the Virus.* Asymptomatic Subject 1, the only subject to clear

the virus, was exposed to the lowest titer inoculum of the group (Table I) in an injury that was exceptionally high risk (direct penetration of the contaminated needle into a vein on the dorsum of the hand). As shown in Figs. 1 and 2, viremia was easily detectable 2 wk after inoculation and it rose very rapidly, reaching a peak titer of  $5.9 \times 10^7$  GE/ml by week 4. The viral titer was remarkably stable for the next 5–6 wk, falling only threefold by week 9 despite a severe and sustained hepatitis (see below). After week 10, however, the viremia rapidly decreased >5 logs by week 12 and it became undetectable ( $<10^2$  GE/ml) by week 13, without a surge in serum ALT activity (see below). Subsequently, the virus rebounded briefly on week 14, and finally became permanently undetectable on week 16. Hepatitis was first detectable as elevated serum ALT activity on week 6, 1 mo after the onset of viremia; it rapidly rose over the next 2 wk to 1374 U/l and it remained in that range until week 11 after which it decreased to normal, then rebounded to 666 U/ml on week 15 in response to the rebound in viremia, and returned permanently to baseline when the virus became permanently undetectable.

Several aspects of this subject's disease course should be noted. First, the virus was present at a very high titer for several weeks in the absence of hepatitis, indicating that HCV is not directly cytotoxic. Second, the high viral titer decreased only slightly during several weeks of rising and very high serum ALT activity, suggesting that the virus was not efficiently contained simply by the destruction of infected cells. Third, the serum ALT activity did not surge when the viral titer decreased >5 orders of magnitude between weeks 10 and 13, suggesting that noncytolytic antiviral mechanisms may have contributed to viral clearance during that period.

The cellular immune response to HCV was monitored



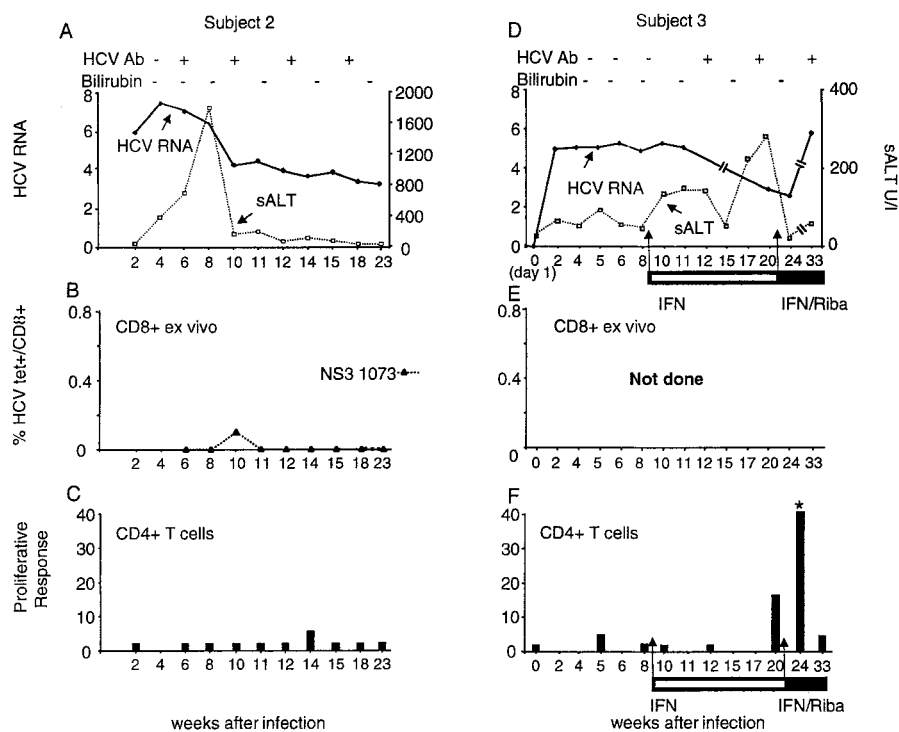
**Figure 2.** HCV-specific T cell responses in Subject 1 during asymptomatic, resolving acute HCV infection. (A) Course of infection (see Fig. 1). (B) Percentage of CD8<sup>+</sup> lymphocytes that were tetramer-positive at each time point. CD8<sup>+</sup> T cell responses were tested directly ex vivo using HLA-A2 tetramers complexed with five different HLA-A2 restricted epitopes (Table II). CD8<sup>+</sup> T cell responses against two epitopes (NS3 1073 and NS3 1406 [VA]) were detectable. (C) Percentage of NS3 1406 (VA)-specific CD8<sup>+</sup> T cells expressing the activation marker CD38. (D) Percentage of CD8<sup>+</sup> T cells that produce IFN- $\gamma$  in response to HCV NS3 1406 (VA) (black bars) and NS3 1406 (SG) (white bars). (E) Proliferative CD4<sup>+</sup> T cell responses against core, NS3, NS4, and NS5 are shown as the sum of all positive stimulation indices (Table III). \*Sum of all specific stimulation indices is 56 (see Table III).

at 1–2 weekly intervals throughout the course of this subject's infection by quantitating the number of HLA-A2-restricted, HCV-specific CD8<sup>+</sup> T cells using the panel of HLA-A2 tetramers shown in Table II. As shown in Fig. 2 B, NS3 1406 (KLVALGINAV) specific CD8<sup>+</sup> T cells were

first detectable in the peripheral blood 7 wk after infection, coinciding with the onset of liver disease, and they remained detectable at levels ranging between 0.1–0.6% of CD8<sup>+</sup> T cells for the duration of the study. In addition, we detected a brief response to NS3 1073 during weeks 14–15, coinciding with a second surge in liver disease activity and the termination of the infection. We did not detect T cells reactive with any of the other HCV tetramers at any time during the infection. This compared favorably with the number of Flu tetramer-specific CD8<sup>+</sup> T cells which were detectable at the 0.1–0.6% level throughout the study (data not shown). Interestingly, as shown in Fig. 2 C, the NS3 1406-specific CD8<sup>+</sup> T cells were CD38<sup>+</sup> (i.e., activated) on weeks 8 and 10, coinciding with the increased serum ALT activity, and they became CD38<sup>-</sup> on week 12 and thereafter, corresponding with the resolution of hepatitis. Surprisingly, at all time points tested during the course of infection, the activated CD8<sup>+</sup> T cells failed to produce IFN- $\gamma$  when they were stimulated by the KLVALGINAV peptide present in the NS3 1406 tetramer (black bars, Fig. 2 D). In contrast, 0.1–0.9% of CD8<sup>+</sup> T cells produced IFN- $\gamma$  in response to the Flu peptide shown in Table II (data not shown).

To investigate the basis for the discrepancy between KLVALGINAV-specific tetramer-binding and IFN- $\gamma$  production, the amino acid sequence of this epitope, as well as all the other HCV epitopes shown in Table II, was deduced by viral nucleotide sequence analysis at week 4 of infection. Importantly, the amino acid sequences of all tested epitopes, except NS3 1406, were identical to the sequences used to produce the peptides and tetramers. For NS3 1406, however, the viral sequence was KLSGLGINAV whereas the peptide used in the tetramer and in the intracellular IFN- $\gamma$  assays was KLVALGINAV, designated NS3 1406 (SG) and NS3 1406 (VA), respectively, hereafter. Therefore, the NS3 1406 (SG) peptide was synthesized and used to detect peptide-specific CD8<sup>+</sup> T cells by monitoring its ability to stimulate them to produce IFN- $\gamma$  directly ex vivo. As shown by the white bars in Fig. 2 D, NS3 1406 (SG)-specific, IFN- $\gamma$ -producing CD8<sup>+</sup> T cells were not detectable during the first 10 wk of the infection, when the liver disease was most pronounced, despite the fact that NS3 1406 (VA) tetramer-binding, activated (CD38<sup>+</sup>) CD8<sup>+</sup> T cells were present during that time (Fig. 2 B and C). Importantly, IFN- $\gamma$ -producing NS3 1406 (SG)-specific CD8<sup>+</sup> T cells suddenly appeared on week 12 (white bars, Fig. 2 D), coinciding with the disappearance of CD38<sup>+</sup> CD8<sup>+</sup> T cells (Fig. 2 C), a 5 log decrease in viremia and resolution of the liver disease (Fig. 2 A). Thus, a CD38<sup>+</sup>IFN- $\gamma$ <sup>-</sup>CD8<sup>+</sup> T cell response to HCV correlated with viral hepatitis but not with viral clearance, while a CD38<sup>-</sup>IFN- $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cell response correlated with viral clearance and resolution of hepatitis.

Note that the number of NS3 1406 (SG)-specific, IFN- $\gamma$ -producing CD8<sup>+</sup> T cells exceeded the number of NS3 1406 (VA) tetramer positive CD8<sup>+</sup> T cells by a large margin (e.g., 1.4 vs. 0.3% of CD8<sup>+</sup> T cells on week 13, respec-



**Figure 3.** HCV-specific T cell responses during asymptomatic acute HCV infection in Subjects 2 and 3 who progressed to chronicity. (A and D) Courses of infection in Subjects 2 and 3 (see Fig. 1). (B) Percentage of CD8<sup>+</sup> lymphocytes that were tetramer-positive at each time point. CD8<sup>+</sup> T cell responses were tested directly ex vivo in Subject 2 using HLA-A2 tetramers complexed with five different HLA-A2-restricted epitopes (Table II). A transient and weak CD8<sup>+</sup> T cell response was only detectable against a single epitope (NS3 1073). Subject 3 was not studied because she was not HLA-A2 positive. (C and F) Proliferative CD4<sup>+</sup> T cell responses against core, NS3, NS4, and NS5 are shown as the sum of all positive stimulation indices (Table III). \*Sum of all specific stimulation indices is 50.1 (see Table III).

tively), suggesting that the tetramer assay underestimated the magnitude of the CD8<sup>+</sup> T cell response, presumably because the subject's NS3 1406 (SG)-specific T cells recognized the NS3 1406 (VA) tetramer with lower affinity than they recognized the cognate peptide sequence itself. Analysis of the subject's CD8<sup>+</sup> T cell response with NS3 1406 (SG)-specific tetramers would be necessary to confirm this hypothesis, and to establish definitively the CD38 status of the IFN- $\gamma$ -producing T cells detectable on week 12 and thereafter during the phase of viral clearance. However, due to limitations in cell numbers, we were unable to perform this experiment in the current study.

As shown in Table III and Fig. 2 E, the subject also mounted a strong proliferative T cell response to HCV, especially to NS4, that was abrogated when CD4<sup>+</sup> T cells were eliminated from the culture in antibody depletion experiments (data not shown). Note that the onset of this response was first detectable 8 wk after exposure, lagging behind the CD8 T cell response and the onset of liver disease, but coinciding with the development of antibodies. Note also that there was a surge in the CD4<sup>+</sup> T cell response between weeks 11 and 13, coinciding with the appearance of CD38-IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells and the beginning of a >5 log decrease in viremia. The CD4<sup>+</sup> T cell response waned briefly toward the end of the infection, after which it rebounded and was maintained at significant but less than peak levels for the duration of the observation period. As shown in Table III, the CD4<sup>+</sup> T cells responded almost exclusively to NS4 throughout the infection except on weeks 12 and 13 when they also responded weakly to NS3.

Collectively, these results suggest that both viral clear-

ance and hepatitis were linked to the kinetics, vigor and quality of the CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in this subject. Specifically, they suggest that viral hepatitis was mediated by the destruction of infected cells by HCV-specific CD38<sup>+</sup> IFN- $\gamma$ <sup>-</sup> CD8<sup>+</sup> T cells but that the infection was only partially controlled by this process and that termination of the infection required additional effector functions provided by CD38<sup>-</sup> IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells and, perhaps, by the CD4<sup>+</sup> T cell population as well. The potential contribution of antibody to viral clearance must also be considered, as the decrease in HCV RNA titers followed with the appearance of anti-HCV antibodies (Figs. 1 and 2).

*The Immune Response to HCV in Subjects who Do not Clear the Virus.* Asymptomatic Subject 2 also displayed a very rapid onset of viremia with  $9.2 \times 10^5$  GE/ml detectable 2 wk after exposure rising to reach a peak titer of  $4.8 \times 10^7$  GE/ml on week 4 and then decreasing to  $3.7 \times 10^6$  GE/ml on week 8 coincident with a severe but asymptomatic episode of hepatitis during which serum ALT activity peaked at 1809 U/l (Table I, Figs. 1 and 3). Note that the viral titer on week 8 (i.e., after several weeks of severe hepatitis) was higher than it was on week 2 (i.e., before the onset of liver disease), suggesting that, as in subject 1, the infection was not controlled despite the destruction of infected cells. Note also that the viremia decreased 100-fold during the next 2 wk, in the absence of a surge in serum ALT activity, again suggesting that, as in Subject 1, the virus was controlled by noncytolytic mechanisms during that period. The virus was not eliminated, however, and viral titers persisted in the  $10^4$  GE/ml range thereafter.

In contrast to Subject 1, the CD8<sup>+</sup> T cell response to

**Table III.** *CD4<sup>+</sup> Proliferative T Cell Response to HCV*

|         | weeks   | core | NS3        | NS4         | NS5        | Sum of all<br>SI > 2 |         | weeks   | core                | NS3         | NS4         | NS5          | Sum of all SI > 2 |              |
|---------|---------|------|------------|-------------|------------|----------------------|---------|---------|---------------------|-------------|-------------|--------------|-------------------|--------------|
| Subj. 1 | week 2  | 0.5  | 1.1        | 1.1         | 1.1        | 0                    | Subj. 3 | week 0  | 0.9                 | 0.8         | 0.9         | 1            | 0                 |              |
|         | week 4  | 1    | 1.7        | 1.6         | 1.3        | 0                    |         | week 5  | <b>2.6</b>          | 2.1         | 2.7         | 1.9          | <b>5.3</b>        |              |
|         | week 6  | 0.8  | 0.8        | 0.7         | 0.6        | 0                    |         | week 8  | 1.1                 | 1.3         | 1.4         | <b>2.3</b>   | <b>2.3</b>        |              |
|         | week 7  | 0.9  | 1          | 1.1         | 1          | 0                    |         | week 9  | IFN treatment start |             |             |              |                   |              |
|         | week 8  | 1    | 0.9        | <b>4.2</b>  | 1.3        | <b>4.2</b>           |         | week 10 | 0.8                 | 0.8         | 0.9         | 0.8          | 0                 |              |
|         | week 9  | 0.8  | 0.9        | <b>7.4</b>  | 0.7        | <b>7.4</b>           |         | week 12 | 0.6                 | 0.8         | 0.9         | 1.2          | 0                 |              |
|         | week 10 | 0.4  | 0.9        | <b>8.4</b>  | 1.1        | <b>8.4</b>           |         | week 20 | <b>4.8</b>          | <b>3.1</b>  | <b>3.1</b>  | <b>3</b>     | <b>14</b>         |              |
|         | week 11 | 0.2  | 1.6        | <b>29.6</b> | 1.2        | <b>29.6</b>          |         | week 21 | IFN/RIBA start      |             |             |              |                   |              |
|         | week 12 | 0.4  | <b>2.7</b> | <b>53.2</b> | 0.7        | <b>55.9</b>          |         | week 24 | 0.9                 | <b>24.8</b> | <b>12.8</b> | <b>12.5</b>  | <b>50.1</b>       |              |
|         | week 13 | 0.5  | <b>3</b>   | <b>36</b>   | 1.2        | <b>39</b>            |         | week 33 | <b>4.7</b>          | 1           | <b>0.8</b>  | 0.9          | <b>4.7</b>        |              |
|         | week 14 | 0.5  | 0.9        | <b>22.3</b> | 0.7        | <b>22.3</b>          |         | Subj. 4 | week 0              | 1.8         | 0.9         | 1.9          | 1.4               |              |
|         | week 15 | 1.2  | 1.5        | 1.2         | 1.4        | 0                    |         |         | week 4              | 0.8         | 0.7         | 1.2          | 1.2               | 0            |
|         | week 16 | 0.2  | 1.7        | <b>4.4</b>  | 0.5        | <b>4.4</b>           |         |         | week 6              | 0.9         | 2           | 11.3         | 1.1               | 0            |
|         | week 19 | 0.1  | 0.5        | 1           | 0.4        | 0                    |         |         | week 7              | 1.2         | 1           | 6.1          | 1.2               | <b>11.3</b>  |
|         | week 20 | 0.6  | 1.6        | <b>10.9</b> | 1.7        | <b>10.9</b>          |         |         | week 10             | <b>2.3</b>  | 0.2         | <b>146.4</b> | <b>3.5</b>        | <b>6.1</b>   |
|         | week 23 | 0.8  | 1.3        | <b>9.5</b>  | 1.7        | <b>9.5</b>           |         |         | week 12             | 1.8         | 0.8         | <b>17.3</b>  | <b>3.3</b>        | <b>152.2</b> |
|         | week 27 | 0.8  | 1.3        | <b>9.6</b>  | 0.7        | <b>9.6</b>           |         |         | week 27             | <b>2.7</b>  | 1.2         | <b>3.3</b>   | 1.9               | <b>6</b>     |
| Subj. 2 | week 2  | 0.5  | 1          | 1.1         | 0.9        | 0                    | week 92 |         | <b>2.7</b>          | 1.3         | <b>6.6</b>  | 1.9          | <b>9.3</b>        |              |
|         | week 6  | 0.5  | 0.3        | 0.4         | 0.4        | 0                    | Subj. 5 |         | week 8              | <b>15.1</b> | <b>8.8</b>  | <b>5.4</b>   | <b>2.8</b>        | <b>32.1</b>  |
|         | week 8  | 0.8  | 0.7        | 0.7         | 0.8        | 0                    |         |         | week 10             | 0.3         | 0.5         | 0.7          | 0.4               | 0            |
|         | week 10 | 0.2  | 0.9        | 1.7         | 1.4        | 0                    |         | week 12 | <b>27</b>           | <b>17.1</b> | <b>37.4</b> | <b>2.6</b>   | <b>84.1</b>       |              |
|         | week 11 | 1.1  | 0.7        | 1.8         | 1.2        | 0                    |         | week 15 | 0.6                 | 0.7         | 1.8         | 0.6          | 0                 |              |
|         | week 12 | 0.1  | 0.5        | 1           | 0.9        | 0                    |         | week 16 | <b>3.1</b>          | 4.8         | <b>5.1</b>  | <b>3.2</b>   | <b>16.2</b>       |              |
|         | week 14 | 0.6  | 1.7        | 1.4         | <b>5.6</b> | <b>5.6</b>           |         | week 36 | 1.3                 | 2           | <b>3.1</b>  | <b>7</b>     | <b>10.1</b>       |              |
|         | week 15 | 0.2  | 0.7        | 1           | 1.4        | 0                    |         |         |                     |             |             |              |                   |              |
| week 18 | 0.3     | 0.8  | 1.3        | 0.8         | 0          |                      |         |         |                     |             |             |              |                   |              |
| week 23 | 0.6     | 0.9  | 1.1        | 0.9         | 0          |                      |         |         |                     |             |             |              |                   |              |

The proliferative T cell response against the HCV synthetic proteins core, NS3, NS4, and NS5 (genotype 1a) was tested at different time points after inoculation. The proliferative response is expressed as SI in which the average [<sup>3</sup>H]thymidine uptake in replicate wells stimulated with the HCV proteins was divided by the average [<sup>3</sup>H]thymidine uptake in replicate wells stimulated with the control protein (SOD). A positive response was determined to be higher than 2.

HCV in this subject was only marginally detectable on week 10 (Fig. 3 B) despite the fact that the dominant viral sequences for all of the epitopes studied in this subject were identical with the peptides used to monitor the CD8<sup>+</sup> T cell response, except NS3 1406. The NS3 1406 sequence in the subject's virus was KLSGLG~~L~~NAV. Accordingly, this peptide was synthesized and used to search for peptide-specific CD8<sup>+</sup> T cells by intracellular IFN- $\gamma$  staining. No specific responses to this peptide, or to any of the other HCV peptides used in this study, were detected by tetramer-binding or IFN- $\gamma$  production at any time point tested (data not shown). In contrast, the number of Flu-tetramer binding cells (0.1–0.6%) and the number of Flu-peptide specific IFN- $\gamma$ -producing cells (0.4–0.5%) were comparable to the response observed in Subject 1, indicating that the failure to

recognize the HCV peptides was not due to a global defect in the PBMCs. Similarly, this subject failed to mount a CD4<sup>+</sup> proliferative T cell response to any of the viral proteins at any time point except a very slight response to NS5 on week 14 (Table III, Fig. 3 C). In contrast, the subject's proliferative response to PHA at each time point (SI range 306–1121) was comparable to the range of PHA responses observed in Subject 1 (range 103–1381).

These results suggest that primary failure of the T cell response to HCV may have been responsible for viral persistence in this subject. If this is correct, the mechanism(s) responsible for the liver disease and the partial control of viremia remain to be determined, although the absence of a vigorous CD4<sup>+</sup> or CD8<sup>+</sup> T cell response suggests that immune responses of undetermined phenotype and specificity

were involved. In keeping with this notion, anti-HCV antibodies appeared 6 wk after infection coinciding with the onset of hepatitis and preceding the fall in viremia. Since these antibody responses are probably CD4 T cell dependent, the results suggest that the subject developed a T cell response that was either too weak to detect or it was directed against antigenic specificities that were not tested in these experiments. Nonetheless, if such a response occurred, it was insufficient to terminate the infection.

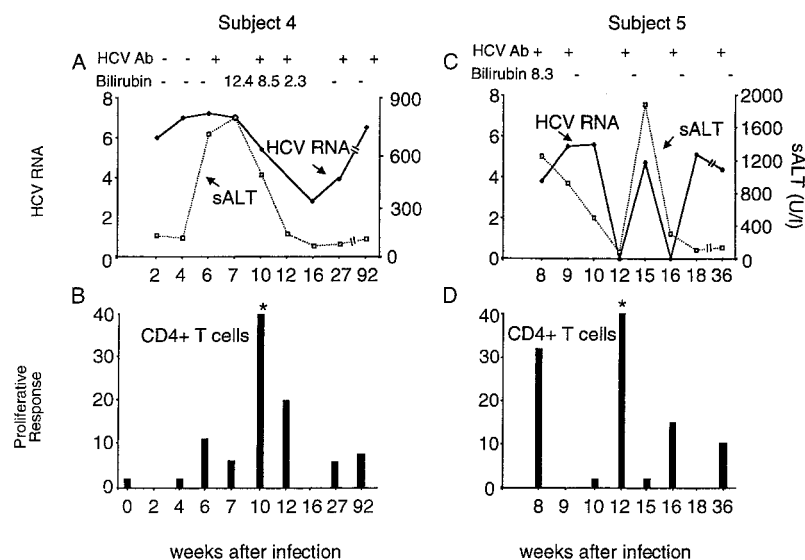
Asymptomatic Subject 3 also displayed a very rapid onset of viremia, reaching a plateau between  $1-2 \times 10^5$  GE/ml 2 wk after exposure. The serum ALT activity was slightly elevated ( $\sim 80$  U/l) and viremia was stable for the first several weeks of the infection. When IFN therapy was instituted on week 8, however, the ALT activity increased slightly to 148 U/l without a change in viral titer. The ALT activity increased again to 299 U/l on week 20 (Table I) by which time the viral titer had already decreased to  $8.2 \times 10^2$  GE/ml. Since serum HCV RNA levels were not measured between weeks 11–20, however, it isn't possible to correlate the decreased viral titer with the small surge in serum ALT activity in this subject. On week 24, however, the serum ALT activity fell and the viral titer rebounded a few weeks later.

The CD8<sup>+</sup> T cell response to HCV could not be monitored in this subject because she was not HLA-A2 positive. However, as shown in Table III and Fig. 3 E, the subject mounted a very weak CD4<sup>+</sup> T cell response to HCV during the first 12 wk of her infection despite the fact that her PHA response (SI range 113–839 stimulation) was comparable to that observed in Subject 1 who mounted a strong proliferative response to HCV. Interestingly, the response strengthened after several weeks of IFN therapy, becoming broadly reactive on week 20 and rising even further on week 24 after the addition of ribavirin to the treatment regimen (Table III). However, the T cell response waned

thereafter, the serum ALT activity declined and the virus rebounded despite the continuation of antiviral therapy (Fig. 3 D). Interestingly, this subject had a very delayed antibody seroconversion (Figs. 1 and 3) consistent with her marginal CD4 response. Thus, like Subject 2, rapid viral spread together with primary failure of the T cell response to HCV appear to have set the stage for the development of an asymptomatic HCV infection that became persistent. Furthermore, it appears that the reasonably vigorous and multispecific CD4<sup>+</sup> T cell response (and perhaps a CD8<sup>+</sup> T cell response that couldn't be monitored with the available tetramers) that was induced by IFN therapy may have caused a very mild exacerbation of hepatitis and temporarily controlled the infection somewhat, but the immune response ultimately waned and the virus returned to its previous high level.

*Host-Virus Interactions in Symptomatic Acute Hepatitis.* The biochemical, virological, and immunological features of Subjects 4 and 5 who became chronically infected after clinically symptomatic acute hepatitis are shown in Figs. 1 and 4 and Tables 1 and 3. We only studied the antiviral CD4<sup>+</sup> T cell response in these subjects because they were not HLA-A2 positive.

Symptomatic Subject 4, like Subjects 1–3, displayed a very rapid onset of viremia (Fig. 4 A) that reached  $10^6$  GE/ml within 2 wk of inoculation, rose to  $1.5 \times 10^7$  GE/ml on week 6 after which it decreased  $>4$  logs by week 16 and then gradually returned toward its initial levels thereafter. The serum ALT activity in this subject was normal for the first 4 wk after inoculation, but it rose on week 6 coincident with the appearance of HCV-specific antibodies and CD4<sup>+</sup> T cells (Fig. 4 B), and it reached a peak of 876 U/l on week 7 after which it declined to between 50–100 U/l where it remained for the duration of the study. Note that the viral titer actually increased between weeks 2–7 when the serum ALT activity was rising, suggesting once



**Figure 4.** HCV-specific T cell responses during symptomatic acute HCV infection in Subjects 4 and 5 who progressed to chronicity. (A and B) Courses of infection (see Fig. 1). (B and D) Proliferative CD4<sup>+</sup> T cell responses were tested against core, NS3, NS4, and NS5 and are shown as the sum of all positive stimulation indices (Table III). \*Sum of all specific stimulation indices is 152.2 in Subject 4 and 84.1 in Subject 5 (see Table III).



again that the virus was not effectively controlled by the destruction of infected cells. Note also that, as in previous subjects, the decrease in viral titer was not associated with a surge in serum ALT activity, suggesting that noncytolytic mechanism(s) may have been controlling the infection, at least transiently, during this period.

Unlike Subjects 2 and 3, Subject 4 developed a relatively strong primary CD4<sup>+</sup> T cell response to HCV. Note that the T cell response was maximal after the peak in serum alanine aminotransferase (sALT) activity suggesting that it wasn't responsible for her liver disease. Also note that the CD4<sup>+</sup> T cell response correlated with the appearance of antibodies to HCV and with the beginning of the major decrease in HCV RNA that wasn't associated with a surge in serum ALT activity. This suggests that the CD4<sup>+</sup> T cells may have controlled the virus noncytolytically in this subject, or that they served as a marker for other noncytolytic antiviral effector mechanisms, e.g., virus-specific CD8<sup>+</sup> T cells that weren't monitored because this subject was not HLA-A2-positive.

Symptomatic Subject 5 did not seek medical advice until 8 wk after inoculation when he was already jaundiced, so we couldn't study the very early events in his infection. As shown in Fig. 4 C, the level of viremia in this subject rose to  $4 \times 10^5$  GE/ml on week 10 after which it fluctuated dramatically and in parallel with wide swings in serum ALT activity. Interestingly, as shown in Table III and Fig. 4 D, the subject mounted a strong, multispecific CD4<sup>+</sup> T cell response to HCV that fluctuated inversely with the viral titers and disease activity. These results suggest that the CD4<sup>+</sup> T cells were not responsible for the liver disease, but that they, or some other mechanisms that they reflected, controlled the viremia at least temporarily until the response waned or the virus became resistant, or both. The extent to which viral sequence evolution played a role in these events remains to be determined.

## Discussion

Acute HCV infection is rarely symptomatic, so acutely infected subjects seldom seek medical attention. Consequently, no information is currently available about the early cellular immune events in the commonest form of acute HCV infection, nor has the incubation phase of HCV infection been studied in any subjects irrespective of their symptomatology. In this study, therefore, we examined the relationship between viral replication kinetics, liver injury, and cellular immune responses during the incubation phase and throughout the course of infection in five subjects after needlestick exposure to HCV-contaminated blood, three of whom were asymptomatic. The CD8<sup>+</sup> T cell response was tested in the two HLA-A2-positive subjects, one of whom was the only subject to clear the infection. The CD4<sup>+</sup> T cell response was studied in all subjects. The results of this study provide the first insight into the host-virus relationship in humans during the incubation phase of acute HCV infection, and they provide the

only insight to date into the virological and immunological characteristics of clinically asymptomatic acute HCV infection, the commonest manifestation of this disease.

Perhaps the most important observations of this study are that HCV infection was completely controlled only in the context of an early, vigorous, and sustained CD4<sup>+</sup> and CD8<sup>+</sup> T cell response that evolved both phenotypically and functionally during the course of the infection, that the ability of the virus to outpace the immune response may explain its tendency to persist, that there are different pathways to viral persistence in asymptomatic and symptomatic acute HCV infection, that the onset of hepatitis coincides with the onset of the CD8<sup>+</sup> T cell response, and that control of the infection may involve more than simply the destruction of infected cells.

The results obtained in the two HLA-A2-positive subjects (Subjects 1 and 2) were extremely informative, in large part because the subjects were so similar to each other in many respects except outcome. Indeed, they were infected by the same HCV genotype (1b), achieved comparable viral titers and similar peak sALT activity, and they experienced asymptomatic infections with similar antibody profiles. Nevertheless, Subject 1 cleared the virus while Subject 2 developed persistent infection. Importantly, the onset and duration of acute hepatitis in subject 1 coincided precisely with the induction of a CD38<sup>+</sup> IFN- $\gamma$ <sup>-</sup> CD8<sup>+</sup> T cell response to NS3 1406 (VA), suggesting that immune-mediated destruction of infected cells by activated virus-specific CD8<sup>+</sup> T cells probably played an important role in the pathogenesis of the disease. Nonetheless, the viral titer decreased less than threefold despite a prolonged episode of acute hepatitis, suggesting that the CD38<sup>+</sup>IFN- $\gamma$ <sup>-</sup>CD8<sup>+</sup> T cell response and the associated destruction of hepatocytes were not sufficient to terminate the infection. This pattern of apparent viral resistance to control during the period of increasing liver cell injury was confirmed in Subjects 2, 4, and 5 as well, suggesting the generality of this notion.

Surprisingly, the NS3 1406 (VA) specific, HLA-A2 tetramer-binding CD8<sup>+</sup> T cells failed to produce IFN- $\gamma$  when they were stimulated by the peptide present in the tetramer at any time during the infection. Since the dominant viral sequence in this subject was found to be KLS-GLGINAV, that peptide was synthesized and used to detect IFN- $\gamma$ -producing virus-specific T cells. Even more surprisingly, during the peak of acute hepatitis the subject's CD8<sup>+</sup> T cells did not produce IFN- $\gamma$  in response to that peptide either, indicating the absence of this effector function during the early, disease-associated, immune response to HCV. However, during the resolution phase of the hepatitis, the subject's CD8<sup>+</sup> T cells lost the CD38 activation marker, as described previously by Lechner et al. (14). Surprisingly, as the CD8<sup>+</sup> T cells lost the activation marker they started to produce IFN- $\gamma$  when they were stimulated by the native peptide. This new information demonstrates for the first time that a switch in the phenotype of HCV-specific CD8<sup>+</sup> T cells can be associated with a switch in antiviral functions (killing versus cytokine production).

Whether this is a cause or consequence of the lower level of viral antigen remains to be determined.

It is very interesting to note that the switch to IFN- $\gamma$  production coincided precisely with a rise in the CD4<sup>+</sup> T cell response and a 5 log decrease in viremia that occurred without a surge in serum ALT activity. The pattern of massive reduction of viral titer in the absence of an exacerbation of liver disease was also observed during the episodes of partial and temporary clearance in the other subjects in this study. This pattern is compatible with the hypothesis that noncytopathic IFN- $\gamma$ -induced, antiviral events may have contributed to the control of the virus in addition to the destruction of infected hepatocytes.

Furthermore, the results point out the importance of matching the synthetic peptides used for functional analysis of the T cell response with the sequences encoded by the infecting virus. Indeed potential discrepancies between these sequences may account for some of the functional T cell deficiencies observed in HCV-infected patients in recent years (34). Furthermore, it is conceivable that the binding of tetramers containing peptides that did not select the T cell response *in vivo* might downregulate or antagonize the functional responsiveness of those T cells *in vitro*.

Collectively, these results suggest that viral clearance during HCV infection is due to a vigorous CD4<sup>+</sup> and/or CD8<sup>+</sup> T cell response to the virus and that CD4<sup>+</sup> T cells may have contributed to the functional maturation of the CD8<sup>+</sup> T cell response such that it acquired the ability to respond to the virus by doing more than simply destroying infected cells. Based on these findings, we suggest that disease pathogenesis and viral clearance may be mediated by different effector mechanisms and T cell populations during acute HCV infection. Specifically, acute hepatitis appears to be mediated by activated CD38<sup>+</sup>CD8<sup>+</sup> T cells that don't produce IFN- $\gamma$  when they recognize antigen, yet these T cells fail to eradicate the infection despite their apparent ability to destroy infected cells since they are associated temporally with a severe and prolonged acute liver disease. In contrast, the virus appears to be very efficiently controlled noncytolytically when CD38<sup>-</sup>CD8<sup>+</sup> T cells appear that produce IFN- $\gamma$  when they recognize viral antigen. This raises the possibility that IFN- $\gamma$  might play a noncytolytic effector role in HCV infection, similar to its ability to control HBV replication in the liver of transgenic mice (35) and infected chimpanzees (36). Proof of this concept must await the performance of similar studies in chimpanzees in which the extent of infection and the nature of the immune response to the virus can be evaluated in the liver.

In contrast to these findings, HCV persisted in the second asymptomatic HLA-A2-positive subject who failed to mount a significant virus-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cell response and in a third subject asymptomatic HLA-A2-negative whose CD8 response was not tested but who also failed to mount a CD4<sup>+</sup> T cell response to the virus. It is important to emphasize that the early phase of the infection has never been studied until now in humans and, therefore, it has not been possible to distinguish between primary failure of the T cell response and secondary failure in the con-

text of continuous antigen stimulation. The current results provide the first evidence that primary failure of the CD4<sup>+</sup> and CD8<sup>+</sup> T cell response is an important pathway to viral persistence during acute HCV infection, raising the possibility that HCV was either not efficiently presented to the immune system in these subjects, or that a T cell response was induced but it was rapidly eliminated.

Primary failure of the antiviral T cell response in the early phase of infection is not the only pathway to HCV persistence, however, since the two symptomatic subjects (Subjects 4 and 5) who became chronically infected mounted vigorous antiviral CD4<sup>+</sup> T cell responses early in the infection that were associated with transient reductions in viral titer. However, the T cell responses eventually waned and the subjects developed chronic infection, suggesting that although the virus was susceptible to immune control early in the course of infection, it may have become resistant later, perhaps by acquiring mutations that enabled it to escape immune recognition or to resist the cytolytic and/or noncytolytic effector functions of the immune response. Alternatively, the persistent infection may have exhausted the immune response. Indeed, in both subjects, the CD4<sup>+</sup> T cell response was either only briefly vigorous (Subject 4) or it was episodic (Subject 5) and the virus returned to baseline in concert with waning of the T cell response. These results, together with the sustained CD8<sup>+</sup> T cell response we observed in subject 1 (Fig. 2), support the notion that a vigorous virus-specific T cell response must be permanently maintained in order to achieve long-term control of HCV (6–10, 14).

A striking observation in this study was the extreme rapidity of viral spread after percutaneous exposure to HCV, which reached maximal titers several weeks before the induction of a humoral or cellular immune response to the virus and the onset of liver disease. This contrasts with the much slower spread of HBV infection (36, 37) which allows the immune response to precede or coincide with the spread of the virus (unpublished data). It is tempting to speculate, therefore, that the different intrinsic replication kinetics of these viruses may contribute to the relative propensity of HCV to become chronic and for HBV to resolve in adult-onset infections.

Another intriguing finding in this study was that HCV was cleared by Subject 1 who produced the highest viral titer, raising the possibility that this may have played a role in the outcome, perhaps by inducing a stronger or more effective immune response or by making the virus more visible to the immune system. This is supported by the observation that Subject 3, who produced the lowest viral titer, also displayed the lowest serum ALT activity and virtually no detectable T cell response before initiation of immunostimulatory therapy with IFN and ribavirin.

In conclusion, the results obtained in this study demonstrate that the T cell response plays a critical role in the pathogenesis of liver disease and the control of HCV during the incubation phase of the infection. Further, they suggest that disease pathogenesis and viral clearance may be mediated by different effector T cell populations, and that

HCV may be controlled not only by the destruction of infected cells but also by CD8<sup>+</sup> T cell-induced noncytolytic antiviral mechanisms the nature of which remain to be defined. The results also suggest that different immunological pathways may be responsible for viral persistence in asymptomatic and symptomatic acute HCV infection, and that the ability of HCV to outpace the T cell response may explain its tendency to persist. Collectively, these observations imply that HCV infection triggers not one but multiple etiologically related but pathogenetically different diseases, and that this heterogeneity may pose a significant challenge for the development of immunotherapeutics and vaccines.

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