Hepatitis C Virus–Specific Cytolytic T Lymphocyte and T Helper Cell Responses in Seronegative Persons

Margaret James Koziel, David K. H. Wong, Darryll Dudley, Michael Houghton, and Bruce D. Walker

Hepatitis C virus (HCV) is a common infection worldwide, and in most persons, it leads to persistent viremia and liver damage. Efforts to identify the correlates of protective immunity are hampered by this high rate of persistent infection in both infected humans and the only animal model, the chimpanzee. Peripheral blood mononuclear cells from seronegative persons were stimulated with synthetic peptides that represent epitopes recognized by HCV-specific cytotoxic T lymphocytes (CTL) after natural infection. In addition, CD4⁺ proliferative responses to recombinant HCV proteins were examined in these same persons. CTL responses directed against a peptide epitope of HCV and proliferative responses in 2 HCV-seronegative persons with possible occupational exposure to HCV were found. These otherwise healthy persons were not viremic, suggesting that they may have recovered from acute HCV infection. Characterization of virus-specific immune responses in exposed but seronegative persons may provide important clues as to the nature of protective immunity in HCV.

Hepatitis C virus (HCV) continues to cause a major proportion of chronic liver disease worldwide despite the advent of highly efficacious blood-screening programs to reduce transfusion-associated transmission [1]. Current estimates are that in the United States alone 1.5%–4.4% of the general population is seropositive for antibodies to HCV. Infection with HCV progresses to chronic liver disease in at least 50% of infected persons [2], and a high proportion of the remainder are viremic even without overt clinical evidence of liver destruction [3]. In juxtaposition, certain individuals have sustained exposure to HCV without apparent infection. For example, HCV transmission through sexual intercourse is inefficient in the absence of human immunodeficiency virus (HIV) coinfection [4], as is vertical transmission [5]. Among intravenous drug users, up to 80% of a given cohort may seroconvert to anti-HCV when followed over 10 years; however, among the remaining 20%, there is no seroconversion, despite repeated exposure to the virus through needle-sharing and sexual exposure [6]. This suggests that there may be large numbers of persons who are repetitively exposed and who may possibly develop protective immunity.

Attempts to identify the correlates of protective immunity in HCV infection have been hampered by an apparent lack of protective immunity in the only animal model of HCV infection, the chimpanzee, and by the high rate of chronicity among infected persons. It is likely that cellular immune responses play a critical role in protective immunity as well as in controlling viral replication. Recent evidence suggests that a quantitatively more vigorous CD4⁺ proliferative T cell response to one or more HCV antigens may be associated with a healthy carrier state or complete recovery from HCV infection [7–9, 34]. Studies of the cytotoxic T lymphocyte (CTL) response have failed to show a clear correlation between HCV-specific CTL responses and clearance of infection. In chronically infected persons, multiple proteins serve as targets of the CTL response, yet virus persists [10–13]; however, it has been suggested that a positive CTL response may be associated with lower titers of virus [14–16].

As with hepatitis B virus infection, investigators have been unable to detect HCV-specific CTL in peripheral blood mononuclear cells (PBMC) without specific antigenic stimulation, usually by peptides that bind to HLA molecules with high affinity [12, 17–19]. In the course of studying HCV-specific CTL responses in the PBMC, we identified 2 persons with likely occupational exposure to HCV who appear to have HCV-specific CD4⁺ and CD8⁺ responses in the absence of either a humoral response or viremia. In this report, we describe for the individual subjects the immune responses that suggest possible exposure to HCV without the development of chronic infection.

Materials and Methods

Subjects. HCV-seronegative subjects were recruited as negative controls for ongoing studies of HCV- and HIV-1–specific cellular immunity. All subjects were laboratory personnel who had either worked in clinical settings or laboratories for at least 1 year. All subjects were seronegative for HIV and HCV antibodies by the ELISA 2 (Ortho Diagnostic Systems, Raritan NJ). In addition, subjects A and B were tested for the presence of HCV RNA by
multiple primers, including primers from the highly conserved 5' untranslated region, HCV core, and NS5 [20–23]. Subjects A and B were repeatedly negative for serum HCV RNA, whereas HCV-infected subjects with chronic infection were positive for serum NS3, NS4, and NS5 were expressed as C-terminal fusions with human Recombinant HCV antigens. B were repeatedly negative for serum HCV RNA, whereas HCV-dase gene (vv-Lac) was used as a control [25]. scribed [30], without further need for peptide stimulation.

Escherichia coli amino acid sequences of the HCV-1 isolate of Choo et al. [26] or with glutamine, antibiotics, HEPES, and 10% heat-inactivated fetal calf serum). Additional B-LCL were obtained from the American was added at a final concentration of 10% dimethyl sulfoxide and 1 mM dithiothreitol (both from Sigma).

Recombinant HCV antigens. The HCV proteins core, E1, E2, NS3, NS4, and NS5 were expressed as C-terminal fusions with human superoxide dismutase (SOD) in yeast (Saccharomyces cervisiae) and purified as described [8]. Recombinant tetanus toxoid (TT), candida (both from Connaught, Swiftwater PA), and superoxide dismutase antigens were used as positive or negative controls.

Stimulation of PBMC with synthetic peptides. PBMC were isolated by ficol-hypaque density gradient centrifugation (Sigma), washed twice in RPMI 1640, and resuspended at 4 x 10^6 cells/mL in RPMI 1640 supplemented with 10% fetal calf serum (R-10), and peptide (10 μM). The peptides used for this study are shown in table 2. All peptides were isolated from specific epitopes recognized by CTL from persons with chronic HCV or HIV-1 infection (table 2). Peptides (final concentration, 10 μM) were added to the cells (4 x 10^6 in a 24-well plate), and TT was added at a final concentration of 1 μg/mL. On day 3, 1 μL of R-10AB supplemented with 20 μg/mL recombinant interleukin (IL)-2 (provided by Maurice Gately, Hoffman–La Roche, Nutley, NJ) was added. On day 7, the cell population was stimulated with 4 x 10^5 autologous PBMC in fresh R-10AB supplemented with 10 μg/mL synthetic peptide, 1 μg/mL TT, and 20 μ/mL IL-2. Cells were tested for cytolytic activity on day 14 of culture. For bulk cultures in which peptide-specific activity was detected, clones were derived after stimulation with irradiated allogeneic feeder cells and the anti-CD3 monoclonal antibody 12F6, as described [30], without further need for peptide stimulation.

Cytotoxicity assay. B-LCL were pelleted, resuspended in fresh R-20, and labeled overnight with Na_2^{145}CrO_4. Cells (1 x 10^6) were then resuspended in 0.2 mL of RPMI 1640 supplemented with glutamine, antibiotics, HEPES, and 10% heat-inactivated fetal calf serum (R-10), and peptide (10 μg/mL) was added. After a 1-h incubation at 37°C, cells were washed three times and used as targets (5 x 10^3 cells/well) in the cytotoxicity assay. Supernatants were harvested at 4 h, and the percent specific cytotoxicity was

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Table 1. HLA haplotypes of HCV-seronegative study subjects and peptides used to type patient peripheral blood mononuclear cells.

<table>
<thead>
<tr>
<th>Subject</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>DR</th>
<th>DQ</th>
<th>DP</th>
<th>Peptides</th>
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<td>11, 24</td>
<td>35, 51</td>
<td>4</td>
<td>7</td>
<td>1, 2</td>
<td>E234, E489, E621, Env 611, RT508</td>
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</tr>
<tr>
<td>B</td>
<td>2, 31</td>
<td>8, 35</td>
<td>4</td>
<td>11</td>
<td>7</td>
<td>E234, NS1395, Env 611</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1, 11</td>
<td>35, 57</td>
<td>4</td>
<td>5, 6</td>
<td>1, 3</td>
<td>E234, E621, NS1073, RT508, Env 611</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>3, 11</td>
<td>35</td>
<td>3, 6</td>
<td>11</td>
<td>1, 3</td>
<td>E234, E621, RT508, Env 611</td>
<td></td>
</tr>
<tr>
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<td>3, 7</td>
<td>2</td>
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<td></td>
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<tr>
<td>F</td>
<td>1, 32</td>
<td>8, 44</td>
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<td></td>
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<td>1, 2</td>
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<td></td>
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<tr>
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<td>7</td>
<td>7</td>
<td>2, 5, 1, 3</td>
<td>C41, E621, RT476</td>
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<td></td>
</tr>
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<td>3, 4</td>
<td></td>
<td>3</td>
<td>NS1073, RT476</td>
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<tr>
<td>J</td>
<td>26, 68</td>
<td>35</td>
<td>4, 7, 11</td>
<td>7</td>
<td>E234, Env 611</td>
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</tbody>
</table>

NOTE. E = envelope protein, Env = envelope, C = core, RT = reverse transcriptase.
Table 2. Peptides used for stimulation of peripheral blood mononuclear cells from HCV-seronegative study subjects.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Source</th>
<th>Amino acids</th>
<th>Sequence</th>
<th>HLA restriction</th>
<th>Reference</th>
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<td>NASRCWVAM</td>
<td>B35</td>
<td>[25]</td>
</tr>
<tr>
<td>Env 611</td>
<td>HIV E</td>
<td>611–619</td>
<td>TAVPNASW</td>
<td>B35</td>
<td>[28]</td>
</tr>
<tr>
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<td>TINYTIKF</td>
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<td>[10]</td>
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<tr>
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<td>[29]</td>
</tr>
<tr>
<td>C41</td>
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<td>41–49</td>
<td>GPRLGVRAT</td>
<td>B7</td>
<td>[11]</td>
</tr>
<tr>
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<td>1395–1403</td>
<td>HSKKKDEL</td>
<td>B8</td>
<td>[10]</td>
</tr>
<tr>
<td>RT476</td>
<td>HIV RT</td>
<td>476–484</td>
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<td>[30]</td>
</tr>
<tr>
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<td>1073–1081</td>
<td>CINGVCWT</td>
<td>A2</td>
<td>[10]</td>
</tr>
<tr>
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<td>HCV core</td>
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<td>PRLGVRA</td>
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</table>

NOTE. E = envelope protein, Env = envelope, HIV = human immunodeficiency virus, RT = reverse transcriptase, C = core.

determined as follows: (release in assay – spontaneous release)/(maximum release – spontaneous release)] × 100. Assays were excluded from analysis if the spontaneous release value was >30%. Results are reported as the mean of triplicate values, with a standard deviation of <5%.

For selected clones, additional cytotoxicity assays were done using vv-infected target cells. B-LCL were infected with either the recombinant vv–HCV vector that contained the relevant epitope (vv-C/E1, which expresses aa met1–ile340 of HCV [25]) or a control vv at an MOI of 5–10 pfu/cell. Cells were then incubated overnight at 37°C in 5% CO₂, labeled with Na₂(51CrO₄), and used as target cells in a chromium release assay [24].

Proliferative responses. Proliferative assays were done using 10⁴ fresh isolated PBMC per well of a 96-well plate in R-10AB supplemented with various amounts of recombinant HCV antigens. For those subjects with positive responses, the assays were repeated with at least 2 separate blood samples using several dilutions of the test-positive antigen. On day 6, 1 mCi of [³H]thymidine was added to the wells, and 18 h later (day 7), incorporated counts per minute (cpm) were harvested onto glass-fiber filters, using an automated harvesting device (Packard Instruments, Meriden CT). The stimulation index (SI) was calculated as follows: (mean cpm in the presence of HCV antigen)/(mean cpm in the presence of no antigen).

Limiting dilution analysis. Serial dilutions of PBMC were plated in 24 replicate cultures into 96-well plates along with 10⁶ irradiated autologous PBMC, 10 µg/mL peptide, 20 U/mL IL-2, and 1 µg/mL TT in R-10AB. Cells were restimulated on day 7 with 10 µg/mL peptide and 20 U/mL IL-2. After 2 weeks, the cells were used as effector cells in a standard cytotoxicity assay, using B-LCL sensitized with relevant or irrelevant peptide as target cells. The precursor frequency of PBMC against a specific target was calculated (using a computer program developed by S. A. Kalam [31]) by plotting the log of the fraction of negative wells (Fe) versus the number of input wells and solving for Fe = 0.37, using the maximum likelihood method [32].

Results

HCV-specific cytolytic T cells in seronegative persons. PBMC from healthy HCV- and HIV-1-seronegative persons were stimulated with synthetic peptides representing CTL epitopes identified from HCV- or HIV-1–infected persons. PBMC from each individual were stimulated with one or more peptides, depending on the HLA haplotype of the subject. These synthetic peptides represented epitopes recognized by HIV-1– or HCV-specific CTL identified after cloning in the absence of specific antigenic stimulation [10, 11, 25, 28–30]. The peptides were not selected on the basis of binding to purified HLA molecules or on the basis of the prediction of sequence-specific motifs, which has been used to identify virus-specific CTL in other circumstances [12, 19, 33]. PBMC were stimulated for 2 weeks in vitro with peptide, TT as a source of T cell help, and IL-2 and then tested for cytolytic activity against targets sensitized with either the stimulatory peptide or an irrelevant peptide. For subjects A and B, peptide stimulations were repeated from 3 separate blood samples, which were obtained over a 6-month period in the absence of any intercurrent illness.

Stimulation of PBMC from subjects A and B revealed cytolytic activity specific for an epitope (E234) in HCV E1 (figure 1). This epitope had been previously shown to be an epitope recognized by an HLA B35–restricted clone derived from the liver-infiltrating lymphocytes of a subject with chronic HCV infection [25]. For subject A, stimulation with an HCV peptide epitope recognized in the context of HLA B51 (E489), an HIV-1 B35–restricted epitope (Env 611), or a control peptide (4C) did not result in peptide-specific cytolytic activity. For subject B, stimulation with Env 611, an HCV A2–restricted epitope (NS1073), or the control peptide 4C did not result in HCV-specific cytolytic activity. For subjects A and B, bulk cultures were established with two rounds of peptide E234 stimulation, followed by limiting dilution cloning in the absence of specific antigenic stimulation [30]. In each case, these CTL lines or clones recognized only B-LCL expressing HLA 35, which were sensitized with E234 not Env 611, and not non-HLA–matched B-LCL (figure 1 and data not shown). PBMC for 8 additional seronegative control subjects were also stimulated with one or more synthetic peptides (table 2), and in no case was peptide-specific CTL activity detected (data not shown).
Figure 1. Cytotoxic T lymphocyte (CTL) activity in peripheral blood mononuclear cells (PBMC) of HCV-seronegative persons. Freshly isolated PBMC were stimulated with indicated peptide, interleukin 2, and tetanus toxoid (TT), as indicated in Materials and Methods. Cells were restimulated once, and then tested against target cell lines sensitized with indicated peptide. Subjects A (A) and B (B) had E234-specific cytolytic activity in stimulated PBMC lines. Representative control subject (C) did not have detectable CTL activity against any target cell line. D, PBMC from subject A were stimulated with peptide E234 in presence or absence of TT or with TT alone, and they were tested against E234-sensitized target cells or target cells sensitized with an irrelevant peptide. Stimulated PBMC recognized target cells sensitized with peptide E234, even in absence of TT as source of T cell help. Effector-to-target cell ratios are indicated on x axis. E = envelope protein, Env = envelope, RT = reverse transcriptase, stim = stimulated, C = core, TT = tetanus toxoid.

Since it is possible that primary immune responses may be generated using TT as a source of T cell help, PBMC from subject A were stimulated with peptide, TT, and IL-2; peptide and IL-2; or TT and IL-2. Although in all cases HCV E234–specific CTL lines could be generated, this activity was present even in the absence of peptide (figure 1D), which suggests that the cytolytic activity was not secondary to in vitro priming.

Limiting dilution analysis was done for subjects A and B and a control subject to estimate the precursor frequency (CTLp) of HCV E234–specific CTL. As shown in figure 2, subject A had a high frequency of HCV E234–specific CTL compared with that for the control subject and compared with CTL specific for the control peptides. The CTLp after HCV E234 stimulation of PBMC from this subject was 1/11,391, compared with <1/70,000 for all other targets was <1/590,000 (data not shown). From both subjects A and B, it was also possible to derive HCV E234–specific clones from the stimulated cultures, using only an anti-CD3 monoclonal antibody as a nonspecific source of T cell stimulation, and ongoing peptide-specific stimulation was not required [30] (data not shown).

HCV-specific proliferative responses in seronegative persons. T cell proliferative responses to HCV and control antigens were measured in 9 subjects (table 3). All subjects had an SI ≥ 2.0 to two or more control antigens. However, only subjects A and B, who also had E234-specific responses, demonstrated specific proliferative responses against one or more recombinant HCV proteins. Positive responses were retested with variable amounts of antigen on multiple occasions, and increasing amounts of antigen resulted in more vigorous SIs in the positive responders, whereas subjects C–I had SIs < 2.0 even when the concentration of antigen was raised to 10 μg/mL (data not shown).
Discussion

In this report, we describe CTL responses directed against a peptide epitope of HCV and proliferative responses in 2 persons with possible occupational exposure to HCV. Testing over a 12-month period revealed that neither individual seroconverted nor developed viremia. These persons were repeatedly tested for the presence of anti-HCV antibodies by use of a second-generation ELISA assay thought to be highly sensitive and for HCV RNA by use of multiple primers against well-conserved regions of the virus. Since these persons were clinically well, liver tissue was not available; therefore, the presence of virus in the liver tissue cannot be definitively excluded. However, on the basis of commonly accepted criteria for HCV infection, these persons did not appear to have ongoing HCV infection.

Despite the absence of HCV-specific humoral responses and virus, these persons had CTL responses directed against an epitope in HCV E1 and proliferative responses against two or more HCV proteins. This epitope was identified by CTL from liver-infiltrating lymphocytes of a person with chronic HCV infection and was not chosen on the basis of binding to purified HLA B35 molecules or a sequence motif [25]; thus, it is clear that this is an epitope that is presented in natural HCV infection in the context of HLA B35. This HCV E1 epitope does bind to purified HLA B35*01, although with lower affinity than the peptides used in most studies [12] (Sidney J, personal communication). HCV E1-specific CTL could be isolated after two rounds of stimulation with peptide, and in one case with TT and no specific peptide stimulation.

The HCV-specific CTL lines and clones obtained from subjects A and B had high levels of peptide-specific activity yet failed to recognize endogenously processed proteins (data not shown). This failure to recognize target cells expressing endogenously processed HCV proteins has been reported by other investigators who have used this stimulation strategy, and it may be due to the selection of CTL with low avidity for the...
### Table 3. Proliferative responses to HCV and control antigens in HCV-seronegative subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Assay</th>
<th>Candida</th>
<th>TT</th>
<th>PPD</th>
<th>Core</th>
<th>E1</th>
<th>E2</th>
<th>e33c</th>
<th>e100</th>
<th>NS5</th>
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<tbody>
<tr>
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<td>34</td>
<td>264</td>
<td>86</td>
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<td>9</td>
<td>14</td>
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<tr>
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<td>2</td>
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<td>223</td>
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**NOTE.** Freshly isolated peripheral blood mononuclear cells from each subject were cultured in presence of recombinant HCV proteins, control proteins (candida, tetanus toxoid [TT], and mycobacterial purified protein derivative [PPD]), or media alone. Concentration of recombinant HCV antigens was 5 μg/mL, and concentration of control antigens was 2 μg/mL. Stimulation index (SI) was calculated as follows: (mean cpm in presence of HCV antigen)/(mean cpm in presence of no antigen). Only subjects A and B had detectable proliferation in presence of HCV proteins. Results shown are representative of 4 assays for subject A and 3 for subject B. All other subjects were tested once. NT = not tested. Bold figures = SI ≥ 3 in presence of HCV proteins.

peptide-HLA complex [12]. These same subjects had CD4+ T cell responses against several HCV proteins, even when the criterion for a positive response was an SI cutoff of 4. The complete absence of a humoral response by what is thought to be a highly sensitive assay remains unexplained; however, healthy carriers of HCV appear to have a weak humoral and strong T helper response against HCV compared with that for persons with chronic liver disease caused by HCV [34].

There are several possible reasons for the presence of HCV-specific immune responses in otherwise seronegative persons. The first explanation is that these responses represent primary in vitro responses and have no meaning in terms of relevance to HCV pathogenesis. The use of peptides that represent potential epitopes to stimulate PBMC responses has been used to define CTL epitopes recognized in both acute and chronic HBV infection, using peptides that bind tightly to HLA molecules and hepatitis B core antigen as a source of T cell help [35, 36]. This approach has been adopted by others to facilitate identification of CTL epitopes in chronic HCV infection [12, 17–19]. However, at least 1 of these groups has also reported the presence of CTL responses against peptides in seronegative subjects, although estimates of the precursor frequency of the response in seronegative versus seropositive persons revealed a log or more difference in CTL precursor frequency, and generation of primary responses required multiple rounds of stimulation in vitro [12, 37]. However, in the subjects reported in our study, HCV peptide-specific responses were identified after only two rounds of stimulation, and other HLA B35–positive persons did not have a peptide-specific response. Of additional significance is the existence of HCV-specific proliferative responses, which would seem to indicate that two arms of the acquired immune system were activated.

A second possibility is that these CTL responses represent cross-reactive responses against heterologous proteins. Since the CTL recognizes viral protein as part of a complex consisting of the HLA molecule and a peptide fragment, a peptide fragment generated from proteolytic degradation of any protein with sufficient homology to this sequence could have primed the immune response in these persons. Cross-reactivity of CTL responses against heterologous proteins has been reported [38, 39]. Although in many circumstances cross-reactivity has been noted to be on the basis of sequence homology, in others the cross-reactivity was not due to homologous sequences [40]. An extensive search for this sequence in the SWISSPROT and European Molecular Biology Laboratory databases failed to reveal any other source for the peptide sequence NASRCW-epitope other than HCV, but the CTL responses could have been primed by another viral infection or exposure to antigen. The presence of vigorous HCV-specific CD4+ responses suggests that the CTL response to HCV was not solely due to molecular mimicry and that there was at least a transient infection from which these persons recovered. A preexisting immunity to another infectious agent could have provided some level of protective immunity against HCV that facilitated recovery from acute HCV infection.

A third possibility is that these immune responses may represent persons who have been exposed to HCV but cleared the infection. The subjects had robust T helper cell responses against HCV. Several groups have recently reported that a T helper cell response against one or more HCV antigens that is quantitatively more vigorous than that for chronically infected persons facilitates recovery from acute HCV [7–9, 34]. To date, no group has reported CTL responses in persons who cleared acute HCV infection. However, studies of persons ex-
posed to HIV-1 who do not themselves become infected have shown similar results to the present study. For example, some uninfected children born to HIV-infected mothers develop HIV-specific CTL responses [41, 42]. Among commercial sex workers in The Gambia with presumed multiple exposures to both HIV-1 and -2, CTL responses were HLA B35 restricted and HIV specific [43]. Similarly, HIV-1–specific CTL and T helper cell responses have been detected in health care workers with occupational exposure to HIV-1–infected blood [44, 45] and in seronegative partners of HIV-1–infected persons [46, 47]. It is not clear in these studies of HIV-1–exposed persons whether the individuals never became infected or whether there was transient replication of HIV in tissue sites, such as lymph nodes, that was subsequently cleared.

In this study, subjects A and B had potential occupational exposures to HCV-infected blood in the absence of other risk factors. Neither person reported any history of hepatitis or symptoms of liver disease, although in many infected persons, acute HCV infection is subclinical [2]; thus, it cannot be ascertained whether these subjects had a transient infection that cleared or were never infected. The presence of both CTL and vigorous T helper cell responses in subjects in the present study suggests that they sustained an infection that was subsequently cleared or have a true healthy carrier state, in that virus cannot be detected in the serum by the polymerase chain reaction. Understanding of the correlates of protective immunity in HCV infection will likely be critical to the design of an effective vaccine or immunotherapeutic strategy. Given the difficulty in identifying persons who successfully clear acute HCV infection, examination of immune responses in large numbers of exposed but uninfected persons may provide an alternate strategy for identification of protective immune responses.

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References