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Hepatitis C Virus (HCV)-Specific Cytotoxic T Lymphocytes Recognize Epitopes in the Core and Envelope Proteins of HCV

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Hepatitis C virus (HCV) is a major cause of posttransfusion and community-acquired hepatitis, and a majority of individuals infected with this virus will subsequently develop chronic hepatitis. Characterization of the host immune response to this infection is an important first step that should facilitate the development of immunomodulatory agents and vaccines. Cellular immune responses, especially those mediated by cytotoxic T lymphocytes (CTL), are important in the control of many viral diseases. In this study, liver-infiltrating lymphocytes from persons with chronic HCV hepatitis were examined for evidence of HCV-specific CTL by using target cells infected with recombinant vaccinia viruses expressing the HCV core, E1, E2, and part of the NS2 proteins. Bulk expansion of liver-derived $CD8^+$ lymphocytes resulted in the detection of HCV-specific CTL activity, whereas activity could not be found in $CD8^+$ lymphocytes expanded from peripheral blood. Epitopes recognized by these CTL were defined by using CTL clones obtained by limiting dilution and target cells sensitized with synthetic HCV peptides. Four distinct HLA class I-restricted epitopes were identified, including two epitopes in the amino-terminal portion of the core protein. These studies provide evidence that the highly conserved core protein is a target for HCV-specific CTL and identify CTL epitopes within the more highly variable E2 envelope protein. Our studies also suggest that HCV-specific CTL are localized at the site of tissue injury in infected persons with chronic hepatitis. Identification of the epitopes recognized by HCV-specific CTL will facilitate exploration of their role in disease pathogenesis and may provide information useful in development of therapeutic interventions or vaccines.

Hepatitis C virus (HCV) is a major etiologic agent of posttransfusion and sporadic community-acquired non-A, non-B hepatitis (7, 12, 43). Although viral particles have yet to be isolated, there has been a rapid accumulation of data regarding the molecular biology of the virus since it was first cloned (12, 13). On the basis of sequence homology with pestiviruses and flaviviruses (12, 48), it appears that there is a single open reading frame encoding a polyprotein precursor of 3,011 amino acids (aa), which undergoes cotranslational or posttranslational processing to the final structural and nonstructural proteins (32). The structural proteins consist of a core protein (p19), a highly glycosylated envelope protein (E1 or gp33), and a 72-kDa glycoprotein that most likely is a second envelope protein [E2 or E2(NS1)] (30, 31, 66, 67). The remaining two-thirds of the genome encodes nonstructural proteins (NS2, NS3, NS4, and NS5), with NS3 thought to be the viral helicase (21, 29) and NS5 thought to be the RNA-dependent RNA polymerase (15).

Of those infected with HCV, 50 to 70% will develop chronic liver disease (4, 19), with subsequent morbidity and mortality due to cirrhosis and hepatocellular carcinoma (61, 65). Given that screening of blood donors will reduce the number of cases of posttransfusion HCV hepatitis (20, 47) but not eliminate sporadic community-acquired HCV hepatitis, there is a need for a vaccine to prevent infection. The immune responses necessary to elicit protective immunity are presently unknown. Although a humoral response directed against multiple HCV antigens is present, the specificity or titer of the antibody response does not correlate with the disease course in either chronically infected chimpanzees (1) or humans (3, 7). High titers of antibody have been associated with disease transmission and persistent viremia in chimpanzees (1, 24, 58) and humans (3, 7). In the absence of an in vitro system for measuring virus replication, however, the presence or absence of neutralizing antibody cannot be readily evaluated.

Cellular immune responses, particularly those mediated by cytotoxic T lymphocytes (CTL), are important components of protective immunity against many viral infections (for a review, see reference 55). In some circumstances, however, CTL may mediate tissue damage in an attempt to limit viral replication (52, 53, 80). In HCV infection, the role of CTL in protecting against viral infection versus causing tissue damage is unknown. Characterization of the $CD8^+$ lymphocyte response to HCV is an important step in defining the role of these cells in disease pathogenesis. Previously, this laboratory has reported HLA class I-restricted CTL specific for the E1 and NS2 proteins among liver-infiltrating lymphocytes of persons with chronic HCV infection, establishing the existence of a CTL response to this virus in chronically infected humans (42). In addition to these class I-restricted responses, a cellular immune response mediated by class II-restricted, $CD4^+$ lymphocytes has recently been reported (8). In this report, we extend the findings regarding cellular immunity to HCV by demonstrating that the highly conserved core protein is a target for HCV-specific CTL and by identifying additional CTL epitopes within the more highly variable E2 protein. In addition, identification of minimal epitopes recognized by these class I-restricted CTL suggests possible allele-specific binding motifs.

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MATERIALS AND METHODS

Subjects. Three HCV-seropositive subjects with evidence of chronic liver disease (at least fourfold elevations in serum transaminases for at least 6 months) underwent liver biopsy by modified Klatskin technique prior to alpha interferon therapy. All subjects were hepatitis B virus (HBV) and human immunodeficiency virus type 1 (HIV-1) seronegative and showed evidence of mild to moderate chronic active hepatitis on histologic examination of liver tissue. All subjects gave informed consent, and the study was approved by the Massachusetts General Hospital Human Studies Committee. Peripheral blood for Epstein-Barr virus transformation, HLA typing, and expansion of $CD8^+$ cells was obtained at the time of the liver biopsy.

Cell lines. Epstein-Barr virus-transformed B-lymphoblastoid cell lines (B-LCL) were established and maintained as described previously (74) in RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 2 mM L-glutamine, 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), in addition to 20% heat-inactivated fetal calf serum (R-20 medium). Additional B-LCL were obtained from the American Society for Histocompatibility and Immunogenetics B-cell line repository.

HLA typing. HLA typing of subjects was performed on fresh peripheral blood mononuclear cells (PBMC) by the Massachusetts General Hospital Tissue Typing Laboratory, using standard serologic techniques.

Vaccinia virus constructs. Vaccinia virus-HCV recombinant viruses were constructed to express the core, E1, E2, and part of the NS2 proteins of the HCV-1 strain of virus (11a, 41, 42, 67). Insertion of genomic sequences coding for the HCV proteins was achieved by ligation of the nucleotide sequences into the psc11 vector by standard techniques (10). (The designation vv stands for vaccinia virus in construct names below.) vv-C/E1 expresses amino acids Met-1 to Ile-340 of HCV, which constitute the core (C) and E1 proteins, and vv-E2/NS2 expresses amino acids Met-347 to Leu-906, which constitute the carboxy terminus of E1, the entire E2 protein, and one-half of the NS2 protein of HCV (31, 32). A vaccinia virus construct expressing only the *Escherichia coli* β-galactosidase gene (vv-Lac) was used as a control (10). An additional vector, vv-E2/NS2/NS3, which expresses amino acids Met-364 to His-1619, was available for screening of clones from one subject (92N), but no clones with specific activity for this vector were identified (data not shown). All vaccinia virus-HCV recombinant viruses were demonstrated to express the appropriate HCV proteins by radioimmunoprecipitation (11a, 67).

Synthetic peptides. Synthetic peptides corresponding to the amino acid sequences of the HCV-1 strain of Choo et al. (13) were synthesized by Cambridge Research Biochemicals by the Fmoc method (5). Peptide 1 corresponds to aa 1 to 20, and adjacent serial peptides overlap by 10 aa. Fine mapping was achieved by using additional smaller peptides 5 to 13 aa in length (IDEC Pharmaceuticals, La Jolla, Calif., and Research Genetics, Huntsville, Ala.). All peptides were reconstituted in sterile distilled water containing 10% dimethyl sulfoxide (Sigma) and 1 mM dithiothreitol (Sigma).

Cloning of liver-infiltrating lymphocytes. Liver-infiltrating lymphocytes were cloned from the liver biopsy specimens as previously described (42). In brief, a 2- to 4-mm section of liver tissue obtained by percutaneous needle biopsy was placed directly in R-10 medium (RPMI 1640 plus 10% heat-inactivated fetal calf serum, antibiotics, L-glutamine, and HEPES buffer) plus 100 U of recombinant interleukin-2 (rIL-2) (Hoffman-La Roche, Nutley, N.J.) per ml in one well of a 24-well plate. The bispecific monoclonal antibody CD3,4B (76) (kindly supplied by Johnson Wong) was added at 0.5 μg/ml. This antibody has one arm which is specific for the CD3 cell surface antigen and one arm specific for the CD4 cell surface antigen, with a common Fc portion (76), leading to the selective expansion of the $CD8^+$ lymphocytes (77). Fluorescence-activated cell sorter (FACS) analysis demonstrated that the lymphocytes stimulated in this manner were 95 to 98% $CD8^+$ (data not shown).

Once lymphocytes reached confluence (2×10^6 to 3×10^6 cells in a 24-well plate), 1×10^6 cells were restimulated with 4 ml of feeder cell suspension, which consisted of 10^6 irradiated (30 Gy) allogeneic PBMC per ml of R-10 medium supplemented with 100 U of rIL-2 per ml and 0.1 μg of the CD3-specific monoclonal antibody 12F6 per ml as a nonspecific stimulus to T-cell proliferation (74). After being fed with fresh rIL-2-containing medium on days 3 and 7, cells were tested for HCV-specific cytolytic activity on day 10. At that time, cells had expanded 10- to 15-fold from the original 10^6 cells and were 95 to 98% $CD8^+$ as determined by FACS analysis (data not shown). In addition, cells were cloned at limiting dilutions at 300, 100, 50, and 25 cells per well into 96-well microtiter plates in 200 μl of feeder cell suspension (74). These cell concentrations have been shown to approximate limiting dilutions by this technique (36, 42, 74). Developing cells were selected from 96-well plates in which less than 33% of the wells displayed visible growth. These cells were restimulated with irradiated feeder cells and 12F6 as previously described (74) and then tested for HCV-specific cytolytic activity.

Expansion of $CD8^+$ lymphocytes in PBMC. For subjects 92G and 92H, PBMC were expanded with CD3,4B at the same time as liver-infiltrating lymphocytes. PBMC were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation. Then 10^6 cells were suspended in R-10 medium supplemented with 100 U of rIL-2 per ml, and the bispecific antibody CD3,4B was added at 0.5 μg/ml. Cells were fed with fresh medium containing rIL-2 on days 3 and 7 and tested for HCV-specific cytolytic activity on day 10. At that time, cells had expanded 10- to 15-fold from the original 10^6 cells and were 95 to 98% $CD8^+$ by FACS analysis (data not shown).

Cytotoxicity assay using vaccinia virus-infected target cells. B-LCL were infected with either recombinant vaccinia virus-HCV vectors or a control vaccinia virus at a multiplicity of infection of 5 to 10 PFU per cell, incubated overnight at 37°C in 5% CO_2, labeled with $Na_2{}^{51}CrO_4$, and used as target cells (5×10^3 cells per well) in a chromium release assay (73). For cloned T cells, supernatants were harvested at 4 h, and for assays with bulk liver-infiltrating lymphocytes, supernatants were harvested at 5 h. Percent specific cytotoxicity was determined by the formula [(release in assay − spontaneous release)/(maximum release − spontaneous release)] × 100. For subjects 92G and 92H, results are shown for autologous target cells or partially HLA-matched target cells expressing HCV antigens as indicated below. For subject 92N, it was not possible to establish and maintain a B-LCL despite repeated attempts; for this reason, all assay results shown are for HLA-matched B-LCL expressing HCV antigens. Assays were excluded from analysis if the spontaneous release value was >30%. Results are reported as the means of triplicate values, with a standard deviation of less than 5%.

Cytotoxicity assay using synthetic peptide-sensitized target cells. B-LCL were pelleted, resuspended in fresh R-20, and labeled overnight with ^{51}Cr. Cells (10^6) were then resuspended in 0.2 ml of R-10, and peptide was added; after a 1-h

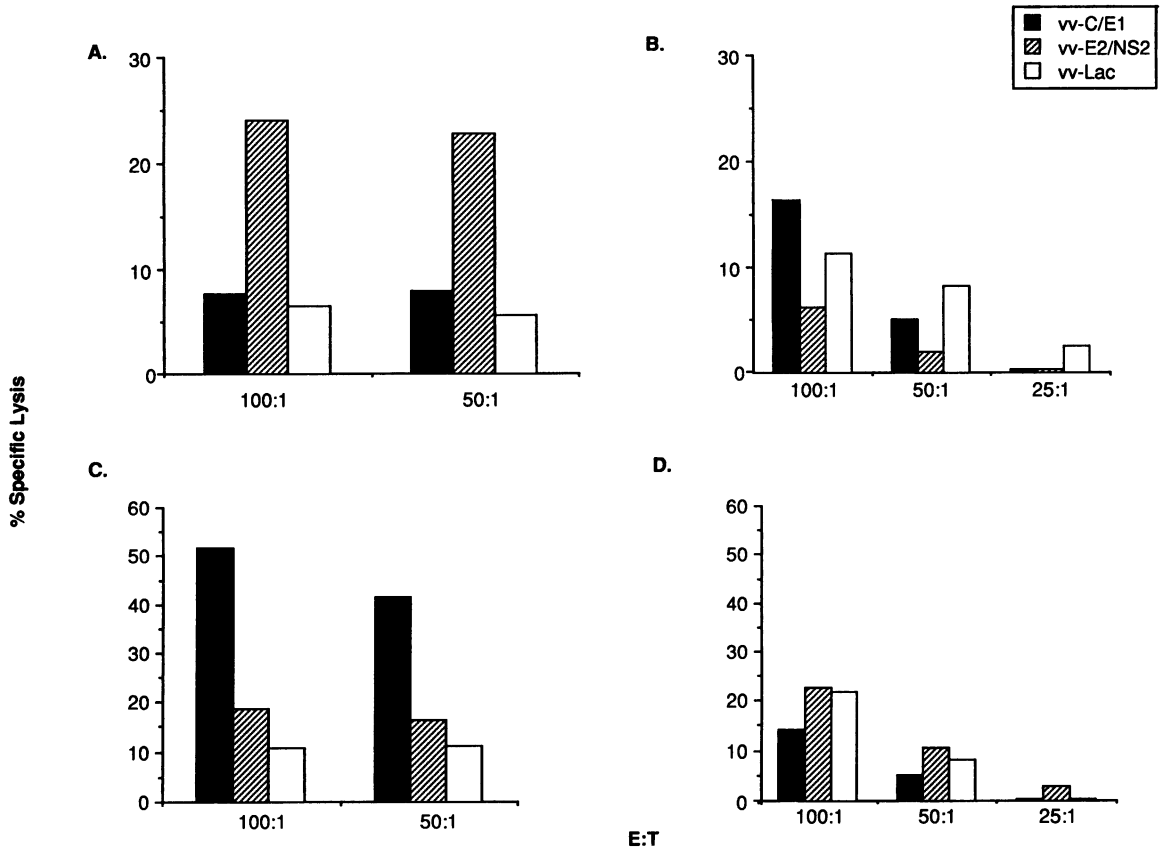

FIG. 1. Specific localization of HCV-specific CTL among the intrahepatic lymphocytes compared with PBMC. (A) Bulk-expanded $CD8^+$ intrahepatic lymphocytes from subject 92G specifically recognized E2(NS1)/NS2-infected target cells. (B) Bulk-expanded $CD8^+$ lymphocytes from PBMC of subject 92G do not specifically lyse autologous B-LCL expressing HCV proteins. (C) Bulk-expanded $CD8^+$ intrahepatic lymphocytes from subject 92H demonstrated specific lysis of target cells expressing the C/E1 protein. (D) Bulk-expanded $CD8^+$ lymphocytes from PBMC of subject 92H do not specifically lyse autologous B-LCL expressing HCV proteins. The effector-to-target cell ratio (E:T) is shown.

incubation at 37°C, cells were washed three times and used as targets in the cytotoxicity assay. The concentration of peptides for screening ranged from 100 to 300 μg/ml unless otherwise indicated. High concentrations of peptide were used for screening because of a previous demonstration that such concentrations may be necessary when peptides longer than the optimal peptide are used for sensitization (reference 36 and data not shown). For screening of large numbers of peptides in some assays, B-LCL were simultaneously sensitized with two noncontiguous peptides, a method which gave results equivalent to those from screening with individual peptides (data not shown).

For fine mapping experiments, the results presented are for the clones which grew best in long-term culture. Because of a laboratory freezer failure, sufficient numbers of clones from subjects 92G and 92H for only a limited number of assays with amino- and carboxy-truncated peptides were available. For clones from subject 92N, sufficient numbers of cells were available to permit construction of dose-response curves for recognition of target cells sensitized with serial 10-fold dilutions of truncated peptides. For these experiments, B-LCL were labeled with ^{51}Cr, washed three times, and then incubated with the indicated concentration of peptide in a 96-well plate (5×10^3 cells per well). After a 1-h incubation, effector cells were added to the wells, and the supernatant was harvested at 4 h in a standard cytotoxicity assay (74).

RESULTS

HCV-specific CTL are detectable among bulk-stimulated liver-infiltrating lymphocytes. Liver-infiltrating lymphocytes were expanded in the presence of rIL-2 and irradiated allogeneic feeder cells, as well as a bispecific monoclonal antibody which results in the proliferation of $CD8^+$ lymphocytes. At no time were these cells exposed to exogenous viral antigens in vitro. The resulting $CD8^+$ lymphocytes were then tested for their ability to lyse autologous target cells expressing HCV proteins, by using vaccinia virus-HCV recombinant viruses. As shown in Fig. 1A and C, subjects 92G and 92H had detectable activity in $CD8^+$ liver-infiltrating lymphocytes even in the absence of in vitro viral antigenic stimulation, with specific lysis of target cells expressing either E2/NS2 (subject 92G) or C/E1 (subject 92H) compared with autologous target cells expressing other HCV proteins or with a control. $CD8^+$ lymphocytes from PBMC from these subjects were expanded in a similar manner and did not have detectable HCV-specific activity (Fig. 1B and D). Although it is not possible to determine the absolute number of $CD8^+$ lymphocytes in the small fragments of liver tissue received, the $CD8^+$ lymphocytes obtained from the liver tissue and peripheral blood had no apparent difference in their ability to proliferate after a nonspecific stimulus to T-cell proliferation such as 12F6. These findings suggest that HCV-specific CTL are concentrated within the hepatic paren-

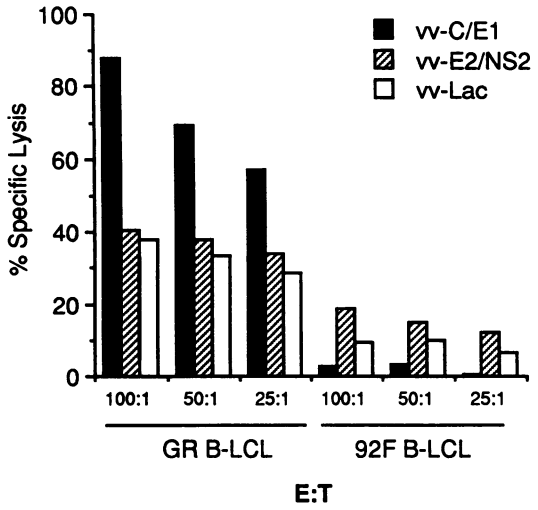

FIG. 2. HCV-specific cytolytic activity of bulk-expanded CD8+ lymphocytes from the liver of subject 92N. CD8+ were expanded from the liver biopsy sample of subject 92N as described above and tested against two HLA-matched lines, as an autologous B-LCL was not available. These CD8+ cells recognized target cells (GR B-LCL) expressing C/E1. The B-LCL GR shares HLA A28, B7, C6, DRw52, and DQw1. The B-LCL 92F shares A3, B51, C4, C7, DR7, DRw53, and DQw1,2. DR6 is the only class I or II molecule expressed by 92N that is not expressed by these lines. The effector-to-target cell ratio (E:T) is as indicated.

TABLE 1. Lysis of HCV-expressing target cells by liver-infiltrating lymphocytes from persons with chronic HCV hepatitis[a]

Subject	Clone[b]	E:T[c]	% Specific cytotoxicity of construct		
			vv-Lac	vv-C/E1	vv-E2/NS2
92G	110	20:1	0.9	0	30.2
	118	20:1	0.9	0	50.3
92H	5	10:1	0.8	35.0	0
	32	2:1	−0.5	59.3	0
92N	2	1:1	4.7	29.6	0
	8	10:1	0	31.3	0
	43	2:1	5.0	23.6	0
	47	2:1	0	54.5	0
	107	10:1	−0.5	0	45.1
	118	10:1	0	0	27.1
	135	2:1	2.3	39.6	0

[a] HCV-specific cytolytic activity of CTL clones derived from liver-infiltrating lymphocytes was tested in a standard 4-h ^{51}Cr release assay. Autologous or, in the case of subject 92N, HLA-matched B-LCL infected with recombinant vaccinia virus-HCV viruses were used as target cells.

[b] CTL clones were derived from CD8+ liver-infiltrating lymphocytes by cloning in the presence of anti-CD3 monoclonal antibody, IL-2, and allogeneic feeder cells. Clones listed are the result of screening the following numbers of clones: for subject 92G, 200 clones; for subject 92H, 100 clones; and for subject 92N, 150 clones.

[c] E:T, effector-to-target cell ratio.

chyma and, if present in the peripheral blood, are present at lower frequencies than in the liver.

CD8+ lymphocytes expanded from the liver biopsy specimen of subject 92N also had detectable HCV-specific cytolytic activity. Because an autologous B-LCL was not available, cytolytic activity was evaluated by using two different partially HLA-matched B-LCL, B-LCL GR and B-LCL 92F, as target cells (Fig. 2). There was specific recognition of the B-LCL GR cells expressing C/E1 compared with those expressing E2/NS2 or the control. For subject 92N, it was not possible to evaluate PBMC in parallel with liver-infiltrating lymphocytes, since an autologous B-LCL was not available despite multiple attempts to establish such a line and since background levels of lysis from using stimulated PBMC against allogeneic target cells were too high.

HCV-specific cytolytic activity in liver-infiltrating lymphocytes. After expansion of the CD8+ lymphocytes from the biopsy specimen, these cells were then cloned in the presence of a CD3-specific monoclonal antibody, rIL-2, and irradiated allogeneic feeder cells. At no time were clones derived from liver-infiltrating lymphocytes exposed to exogenous HCV antigens in vitro. Developing cells were tested for HCV-specific cytolytic activity. In all three subjects, HCV-specific CTL which recognized target cells infected with recombinant vaccinia virus-HCV viruses and grew in sufficient numbers to allow performance of multiple assays on multiple dates (Table 1). were found. For subject 92G, two clones which were stable in long-term culture were isolated. These clones specifically lysed target cells expressing HCV E2/NS2. Clones from subject 92H recognized target cells expressing C/E1. For subject 92N, there was a polyclonal HCV response, with two different populations of HCV-specific clones identified. One population recognized target cells expressing C/E1 proteins, and the other recognized target cells expressing E2/NS2 proteins. All HCV-specific clones were confirmed to be CD8+ by FACS analysis (data not shown).

HCV-specific CTL are restricted by HLA class I molecules. Recognition of endogenously synthesized proteins, such as occurs on the surface of cells infected with viruses, occurs in the context of a short fragment of protein presented as a trimolecular complex with the major histocompatibility complex class I molecule and beta 2 microglobulin (79). In order to further define the characteristics of the observed HCV-specific cytolytic activity, CTL clones were tested for their ability to lyse allogeneic target cells expressing HCV antigens. As shown in Fig. 3, the HCV-specific cytotoxic effector cells recognized allogeneic target cells only in the context of a shared HLA class I molecule, with different HLA molecules restricting recognition of different HCV antigens. Clone 92G-118 recognizes target cells expressing E2/NS2 only in the context of HLA B50 (Fig. 3A). As shown in Fig. 3B, clone 92H-32 recognizes a specific core peptide only in the context of HLA A11. Clones with two different specificities from subject 92N were isolated, with one population directed against C/E1 and the other against E2/NS2. Epitope recognition by these clones occurred in the context of different HLA molecules (Fig. 3C and D). Clone 92N-135 recognizes C/E1 protein only in the context of HLA B7, and clone 92N-107 recognizes E2/NS2 in the context of HLA B51.

HCV-specific CTL recognize HCV core and envelope proteins. Crystallographic resolution of the peptide binding sites of class I molecules (26, 44, 63), as well as elution of naturally processed viral proteins bound to class I molecules (23, 60), has shown that peptide fragments presented to class I-restricted CTL are 8 to 10 aa in length. Incubating target cells with synthetic peptide has been shown to bypass the normal requirements for endogenous processing of viral peptide into fragments of the appropriate length (71) and thus enables definition of the epitopes recognized by virus-specific CTL. Overlapping 20-aa synthetic peptides spanning the appropriate viral proteins were used to map the regions containing epitopes recognized by the HCV-specific CTL. As shown in Fig. 4, clone

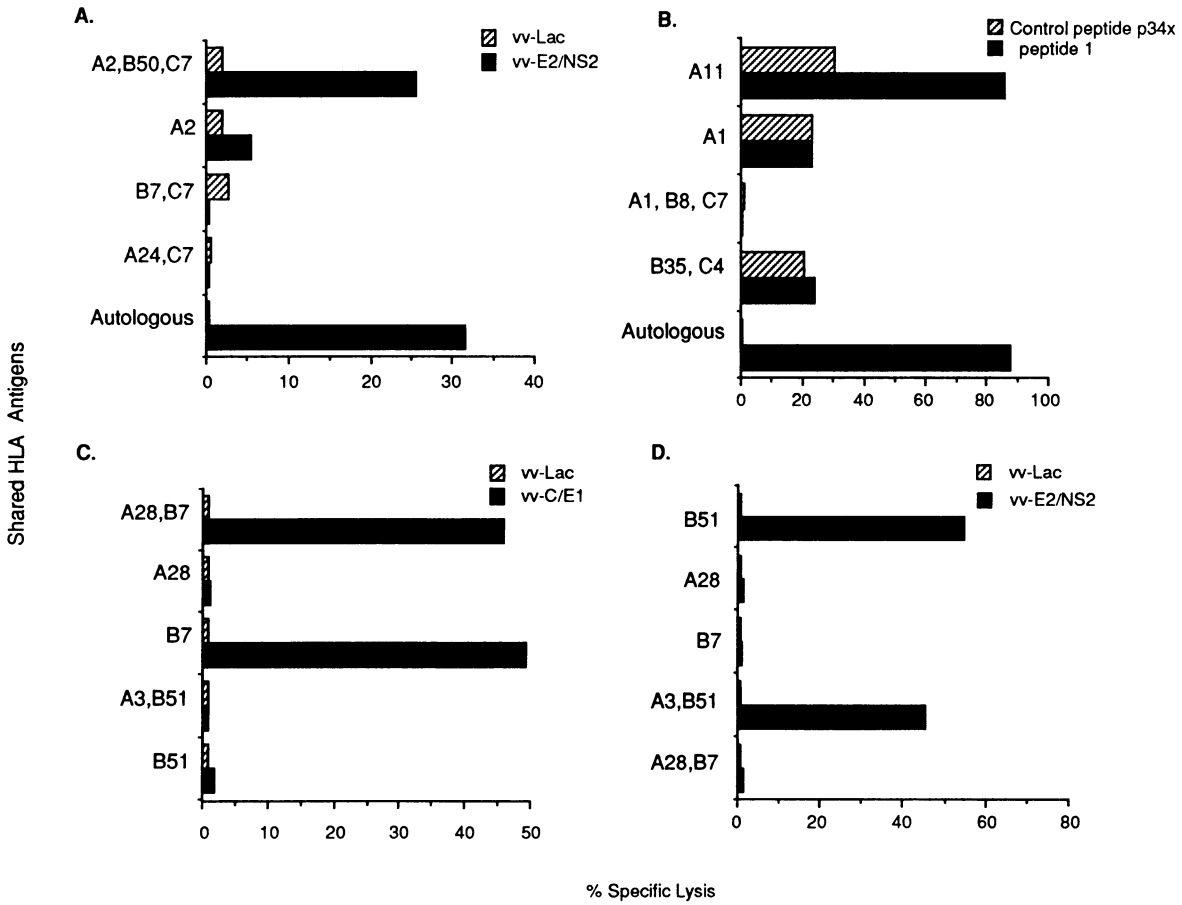

FIG. 3. HCV-specific CTL are HLA class I restricted. Clones were tested for the ability to lyse autologous and allogeneic target cells expressing HCV antigens. Target cells were infected with the vaccinia virus construct expressing the relevant antigen as well as the control virus vv-Lac. Shared HLA molecules are shown. (A) Clone 92G-118 recognized E2(NS1)/NS2 in the context of HLA B50. (B) Clone 92H-32 recognized the HCV core protein in the context of HLA A11. Target cells were sensitized with the HCV core peptide recognized by this clone (see Fig. 7). Both the core peptide and the control peptide p34x (ALVMAQLLRIPQAILDMIAG) were used at a saturating concentration of 100 μg/ml. (C) Clone 92N-47 recognized C/E1 in the context of HLA-B7. (D) Clone 92N-107 recognized E2/NS2 in the context of B51. The complete HLA type of subject 92G is A2,24, B7,50, Bw6, C6,7, DR2,7, DRw53, and DQ1,2. The complete HLA type of subject 92H is A1,11, B8,35, Bw6, Cw4,w7, DR1,3, DRw52, and DQw1,w2. The complete HLA type of subject 92N is A3,28, B7,51, Bw4,6, C4,6, DRw6,7, DRw52,53, and DQw1,2. All results are shown at an effector-to-target cell ratio of 10:1.

92G-118 lysed target cells sensitized with a peptide fragment of the E2 protein. Synthetic 20-aa peptides (57 in all) corresponding to the proteins expressed by the vv-E2/NS2 vector were used to sensitize autologous B-LCL for lysis. Only peptide 57 (aa 561 to 580) sensitized target cells for recognition by this B50-restricted clone (only data for peptides 54 to 60 are shown; no other peptides sensitized cells for specific lysis). Further definition of the epitope was accomplished by using 10-aa peptides which overlapped one another by 8 aa (Fig. 4B). Peptide 57D (CVIGGAGNNT, aa 569 to 578) was sufficient to sensitize target cells for lysis.

Synthetic peptides were also used to define the epitope recognized by clone 92H-32. Overlapping 20-aa peptides defined this A11-restricted epitope as being in the first 20 aa of the core (data not shown); further mapping with 13-aa peptides which overlapped by 10 aa showed the epitope to be in the first 13 aa (Fig. 5A). Amino- and carboxy-terminal truncation of the 13-aa peptide was used to further define the epitope recognized (Fig. 5B). Incubation of target cells with the peptides containing the sequence STNPKPQK (aa 2 to 9) was sufficient to sensitize cells for lysis but not incubation with peptides which did not contain the amino-terminal serine (aa 2) or the carboxy-terminal lysine (aa 9), suggesting that these amino acids are important for binding to the HLA A11 molecule.

In a similar manner, 20-aa overlapping synthetic peptides were used to define the B7-restricted epitope recognized by the core-specific clone 92N-135 to amino acids present within the overlap of two adjacent peptides, peptide 4 (aa 31 to 50) and peptide 5 (aa 41 to 60) (Fig. 6A). By using 10-aa peptides which overlapped by 8 aa, the region recognized by this clone was confirmed to lie within aa 41 to 50 (data not shown). The availability of large numbers of cells permitted a more extensive analysis of the minimum epitope recognized by clones from subject 92N. Fine mapping of this epitope showed that optimal recognition was achieved with the sequence GPRLGVRAT (aa 41 to 49), as evidenced by the ability of this peptide to sensitize target cells for lysis at the lowest concentration of peptide (Fig. 6B). Lysis was reduced when either the amino-terminal glycine or the carboxy-terminal threonine was removed. The peptide GPRLGVRA was still able to sensitize cells for lysis when high peptide concentrations were used. In

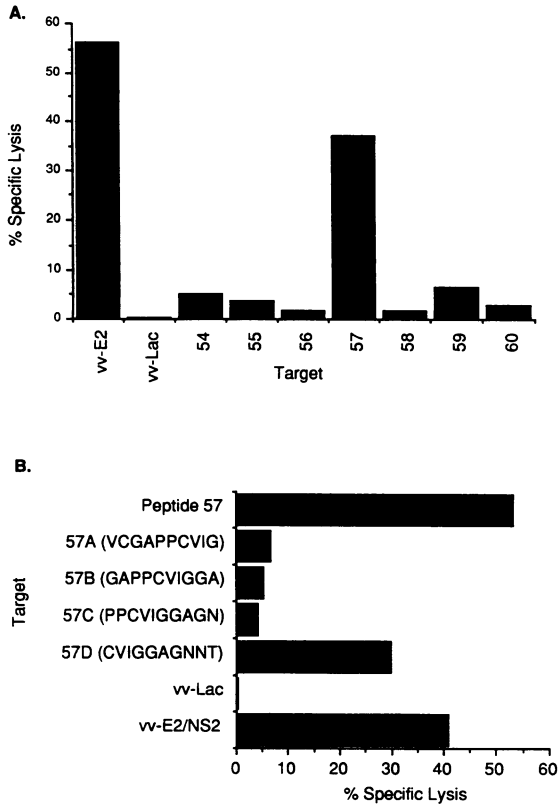

FIG. 4. Mapping of the HCV epitope recognized by the HLA B50-restricted clone 92G-118. (A) Clone 92G-118 was tested for its ability to lyse autologous target cells individually incubated with synthetic 20-aa HCV peptides spanning the E2(NS1) and NS2 proteins. This clone recognized target cells sensitized with peptide 57 (aa 571 to 590) in the E2(NS1) glycoprotein. Lysis of target cells infected with the recombinant vaccinia virus is shown as a control. The effector-to-target cell ratio shown is 10:1. (B) Clone 92G-118 was also tested for its ability to lyse autologous target cells incubated with 10-aa peptides which spanned aa 573 to 588. Peptide 57D (aa 569 to 578) was able to sensitize target cells for lysis. The effector-to-target cell ratio was 5:1, and all peptides were used at a concentration of 50 μg/ml.

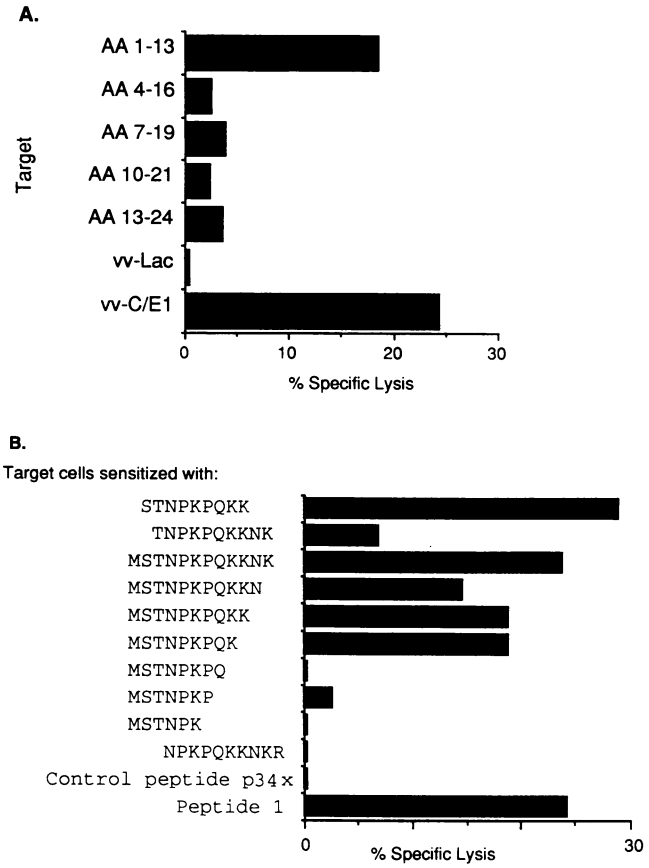

FIG. 5. Mapping of the HCV epitope recognized by the HLA A11-restricted clone 92H-32. (A) Clone 92H-32 recognizes autologous target cells incubated with the 13-aa peptide 1A (aa 1 to 13, MSTNPKPQKKNKR); all other peptides spanning the core and E1 proteins failed to sensitize target cells for lysis (data not shown). Lysis of autologous target cells infected with recombinant vaccinia virus is shown as a control. The effector-to-target cell ratio was 2:1, and the concentration of peptide used for sensitization was 5 μg/ml. (B) Fine mapping of the epitope recognized by clone 92H-32. Recognition of target cells sensitized for lysis with amino- and carboxy-terminal truncations of peptide 1A indicated that the presence of the sequence STNPKPQK (aa 2 to 9) was sufficient to sensitize target cells for lysis. The effector-to-target cell ratio was 5:1, and the concentration of peptide used for sensitization was 5 μg/ml.

a separate experiment using 10-fold dilutions of the 9-aa peptide GPRLGVRAT, half-maximal lysis was achieved even at 100 pg/ml (data not shown).

Clone 92N-107, which recognized target cells infected with the E2/NS2 vector, was tested for its ability to lyse autologous target cells individually incubated with synthetic 20-aa HCV peptides spanning the proteins expressed by vv-E2/NS2 (Fig. 7A). Only peptide 49 (aa 481 to 500) was found to sensitize cells for lysis, suggesting that the B51-restricted epitope recognized by this clone lay within this 20-aa peptide. Further definition of the epitope was accomplished by using 10- to 11-aa peptides which overlapped by 8 aa. Of these, only peptides 49C (WHYPPKPCGI) and 49D (YPPKPCGIVPA) sensitized target cells for lysis (data not shown), suggesting that the epitope lay within the region (YPPKPCGI) shared by these two peptides. Examination of dose-response relationships of peptides spanning this region demonstrated that optimal recognition was achieved when target cells were sensitized with the 8-aa peptide YPPKPCGI (aa 489 to 496), which sensitized cells for lysis even at 10 ng/ml (Fig. 7B). Removal of the amino-terminal tyrosine or the carboxy-terminal isoleucine abrogated recognition. Addition of an amino-terminal histidine resulted in sensitization of target cells but required higher concentrations of peptide. Of note, the 11-aa peptide YPPKPCGIVPA, which contains the entire 8-aa epitope, was less efficient for sensitization of target cells than the 8-aa peptide, as would be expected of peptides longer than the minimal epitope.

DISCUSSION

The role of cellular immune responses in HCV infection is still in the early stages of characterization. In this report, we identify the core and E2 proteins as targets for HCV-specific CTL in chronically infected persons. HCV-specific cytolytic activity is present in bulk-expanded liver-infiltrating lymphocytes in the absence of any in vitro antigenic stimulation, whereas such activity could not be detected in bulk-expanded PBMC, suggesting a tissue-specific localization of HCV-spe-

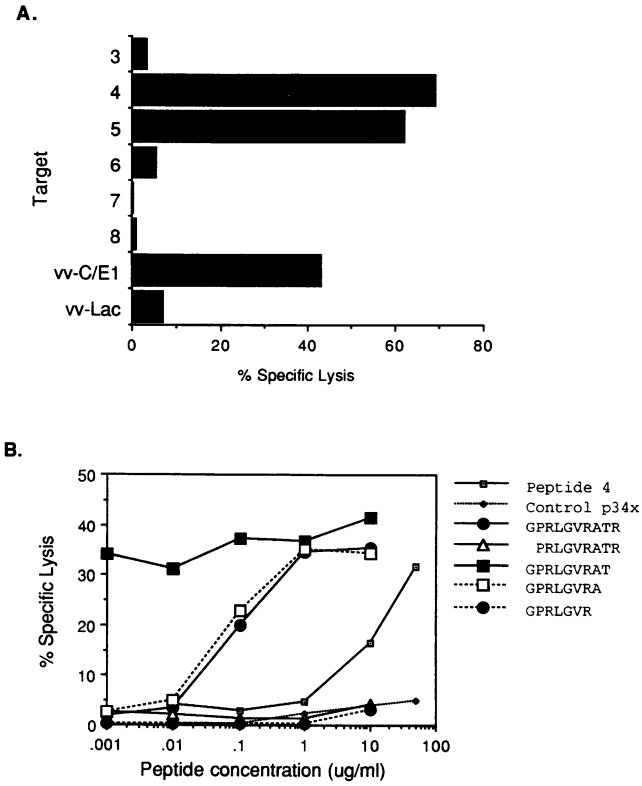

FIG. 6. Mapping of the HCV epitope recognized by the HLA B7-restricted clone 92N-135. (A) Clone 9N-135 was tested for its ability to lyse autologous target cells individually incubated with synthetic 20-aa HCV peptides spanning the core and E1 proteins. This clone recognized target cells sensitized with either of two adjacent core peptides (aa 31 to 50 and 41 to 60); peptide titration curves showed identical recognition of these peptides down to peptide concentrations of 10^{-9} M (data not shown). This suggested that the epitope was contained in the region overlapped by the two peptides, aa 41 to 50 (GPRLGVRATR). The effector-to-target cell ratio was 5:1, and the concentration of peptides used was 50 μg/ml. (B) Amino- and carboxy-terminal truncations of the overlap region of peptides 4 and 5 were tested for their ability to sensitize target cells for lysis. Target cells were sensitized with the indicated concentrations of peptide, and clone 92N-135 was tested for recognition. Optimal recognition was achieved with the sequence GPRLGVRAT (aa 41 to 49). The effector-to-target cell ratio was 1:1.

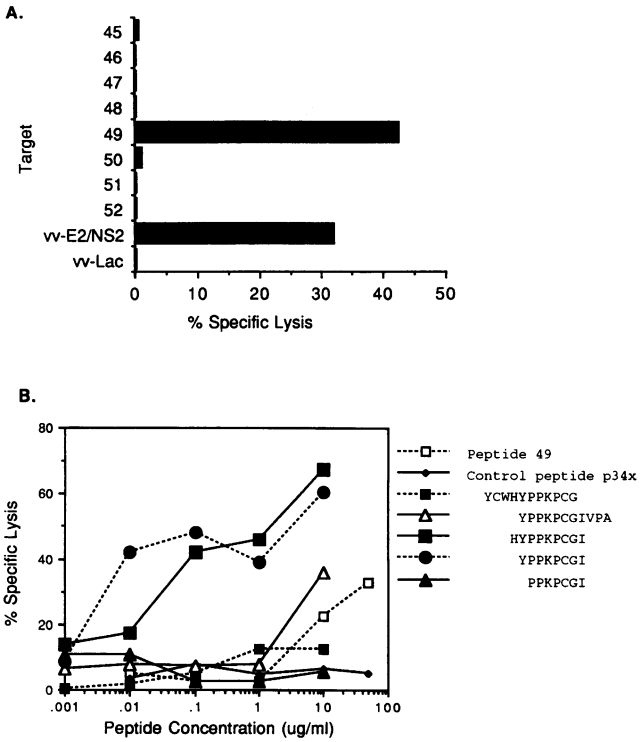

FIG. 7. Mapping of the HCV epitope recognized by the HLA B51-restricted clone 92N-107. (A) Clone 9N-107 was tested for its ability to lyse autologous target cells individually incubated with synthetic 20-aa HCV peptides spanning the E2 and NS2 proteins. Only the E2 peptide 49 (aa 481 to 500) was found to sensitize cells for lysis. The effector-to-target cell ratio was 10:1, and the concentration of peptide used for sensitization was 50 μg/ml. (B) Clone 92N-107 was also tested for its ability to lyse autologous target cells incubated with amino- and carboxy-terminal truncations of peptide 49. Tenfold serial dilutions of amino- and carboxy-terminal truncations of a region of peptide 49 were incubated with target cells, and clone 92N-107 was tested for its ability to lyse target cells. Optimal lysis was achieved with the sequence YPPKPCGI (aa 489 to 496). The effector-to-target cell ratio was 2:1, and the concentrations of peptide used for sensitization were as indicated.

cific CTL. With nonspecific stimulation strategies, such as have been employed in detecting HIV-1-specific CTL (74) as well as HCV-specific CTL (42), it is possible to detect CTL directed against multiple different antigens without influencing the specificities of the CTL in vitro. For subjects 92G and 92H, a single epitope could be defined by using cloned CTL, whereas for subject 92N the CTL response was found to be directed at epitopes in two different viral proteins.

Liver biopsy specimens from three other patients with chronic active hepatitis C during the period of this study were evaluable, and HCV-specific clones could not be isolated from these subjects (data not shown). The clinical characteristics of these patients were no different from those of patients from whom HCV-specific CTL were isolated. The inability to detect such responses may be due to a low frequency or absence of CTL or due to CTL activity directed against portions of the virus not present in the vaccinia virus recombinants. For five of the six subjects studied, vaccinia virus recombinants expressing only the first 906 aa of HCV-1 were available at the time of study. An additional vector, vv-E2/NS2/NS3, which expresses Met-364 to His-1619, was available for the evaluation of clones from subject 92N, although no clones with specific activity solely for NS3 were detected (data not shown). In addition, the vectors used were derived solely from the HCV-1 strain. Since HCV displays considerable sequence heterogeneity among isolates (for a review, see reference 32) and since CTL may be either group or type specific (2, 17), it is likely that our results actually underestimate the frequency and spectrum of HCV-specific CTL in the liver-infiltrating lymphocytes of these chronically infected individuals.

The ability to define both the HLA restriction and the minimum epitope recognized by HCV-specific CTL facilitates the prediction of allele-specific motifs recognized in natural infection (for a review, see reference 59). Identification of epitopes by other methods, such as solid-phase peptide binding assays (14) or amphipathicity profiles (45), has not always predicted the specificities of virus-specific CTL. Allele-specific motifs have been proposed for several murine and human HLA types, including A2, B27, A3, and B8 (18, 23, 33, 35).

TABLE 2. Epitopes recognized by HLA A11-restricted and HLA B7-restricted CTL

CTL	Antigen (aa)	Amino acid at position[a]:												Source or reference(s)
		—	—	1	2	3	4	5	6	7	8	9	—	—	
HLA A11 restricted	HCV core (2–9)				S	T	N	P	K	P	Q	K			This report
	HIV-1 pol (325–333)[b]			A	I	F	Q	S	**S**	M	T	**K**			74
	HIV-1 pol (508–517)		I	Y	Q	E	P	F	**K**	N	L	**K**			34a, 37
	HIV-1 nef (73–82)		Q	V	P	L	R	**P**	M	T	Y	**K**			40
	HIV-1 nef (83–94)		A	A	V	D	L	S	H	F	L	**K**			16
	Epstein-Barr virus EBNA (416–424)			I	V	T	D	F	S	V	I	**K**			78
	HBV pre-S1 (21–28)				P	L	G	F	F	**P**	D	H			34
HLA B7 restricted	HCV E2 (41–49)			G	**P**	R	L	**G**	**V**	**R**	A	T			This report
	HIV-1 nef (126–138)	N	Y	T	**P**	G	P	**G**	**V**	**R**	Y	P	L	T	16

[a] For the peptides for which the minimum epitope has not been defined, alignment was done by comparison of charge among residues, and the best alignment is shown. Amino acids which are identical to the HCV core are shown in boldface type. —, no position number.
[b] Amino acid coordinates for the HIV-1 sequence are based on the LAI sequence (50a).

Recognition of other viral epitopes restricted by HLA A11 has been reported for a number of other viral infections, including HIV-1 (72), Epstein-Barr virus (27), and HBV (34). Comparison of the HLA A11-restricted core peptide (aa 2 to 9) with other peptides containing HLA A11-restricted epitopes indicates similarities among peptides which bind to HLA A11 (Table 2). All but one of the peptides containing A11-restricted epitopes have a lysine at the C terminus (referred to here as position 9). The only exception is an HBV epitope with a histidine at position 9, representing a conservative amino acid substitution. Other shared features of these peptides in this alignment include nonpolar or uncharged polar R groups at positions 2, 5, 7, and 8. A similar motif for peptides binding to the HLA A11 molecule has recently been proposed (78). By using synthetic peptides with amino acid substitutions within an epitope recognized by Epstein-Barr virus-specific CTL, a motif which consists of a hydrophobic amino acid at position 2, amino acids with small side chains at positions 3 and 6, and a positively charged amino acid at position 9 was proposed. The A11-restricted peptides shown in Table 2 suggest that larger side chains such as the lysine in the HCV core protein are possible at position 6 and that polar uncharged amino acids such as serine and glutamine can occupy position 2. Both our proposed motif and that proposed by Zhang et al. (78) indicate the need for a small side chain at position 3 and a lysine or other positively charged R group in position 9. Differences in the motifs, such as the substitution of a large positively charged R group at position 6, might be explained by differences in molecular structure among A11 molecules which share serologic cross-reactivity (6). Although fewer HLA B7-restricted epitopes have been reported, one alignment of the peptide representing the epitope in HCV E2 recognized by an HCV-specific CTL and a peptide representing the epitope in HCV E2 recognized by an HCV-specific CTL and a peptide representing the epitope in HIV-1 nef protein is striking in that 4 aa can be perfectly aligned (Table 2).

The epitopes recognized by the HCV-specific CTL identified in this study are present within both highly conserved and variable regions of the virus. The epitopes within the HCV core recognized by clones from subjects 92H and 92N are relatively highly conserved among reported isolates, although variation still exists in this region (9, 13, 30, 31, 38, 54, 69, 70). Of note, these CTL epitopes present within the core protein also overlap with B-cell epitopes reported in a high percentage of chronically infected subjects (51, 62). The presence of a proliferative response to core proteins was shown to be the only discriminatory T_h-cell response which separated subjects with chronic HCV infection from those who appeared to be healthy carriers of HCV (8). These observations suggest that the HCV core protein may be relatively immunodominant for humoral and T_h-cell responses, in addition to being a target for CTL.

Clones 92G-118 and 92N-107 recognized epitopes within more variable regions of the E2 protein, with multiple conservative and nonconservative amino acid substitutions within these epitopes reported (9, 13, 30, 31, 38, 54, 69, 70). Such sequence heterogeneity among HCV isolates is not randomly distributed, with the highest degree of diversity among isolates observed in the envelope proteins and the least in the core and NS proteins (25, 46, 54, 75). The functional implications of this diversity in HCV are unknown. For other viruses, such as HIV-1, it has been demonstrated that sequence diversity within CTL epitopes may facilitate escape from immune surveillance, since even single amino acid changes within CTL epitopes may abrogate recognition by CTL (17, 35, 57, 68). Although conclusive evidence regarding the clinical importance of this mechanism is lacking, our system will allow us to test the hypothesis that sequence variation in an individual over time correlates with escape from immune recognition in these chronically HCV-infected subjects.

If CTL are important to host defenses against HCV, this response appears to be insufficient to clear virus completely in the majority of HCV infections. In such circumstances, CTL may contribute to disease pathogenesis. Several lines of evidence support this hypothesis with regard to chronic HBV infection (for reviews, see references 49 and 56). $CD8^+$ T cells capable of lysing hepatocytes in a major histocompatibility complex-restricted manner have been isolated from individuals with chronic HBV hepatitis, although the antigenic specificity of these cells could not be defined (22). More recent data have

shown that adoptive transfer of HBV-specific CTL into hepatitis B surface antigen-transgenic mice led to liver cell necrosis (50). This tissue damage is due to direct cell lysis by the CTL, with amplification of liver necrosis by cytokines such as gamma interferon and tumor necrosis factor alpha (11, 28).

Although HCV-specific CTL may mediate liver damage in chronic infections, it remains possible that HCV-specific CTL contribute to either clearance of viremia or lack of progression to chronic disease in some individuals. If HCV-specific CTL are shown to be important in limiting viral replication, the identification of epitopes recognized by HCV-specific CTL may have important implications for vaccine development. Immunization with peptides alone has been shown to lead to the induction of a protective $CD8^+$ CTL response (39, 64), although such approaches may be limited by the need to include multiple peptides in order to confer protection in human populations with diverse HLA backgrounds. Knowledge of the epitopes recognized by HCV-specific CTL should facilitate design of peptide-based vaccines and may be important in understanding the mechanisms of persistent viral infection.

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