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Induction of Cytotoxic T Cells to a Cross-Reactive Epitope in the Hepatitis C Virus Nonstructural RNA Polymerase-Like Protein

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Cytotoxic T lymphocytes (CTL) have been found to mediate protection in vivo against certain virus infections. CTL also may play an important role in control of infection by hepatitis C virus (HCV), but no CTL epitopes have yet been defined in any HCV protein. The nonstructural protein with homology to RNA polymerase should be a relatively conserved target protein for CTL. To investigate the epitope specificity of CTL specific for this protein, we used 28 peptides from this sequence to study murine CTL. Mice were immunized with a recombinant vaccinia virus expressing the HCV nonstructural region corresponding to the flavivirus NS5 gene (RNA polymerase), and the primed spleen cells were restimulated in vitro with peptides. CTL from *H-2*^{d} mice responded to a single 16-residue synthetic peptide (HCV 2422 to 2437). This relatively conserved epitope was presented by *H-2*^{d} class I major histocompatibility complex (MHC) molecules to conventional CD4$^-$ CD8$^+$ CTL but was not recognized by CTL restricted by H-2^b. Moreover, exon shuffle experiments using several transfectants expressing recombinant D^d/L^d and K^d demonstrated that this peptide is seen in association with α1 and α2 domains of the D^d class I MHC molecule. This peptide differs from the homologous segments of this nonstructural region from three other HCV isolates by one residue each. Variant peptides with single amino acid substitutions were made to test the effect of each residue on the ability to sensitize targets. Neither substitution affected recognition. Therefore, these conservative mutations affected peptide interaction neither with the D^d class I MHC molecule nor with the T-cell receptor. Because these CTL cross-react with all four sequenced isolates of HCV in the United States and Japan, if human CTL display similar cross-reactivity, this peptide may be valuable for studies of HCV diagnosis and vaccine development. Our study provides the first evidence that CD8$^+$ CTL can recognize an epitope from the HCV sequence in association with a class I MHC molecule.

Hepatitis C virus (HCV) is the recently recognized causative agent of parenterally transmitted non-A, non-B hepatitis. The RNA genome of the virus has recently been molecularly cloned from the plasma of an experimentally infected chimpanzee (8). Analysis of the sequence of the genome revealed that it is a single-stranded, plus-sense RNA approximately 9,500 nucleotides in length. A single long open reading frame of 9,033 nucleotides coding for a polyprotein of 3,011 amino acid residues was found. These genomic features and sequence comparisons combined with what had been known about the size and lability to lipid solvents indicated that the virus is probably a member of the flavivirus family (6, 22), but it may be more closely related to the *Pestivirus* genus than to the classic flaviviruses (27, 60).

From sequence similarities of both the genome and the predicted polyprotein as well as from certain features of the polyprotein such as hydrophobicity profiles and acidic/basic amino acid content, it is predicted that proteins of HCV are encoded in the same general regions of the genome as has been determined for the flaviviruses. In the flaviviruses and the related pestiviruses, the amino-terminal approximately one-third of the polyprotein constitutes the structural proteins of the virus. These consist of a highly basic nucleocapsid protein termed C, an envelope-associated glycoprotein termed M, and a second envelope glycoprotein, E. The nonstructural proteins are named NS1 through NS5, but the functions of only NS3 and NS5 have been assigned with certainty. NS3 is the viral protease and probably a helicase, and NS5 is the viral RNA-dependent RNA polymerase. The polyprotein is processed in a series of proteolytic digestions by a combination of the viral protease and host signalase. The processing of the pestivirus polyprotein is different in detail from that of the flaviviruses but is similar in general scheme. The proteins of HCV have been determined largely by analogy with the flavivirus proteins, and, like the pestiviruses, they may also differ in details of size, number, and processing from the flaviviruses. With these caveats, we shall, for convenience of nomenclature but not to imply identity in function or processing, hereafter refer to the nonstructural region under study, which is homologous to the flavivirus NS5 region RNA polymerase, as the NS5 region or protein of HCV.

Diagnostic tests based on expressed viral antigens have also been developed, and seroepidemiologic surveys using these assays have been performed (33). HCV not only is the cause of most cases of parenterally acquired non-B hepatitis but also is responsible for a large portion of sporadic community-acquired acute viral hepatitis and chronic hepatitis of unknown origin, as well as cryptogenic cirrhosis and probably hepatocellular carcinoma (2, 16, 31, 50). It is the propensity of this virus to cause chronic infections and chronic liver disease that makes it such a medically impor-

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tant problem. Treatment of chronic HCV liver disease by alpha interferon therapy has recently been approved by the Food and Drug Administration (FDA) in the United States. However, less than half of the patients respond, and of the responders approximately 50% relapse after treatment is stopped (16). Therefore, there is an important need for a vaccine to protect against infection by this virus. While the diagnostic tests are based on the detection of serum antibody to expressed viral proteins, to date all of these proteins have represented either nonstructural viral proteins or internal components of the virus particle. It is not clear at this time whether effective neutralizing antibodies to HCV are commonly produced by individuals infected with the virus.

Cytotoxic T lymphocytes (CTL) have been found to mediate protection in vivo against certain virus infections (17, 47, 48). In hepatitis B infection, CTL are thought to be responsible for the pathogenesis of chronic type B hepatitis and to lyse hepatitis B virus-infected hepatocytes by recognizing the viral antigen expressed on the infected cells (39, 42). In the case of HCV, there is no information on the pathogenesis of HCV infections. The chronicity of infections as well as histopathologic findings indicate that the virus is probably not directly cytopathic (or cytolytic) in hepatocytes. It is possible that the chronic liver disease associated with HCV infections is immune mediated. Therefore, it is not clear whether CTL specific for HCV will be implicated in the pathogenesis of disease or will be important for protection or recovery from infection. Previous studies have reported that $CD8^+$ CTL recognize hepatocytes from patients with chronic non-A, non-B hepatitis (28). However, to date, no epitope of HCV recognized by T cells has been identified in any HCV protein.

In other viral models, internal proteins are the major targets of the CTL response (3, 30, 49, 62, 69). In previous studies from our laboratory and others, peptides from an internal protein, reverse transcriptase, of human immunodeficiency virus (HIV) as well as envelope glycoprotein and *gag* and *nef* proteins were found to be recognized by CTL in mice and humans (10, 14, 24, 32, 43, 57, 65). Immunization with the envelope protein may not be an ideal approach for HCV, because the envelope is relatively highly variable in sequence (27) and CTL clones may distinguish different isolates of HCV, as has been shown in studies of HIV-1 (41, 58, 59). In contrast to the substantial amino acid sequence variation in the predicted envelope glycoproteins (termed E1 and E2/NS1), the internal nucleocapsid proteins, C, NS, NS4, and NS5, of HCV all show greater sequence conservation between isolate groups (27). The coding region of the HCV genome that is analogous to the NS5 region of the flavivirus genomes, both by its location in the genome and by its sequence similarities, is believed to represent the viral replicase. Because of its relative conservation and importance to viral function, the RNA polymerase appears to be worthy of particular interest in this regard.

Therefore, we investigated the specificity of NS5-specific CTL by using 28 peptides from NS5 predicted as potential T-cell epitopes on the basis of amphipathicity. In BALB/c mice, a single, relatively conserved epitope represented by a 16-residue synthetic peptide was presented by D^d class I major histocompatibility complex (MHC) molecules to conventional $CD4^-$ $CD8^+$ CTL. The CTL line specific for the peptide could recognize the two known variants with single conservative substitutions. Our results suggested the possibility that CTL recognize the products of the RNA polymerase gene, NS5, of HCV on the hepatocytes, or any other cells infected with the virus in association with class I MHC molecules. Therefore, if similarly recognized by human CTL, this peptide may be a potentially valuable candidate component of an HCV vaccine to prevent or treat the virus infection.

MATERIALS AND METHODS

Mice. BALB/c mice were purchased from Charles River Laboratories, and BALB.B mice were bred in our own colony from breeders kindly provided by F. Lilly (Albert Einstein College of Medicine, New York, N.Y.). Mice used were 8 weeks old.

Recombinant vaccinia viruses. The region of the HCV genome coding for amino acids 1959 through 2872, representing most of the predicted NS5 region based on analogy to flaviviruses, was cloned into vaccinia virus under the P7.5 promoter as described by Chakrabarti et al. (7) and was given the designation vHCV#3. HCV viral RNA was extracted from the liver biopsy of a chimpanzee acutely infected with the H strain of HCV (FDA isolate of HCV strain H [19]). The RNA was reverse transcribed and amplified by the polymerase chain reaction, using specific HCV primers as previously described (9). The 5′ primer included an ATG sequence at its 5′ end. This polymerase chain reaction product was ligated into the *Stu*I site of the pSC11ss transfer vector and then inserted into vaccinia virus by homologous recombination. The vaccinia virus was amplified in BS-C-1 cells and used for immunizing the mice to generate HCV NS5-specific CTL. vSC8 (recombinant vaccinia virus containing the *Escherichia coli lacZ* gene) and vSC25 (recombinant vaccinia virus expressing the HIV-1 IIIB gp160 envelope glycoprotein without other structural or regulatory proteins of HIV), generous gifts from Bernard Moss, National Institute of Allergy and Infectious Diseases, have been described elsewhere (7) and were used as controls for immunizing the mice.

Peptide synthesis and purification. HCV NS5 peptides were prepared by the simultaneous multiple-peptide method of solid-phase peptide synthesis, in polypropylene mesh "teabags" as described previously (25). Peptides were desalted by reverse-phase chromatography on C18 Sep-Pak columns (Waters Associates, Milford, Mass.) and analyzed by high-performance liquid chromatography (HPLC). Some peptides were prepared by an automated peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, Calif.) using t-Boc chemistry and purified by HPLC. Peptide P18 was prepared under good manufacturing practice conditions by Peninsula Labs (Belmont, Calif.).

CTL generation. Mice were immunized intravenously with 10^7 PFU of recombinant vaccinia virus. Four to 6 weeks later, immune spleen cells (5×10^6/ml) in 24-well culture plates in complete T-cell medium (CTM; 1:1 mixture of RPMI 1640 and EHAA medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 5×10^{-5} M 2-mercaptoethanol) were restimulated for 6 days in vitro with peptides and 10% concanavalin A (ConA) supernatant-containing medium (rat T-cell Monoclone; Collaborative Research, Inc., Bedford, Mass.). Because, in our experience with other antigens, restimulation of recombinant vaccinia virus-immune spleen cells with recombinant vaccinia virus led to a predominant response to the vaccinia virus and difficulty detecting weaker CTL responses to peptides from the inserted recombinant gene (although we did not find this to be the case for P17 [see Table 1]), we restimulated with peptides rather than with the same recombinant vaccinia virus used for immunization.

Logistically, we could not perform an experiment in which all 28 peptides were used to stimulate individual effector populations, so we stimulated with mixtures of three peptides each (at 4 μM) and tested each population of effectors against the three peptides in the corresponding mixture individually. We did not stimulate with a mixture of all the peptides, to avoid missing sites that might compete with each other for binding to MHC (20). In our experience, the frequency of peptides binding to a given class I molecule is low enough, and the frequency of finding a peptide that can compete at a concentration as low as 4 μM is sufficiently low, that it would be unlikely that we would miss a positive response because of competition between two peptides in a mixture of three at 4 μM. Nevertheless, we cannot formally exclude such competition as one of many possible reasons for negative results with some of the peptides. Long-term CTL lines were also generated by repetitive stimulation of immune cells with peptide-pulsed irradiated syngeneic spleen cells (2.5×10^6 cells per ml; pulsed with peptides at 10 μM for 4 h and then irradiated) and 0.5 to 1 μM peptide in 10% rat ConA supernatant-containing medium.

CTL assay. Cytolytic activity of in vitro secondary CTL or CTL lines was measured as previously described (57, 62), using a 6-h assay with ^{51}Cr-labeled targets. For testing peptide specificity of CTL, effectors and ^{51}Cr-labeled targets were mixed with various concentrations of peptide, or effectors were cocultured with peptide-pulsed targets. The percent specific ^{51}Cr release was calculated as 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton X-100. Spontaneous release was determined from targets cells incubated without added effector cells. 18Neo (*H-2*^{d}; class I MHC$^+$, class II MHC$^-$ neomycin resistance gene-transfected 3T3 fibroblasts [57]), L cells (L28; *H-2*^{k}), and EL4 thymoma cells (*H-2*^{b}) were used as targets.

Blocking of CTL response by antibodies. Culture supernatant of hybridoma GK1.5 or 2.43 containing anti-L3T4 (anti-CD4, immunoglobulin G2b [68]) or anti-Lyt 2.2 (anti-CD8 [54]) antibody, respectively, was added to the 96-well plates used for the CTL assay at the indicated concentrations.

Exon-shuffled and wild-type class I transfectants. Mouse L-cell transfectants with D^d, L^d, or exon shuffles between these molecules have been previously described (18, 37, 38, 40) and were the kind gifts of David Margulies, National Institute of Allergy and Infectious Diseases. The transfectant expressing K^d was developed by Abastado et al. (1) and was a gift of Keiko Ozato (National Institute of Child Health and Human Development). All transfectant cell lines were examined by fluorescence-activated cell sorting analysis with an appropriate panel of anti-H-2D^d, anti-H-2K^d, and anti-H-2L^d monoclonal antibodies to confirm their expressed phenotype before the performance of the functional studies reported here.

RESULTS

To generate CTL specific for the peptides from the HCV NS5-like region in BALB/c and BALB.B mice, spleen cells of mice immunized 4 weeks earlier with the NS5-expressing recombinant vaccinia virus (vHCV) (10^7 PFU intravenously) were stimulated in vitro with 4 μM peptides in the presence of interleukin-2 (10% rat ConA supernatant). On the basis of the published sequence of NS5 (26), we synthesized a series of 28 peptides, including some overlapping peptides, covering much of the HCV putative RNA polymerase sequence encoded by the NS5-like region, selected on the basis of amphipathicity (12, 13, 15, 36) as potential T-cell epitopes. Subsequent sequencing of the whole NS5 region of the HCV isolated in the FDA isolate used to construct the recombinant vaccinia virus showed that 11 of 28 peptides had mutations in up to three residues compared with the published sequence (Fig. 1a). BALB/c mice that were immunized with vHCV developed CTL responses to peptide P17 but not to any of the other peptides (Fig. 1b). As we have no antibodies specific for the HCV region cloned into vaccinia virus, we cannot test directly for expression of protein to the carboxy-terminal side of P17. Although we have no reason to suspect that this sequence is not expressed, it remains possible that lack of expression carboxy terminal to P17 could account for the lack of response to the peptides following P17. It is also possible that negative responses are due to the differences in sequence in 11 of the peptides between the isolate of HCV cloned into vaccinia virus and the isolate of HCV whose published sequence was used to synthesize the peptides. Because these and other reasons could account for negative responses, only positive responses can be clearly interpreted. Because class II-negative fibroblast targets were used, and lysis was restricted under MHC-linked control, P17 was probably presented by class I MHC molecules to CTL in the *H-2*^{d} mice. BALB.B (*H-2*^{b}) mice showed no response to any peptide tested. However, because we cannot use a vaccinia virus recombinant to stimulate spleen cells from mice immunized with a recombinant vaccinia virus without activating a vaccinia virus-specific response that overwhelms the response to the inserted gene product when vaccinia virus-infected targets are used, we cannot use BALB.B cells infected with vHCV expressing the whole NS5-like protein to determine whether BALB.B mice might respond to other epitopes of HCV not tested with peptides.

When the FDA isolate of HCV used to make the vaccinia virus recombinant vHCV was sequenced, it was found to differ in the P17 sequence from the Chiron HCV sequence (26) by one residue and from the Japanese sequence (conserved at this site in two independent Japanese isolates [29, 60]) by another residue (Fig. 1a). Because mice were immunized with vHCV expressing the FDA sequence but the CTL were restimulated with and tested on targets pulsed with the P17 peptide made according to the Chiron sequence, the CTL were expected to cross-reactively recognize both variants of HCV. We synthesized two variant P17 peptides with amino acid substitutions at positions 2425 and/or 2431 corresponding to the FDA (P17FDA) and Japanese (P17JPN) isolates (Fig. 1a). Variant peptides, differing at one or two residues, could be used to define the effect of naturally occurring virus mutation on peptide presentation by the H-2^d class I MHC molecules and on CTL recognition.

Specificity of CTL for NS5 was demonstrated at the level of lymphocyte priming in vivo, restimulation in vitro, and expression on the target cells in the CTL assay (Table 1). Only the recombinant vaccinia virus expressing the NS5 gene (vHCV), not the control vaccinia viruses (vSC8 and vSC25), could prime mice for development of CTL specific for P17 or P17 variants (Table 1). Titration of the peptide concentrations used for stimulation of immunized spleen cells demonstrated that lysis specific for P17 was induced at a low concentration of peptide, 0.1 μM (Table 1). P17 required 1 to 10 μM peptide for the stimulation of immune spleen cells to elicit the highest level of lysis against H-2-

a

Peptide	Residue Nos.	Sequence	Amphipathic Score
3	(1958-1977)	RRLHQWISSECTTPCSGSWL	18.0
4	(1969-1988)	TTPCSGSWLRDIWDWICEVL	26.8
5	(1981-2000)	WDWICEVLSDFKTWLKAKLM	22.6
6	(2042-2061)	GTMRIVGPRTCRNMWSGTFP	20.8
7	(2089-2103)	RVSAEEYVEIRQVGDFHYVT R S	25.9
8	(2089-2108)	EYVEIRQVGDFHYVTGMTTD S	23.9
9	(2113-2132)	PCQVPSPEFFTELDGVRLHR I	18.1
10	(2117-2136)	PSPEFFTELDGVRLHRFAPP	24.1
11	(2168-2183)	VAVLTSMLTDPSHITA	14.1
12	(2180-2195)	ITAEAAGRRLARGSP	11.2
13	(2265-2284)	ERAISVPAEILRKSRRFAQA E	19.6
14	(2267-2286)	AISVPAEILRKSRRFAQALP E	19.3
15	(2335-2354)	LTESTLSTALAELATRSFGS	22.1
16	(2348-2362)	ATRSFGSSSTSGITG	8.3
17	(2422-2437)	MSYSWTGALVTPCAAE	13.2
17FDA		MSYTWTGALVTPCAAE	
17JPN		MSYTWTGALITPCAAE	
18	(2438-2455)	EQKLPINALSNSLLRHHN	16.1
19	(2477-2497)	LQVLDSHYQDVLKEVKAAASK	29.8
20	(2531-2550)	HARKAVTHINSVWKDLLEDN V S	28.0
21	(2535-2554)	AVTHINSVWKDLLEDNVTPI A SF	28.7
22	(2583-2598)	PDLGVRVCEKMALYDV	12.2
23	(2593-2607)	MALYDVVTKLPLAVM S	11.1
24	(2668-2683)	QARVAIKSLTERLYVG	11.1
25	(2701-2719)	ASGVLTTSCGNTLTCYIKA	17.5
26	(2721-2736)	AACRAAGLQDCTMLVC R	12.7
27	(2749-2768)	VQEDAASLRAFTEAMTRYSA V V	27.3
28	(2757-2776)	RAFTEAMTRYSAPPGDPPQP V	24.3
29	(2822-2834)	HTPVNSWLGNIIM	11.2
31	(2866-2880)	EIYGACYSIEPLDLP A V	12.0

b

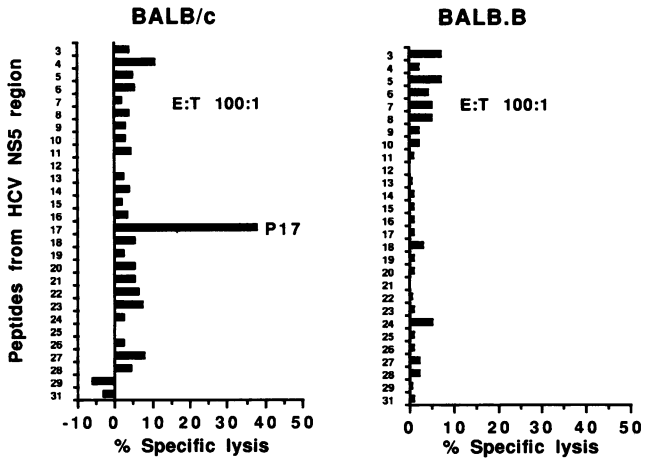

FIG. 1. (a) Series of 28 peptides selected by the amphipathicity algorithm for T-cell epitopes from the HCV NS5 region. The sequences of synthesized peptides except two P17 variants, P17FDA (isolated by the U.S. FDA) and P17JPN (isolated in two Japanese laboratories independently) were based on the isolate in Chiron Corp. (26). Mutated residues at which the FDA isolate differs from the Chiron sequence are shown under the original sequences with underlines. Amphipathic scores (36) of the peptides are indicated. Although the correlation between class II-restricted T-cell epitopes and regions with amphipathic scores of >4 remains highly statistically significant even as evaluated on 92 epitopes known in 1991 (12, 13) ($P < 0.0003$) and is significant on a smaller set of CTL epitopes studied as well, we have not identified a correlation between the magnitude of the score above this threshold and the probability that a site will be recognized by T cells. (b) CTL response to peptides from HCV NS5 in BALB/c and BALB.B mice. Mice were primed intravenously with 10^7 PFU of recombinant vaccinia virus expressing the HCV NS5 region (vHCV). The immune spleen cells were restimulated in vitro with peptides at 4 μM (three different peptides for each culture) or no peptide in the presence of ConA supernatant (interleukin-2). CTL activity was measured against *neo* gene-transfected 3T3 fibroblast cells (18Neo; *H-2*^{d} class I positive, class II negative) in BALB/c mice and EL-4 cells (*H-2*^{b}) in BALB.B mice. Targets were sensitized with 10 μM each peptide or no peptide for 6 h. The experiments were performed in triplicate. E:T, effector/target ratio; 5,000 target cells per well. Lysis in the absence of peptide was 2.2 to 7.7% in BALB/c mice (4.2% for P17-stimulated immune cells) and less than 2% in BALB.B mice. In general, standard errors of triplicates were <5% of the values, and comparable results were obtained in a repeated experiment.

matched peptide-pulsed target cells. P17, P17FDA, and P17JPN were nearly interchangeable both for stimulation of CTL and for sensitization of targets (Table 1). Similar results were obtained in two independent experiments. Thus, complete cross-reactivity between these variants was observed.

Long-term CTL lines specific for P17 and P17FDA were established by repetitive stimulation of spleen cells from vHCV-immunized mice with peptide-pulsed irradiated syngeneic spleen cells and interleukin-2 (ConA supernatant) (Fig. 2). The CTL lines stimulated with P17 or P17FDA (position 2425, S→T) manifested highly specific lysis of targets with each peptide. In the titration study, P17 and P17 variants could each sensitize target cells for similar levels of lysis by the CTL lines, between 0.01 and 10 μM (Fig. 2). The lysis was on plateau in the presence of each peptide at concentrations above 0.1 μM. The maximum lysis achievable with P17 was comparable to the maximum lysis achievable with two other P17 variants at similar concentrations of peptide, using the two different lines specific for P17 and P17FDA. Thus, these mutated residues did not affect the magnitude of response or concentration dependence, and the peptides were fully cross-reactive. CTL stimulated in vitro with any of the three variants of the P17 peptide lysed targets in the presence of all three P17 variant peptides equally well (Table 1). Therefore, the conservative point mutations in the P17 sequence at which the four clones of HCV isolated in the United States and Japan differ did not seem to affect peptide interaction with H-2^d class I MHC molecules or recognition by the T-cell receptor.

Because the CTL lines were generated by repeated in vitro stimulation with peptide, we wanted to be sure that they recognized the processed products of endogenously synthesized NS5 protein, not just peptide. The CTL lines restricted by H-2^d (BALB/c) were able to kill the vHCV-infected syngeneic target cells (18Neo cells [BALB/c 3T3 fibroblasts transfected with the neomycin resistance gene]) endogenously expressing NS5, as well as 18Neo cells pulsed with P17 or P17FDA, but not the control targets, unpulsed or P18IIIB-pulsed 18Neo cells (Fig. 3). Therefore, these CTL were specific for processed products of endogenously synthesized NS5, not just exogenous peptide.

Treatment of the CTL cell lines specific for P17 with an anti-CD8 monoclonal antibody, but not an anti-CD4 antibody, reduced or abrogated cytotoxic activity on target cells (Fig. 4). These data indicate that the effector cells which recognize P17 are conventional CD8$^+$ CD4$^-$ (Lyt2$^+$ L3T4$^-$) CTL. For H-2^d-restricted peptide-specific CTL lines in

TABLE 1. Priming and boosting requirements for CTL induction in *H-2*^{d} mice[a]

Immunization	Restimulation	% Specific lysis						
		vHCV	vSC8	P17	P17FDA	P17JPN	P18IIIB	Unpulsed
None	P17	0.7	0.6	1.4				0.5
	P17FDA	0.8	1.0		2.9			2.9
	P17JPN	0.0	0.2			1.2		3.2
	vHCV	0.1	0.2	1.3	0.4	2.0		2.9
vSC8	P17	1.2	0.4	3.0				0.3
	P17FDA	1.1	0.8		2.3			3.2
	P17JPN	0.8	0.6			2.6		0.6
	vHCV	41.6	37.1	0.9	0.3	−0.1		0.9
	vSC8	47.6	44.5	2.8	0.5	1.9		0.4
vSC25	P18IIIB			0.8	0.5	1.8	63.3	2.3
	P17				0.1		1.7	0.2
	P17FDA				0.4		1.7	0.3
	P17JPN					1.4	1.8	0.3
	vHCV	37.9	40.5	−0.3	0.4	0.1	3.2	0.8
vHCV	P17		3.4	42.7	38.0	37.9	3.3	0.5
	P17, 1 μM		3.7	33.8	32.4	31.9	1.4	0.7
	P17, 0.1 μM		1.1	17.1	12.8	12.9	3.3	0.0
	P17FDA		5.8	34.9	39.7	36.1	2.2	2.0
	P17JPN		4.5	39.7	38.7	35.8	2.5	1.8
	vHCV	62.4	41.2	33.4	31.9	32.7	2.4	0.5
	vSC8	48.1	41.7	−1.1	1.6	0.0	2.2	0.5
	P18IIIB	2.2	−0.8	1.8	0.8	1.6	1.6	0.5

[a] The ability of recombinant vaccinia viruses to prime and stimulate CTL specific for the products of inserted viral genes was used to generate CTL specific for HCV NS5 in BALB/c (*H-2*^{d}) mice. Nonimmune or immune spleen cells were restimulated with P17 or P17 variants at 10 μM (or at the indicated concentration [0.1 or 1 μM P17]), P18IIIB at 0.1 μM, or vaccinia virus (vHCV, vSC8, or vSC25) and tested against vaccinia virus-infected 18Neo, 18Neo pulsed with P18IIIB at 1 μM (an immunodominant CTL site, 315 to 329, of the HIV-1 gp160 IIIB isolate), and unpulsed 18Neo in the presence of the P17 or P17 variant peptides (10 μM) or no peptide at an effector/target ratio of 100:1. Lysis in the absence of peptide was <4%. No toxicity of peptides against targets was observed. Spontaneous release was less than 20% of maximum release. vHCV, recombinant vaccinia virus expressing NS5 of HCV; vSC8 and vSC25, control vaccinia virus and recombinant vaccinia virus expressing HIV-1 gp160, respectively; 18Neo, BALB/c 3T3 fibroblast, *H-2*^{d}.

BALB/c mice, 18Neo cells expressing class I but not class II MHC gene products were used as targets. Therefore, these H-2^d-restricted CTL lines are likely to be class I MHC restricted, as expected for Lyt2$^+$ CD8$^+$ effector T cells. The P17FDA-specific line showed similar results (not shown).

We used transfectants expressing the K^d, D^d, or L^d molecule to determine which molecule was specifically required for the presentation of P17 in H-2^d. The targets were pulsed with the indicated peptide and labeled with ^{51}Cr at same time. T37.2.1 (α1 α2 of D^d) and T4.8.3 (D^d) were found to present P17 nearly as well as did the positive control,

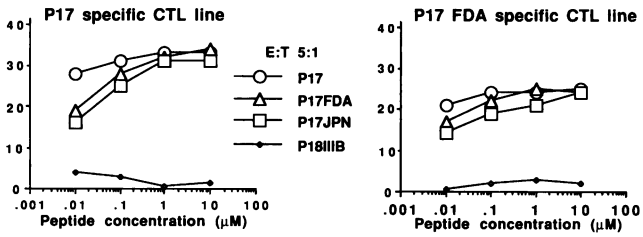

FIG. 2. Cytotoxicity of CTL lines specific for P17 and P17FDA against each specific peptide and other P17 variants. The effects of a single amino acid mutation on recognition of variant epitopes was examined. A total of 5 × 10^3 ^{51}Cr-labeled target cells (18Neo) were cultured with effector cells from long-term CTL lines, repetitively stimulated with specific peptides, in the presence of each specific peptide, other P17 variants, P18IIIB (negative control peptide), or no peptide. E:T, effector/target ratio. Lysis in the absence of peptide was <1%. Standard errors of triplicates were generally <5% of the values, and comparable results were obtained in three independent experiments.

18Neo BALB/c 3T3 fibroblasts that express all three H-2^d class I MHC molecules (Fig. 5). Neither K^d nor L^d nor any other D^d/L^d exon-shuffled class I molecule presented P17 to the CTL. Therefore, both α1 and α2 domains of D^d were necessary and together sufficient to present this peptide. Identical restriction was found for CTL recognizing P17FDA in BALB/c mice (not shown).

DISCUSSION

HCV is probably a member of the *Flaviviridae* family, which includes both the classic flaviviruses such as yellow fever virus and the pestiviruses of animals such as bovine viral diarrhea virus (11). The classic flaviviruses have arthropod vectors and cause only acute disease. The pestiviruses, on the other hand, have no known arthropod vectors and may cause chronic infections as well as acute disease. HCV causes acute, self-limited infections, but like pestiviruses, also commonly causes chronic infections and liver diseases that include chronic active hepatitis, cirrhosis, and probably hepatocellular carcinoma. The mechanism of chronicity and the pathogenesis of the chronic liver disease are not understood. It does not seem likely that the chronic virus infection is directly cytopathic, as it would result in much greater liver destruction. Therefore, as in hepatitis B infections, an immune mechanism has been proposed.

Studies on the immune response to HCV infections are still at a formative stage. The commercially available antibody assay measures antibody to an HCV antigen expressed in yeast cells by recombinant DNA technology. This antigen, termed C100-3, is derived from the nonstructural protein-coding region of the viral genome and probably represents a portion of NS4 (33). Newer versions of the assay

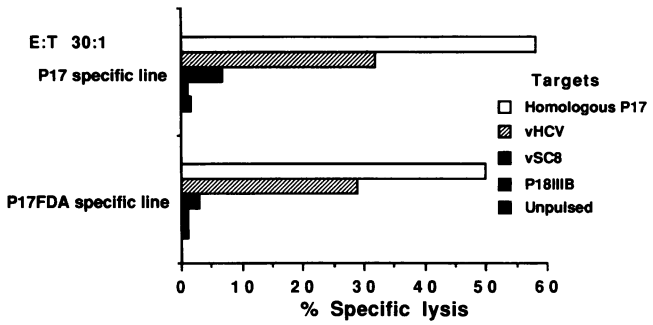

FIG. 3. CTL lines specific for P17 and P17FDA restricted by H-2^d were tested for cytotoxicity against 18Neo cells infected with vHCV vaccinia virus expressing NS5 (1 h, 37°C, multiplicity of infection of 10:1, three washings before use) as well as 18Neo cells pulsed with each specific peptide (10 μM). As negative control targets, 18Neo cells infected with control vSC8 vaccinia virus and P18IIIB (1 μM)-pulsed and unpulsed 18Neo cells were used. Standard errors of triplicates were generally <5% of the values, and comparable results were obtained in three independent experiments. E:T, effector/target ratio.

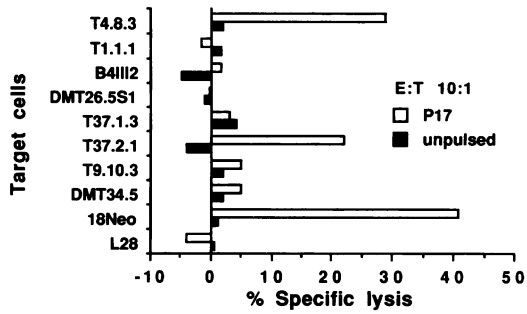

FIG. 5. MHC class I molecules responsible for the presentation of P17 to CTL line in the *H-2*^{d} strain. Target cells were pulsed overnight with P17 (10 μM) and washed three times. E:T, effector/target ratio. The origins of the α1 α2 α3 and transmembrane portions of the class I molecule for the various transfectants are D^d D^d D^d D^d for T4.8.3, L^d L^d L^d L^d for T1.1.1, −D^d D^d D^d for DMT26.5S1, D^d D^d L^d L^d for T37.2.1, L^d L^d D^d D^d for T37.1.3, D^d L^d L^d L^d for T9.10.3, and L^d D^d D^d D^d for DMT34.5. Standard errors of triplicates were generally <5% of the values, and comparable results were obtained in two independent experiments. L28, DAP3 L cell (*H-2*^{k}) transfected with pSV2neo alone; 18Neo, BALB/c 3T3 fibroblasts (*H-2*^{d}) transfected with pSV2neo alone. E:T, effector/target ratio.

include antigens from NS3 (protease/helicase) and the internal nucleocapsid protein, C (64). None of these antibodies is thought to be protective, but each is commonly found in chronically infected individuals. Neutralizing antibodies to most flaviviruses are directed against epitopes in the envelope glycoprotein. Either these antibodies are not commonly made by people infected by HCV or we do not yet have an assay available to measure them. Sequence analysis of the two putative envelope glycoproteins of HCV has revealed considerable strain variability (23, 27, 67). This may again confound attempts to produce an antigen that induces neutralizing antibodies of broad reactivity. For these reasons, we have begun an investigation of potential T-cell epitopes that may be important in pathogenesis or could have utility as immunogens in a vaccine.

Previous studies have reported that CD8$^+$ CTL recognize hepatocytes from patients with chronic non-A, non-B hepatitis in a non-MHC-restricted manner (28). It has been thought that CTL against virus-infected cells most often recognize the nucleoprotein of the virus expressed on the infected cells and lyse the cells (52). In hepatitis B infection, CTL are thought to be responsible for the pathogenesis of chronic type B hepatitis and to lyse hepatitis B virus-infected hepatocytes by recognizing the viral antigen expressed in the infected cells (39, 42). Our results of studies using mice suggested the possibility that CTL recognize the product of the RNA polymerase gene, a nonstructural region, of HCV on cells infected with the virus in association with class I MHC molecules. Because murine cells cannot be infected with HCV, we cannot test this possibility directly on infected cells in BALB/c mice.

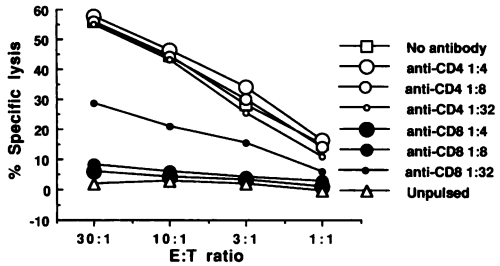

FIG. 4. Phenotype of the *H-2*^{d} CTL line specific for P17. The CTL assay was performed as described for Fig. 2 in the presence of an anti-L3T4 (GK1.5) (anti-CD4) or anti-Lyt 2.2 (2.43) (anti-CD8) monoclonal antibody (culture supernatant) at the indicated dilution, or no antibody, for 6 h. 18Neo cells were pulsed overnight with P17 (10 μM) and washed three times. Standard errors of triplicates were generally <5% of the values, and comparable results were obtained in three independent experiments. E:T, effector/target.

Class I and class II MHC molecules allow T cells to recognize polypeptide fragments of protein following processing of foreign antigens (4, 51, 55, 61, 70). Synthetic peptide vaccines may elicit fewer deleterious immune responses than does the whole protein or attenuated or killed virus (5).

We have produced murine CTL lines with ability to kill syngeneic target cells expressing the HCV NS5 as well as target cells pulsed with peptide P17 (HCV residues 2422 to 2437 within NS5) in *H-2*^{d} mice but not in *H-2*^{b} mice. We conclude that *H-2*^{d} is an immune response gene responder haplotype to P17, whereas *H-2*^{b} is not a responder. To determine whether the response to P17 of HCV NS5 depends on both the α1 and α2 domains of the D^d class I molecules, we used eight L-cell (*H-2*^{k}) transfectants with different exon shuffles between D^d and L^d. The results revealed that the P17 peptides required both α1 and α2 domains of the D^d molecule for effective peptide presentation, as shown in the presentation of peptides P18 (58) and HP53 from HIV-1 gp160 (56). It is of interest that P17, predicted to be a short, highly amphipathic alpha-helical site, P18, and HP53 (56), which share no striking sequence similarity but have similar amphipathic hydrophobicity profiles when folded as an alpha helix, were all presented by the same D^d class I MHC molecule. Although insufficient homology is present to define an obvious motif for D^d binding, analysis of residues involved in D^d binding (58) for each peptide may shed new light on the structural requirements for the D^d specificity.

To obtain maximal lysis, the peptide concentration required to stimulate CTL in vitro secondarily or to sensitize

targets appeared to be very low (0.1 to 1 μM) for P17. This result suggests that P17 binds with a relatively high affinity to class I MHC molecules in H-2^d. Also, P17 showed no toxicity (data not shown), which might affect its activity in CTL stimulation.

Recent reports of HCV sequence diversity allow comparison of several isolates (9, 23, 35, 44, 67; reviewed in reference 27). It is noteworthy that C, NS3, NS4, and NS5 exhibit greater sequence conservation in contrast to the hypervariability of the putative envelope glycoproteins encoded by the E1 and E2/NS1 genes and the greater heterogeneity of NS2. This hypervariability of the HCV envelope protein suggests that this region may be under selective pressure for variation of a protective B-cell or T-cell epitope, as suggested in the case of the HIV-1 envelope protein V3 loop, which is the principal neutralizing domain as well as an immunodominant determinant for CTL in both humans and mice (10, 21, 46, 53, 57). The hypervariability suggests an ability of this virus to escape the immune system by rapid mutation. Within the groups of isolates, broadly subdivided by comparison of all the reported HCV sequences, NS5 shows 95 to 100% homology (27). The variability is also relevant to the issue of multiple infection with different HCV isolates. Most recent sequence analysis of the HBV genome from fulminant hepatitis suggested that naturally occurring viral mutations may predispose the infected host to more severe liver injury (34, 45). Thus, cross-neutralization of variants by antibody and cross-recognition of variants by T cells represent an important issue in the development of vaccines to prevent the rise of escape mutants from the immune system.

Availability of two different sequences of other variants (United States and Japan) of this relatively conserved epitope (26, 29, 60) allowed us to synthesize two variant peptides, each differing at one or two residues from P17, in order to define the effect of naturally occurring viral mutation on the peptide presentation by the D^d molecule and CTL recognition. The single amino acid substitution at position 2424 (T→S) and at position 2431 (V→I) did not reduce the CTL recognition of peptide P17 in BALB/c mice. Therefore, these point mutations, at which four cloned HCV isolates from the United States and Japan differ, seem to affect peptide interaction with neither the D^d class I MHC molecule nor the T-cell receptor. If human CTL display similar cross-reactivity for this site, it may be useful as a CTL epitope for vaccine development despite the few conservative substitutions found in nature.

There are many lines of evidence that CTL can block outgrowth of virus (17, 28, 39, 42, 47, 48, 63, 66). Likewise, it is thought that a vaccine eliciting HCV-specific CTL may be protective against HCV. In this study, P17 from the HCV NS5 region was found to be presented by class I MHC molecules to CD8$^+$ CD4$^-$ CTL. The high conservation and cross-reactivity of peptide P17 suggests that this peptide could play a role as a component of a broadly effective vaccine for HCV if also recognized by human CTL. We are presently investigating whether this site is presented by a diversity of human class I MHC alleles and whether P17 is also able to be presented by class II MHC molecules to CD4$^+$ CTL, not only from the point of view of vaccine development but also for analysis of the versatility of viral peptides for binding MHC molecules. In our previous experience with HIV-1 proteins gp160 and reverse transcriptase, the epitopes recognized by murine CTL were also recognized by human CTL (10, 24, 57). We have observed T-cell proliferation but not cytotoxicity to P17 stimulation in one chronically infected chimpanzee. Future studies of critical amino acids in the peptide for binding to MHC molecules and the T-cell receptor may yield new information on the molecular basis of escape mutation by HCV and the chronicity of HCV infection.

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