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Identification and characterization of a cytotoxic T cell epitope of hepatitis C virus presented by HLA-B*3501 in acute hepatitis

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In order to clarify the role of cytotoxic T lymphocytes (CTL) in hepatitis C virus (HCV) infection, an HLA-B35-restricted cytotoxic T cell epitope of HCV was identified using a strategy called reverse immunogenetics. Twenty-eight of 53 HCV peptides carrying two anchor residues were selected as HLA-B*3501 binding peptides. These peptides were used to induce the specific cytotoxic T cells in peripheral blood lymphocytes from a patient with acute hepatitis C. Six HLA-B*3501 binding peptides induced the peptide-specific CTL. One (HPNIEEVAL) of five peptides was confirmed as the epitope by the specific T cell clones. A sequence identical to the epitope was detected in isolates of the virus from the patient and a strong CTL response to this epitope was observed in the acute phase of hepatitis C but not in the recovery phase. The specific CTL for this epitope were not detected in peripheral blood lymphocytes from patients with chronic hepatitis C. Together these results suggest that the CTL specific for this epitope have an important role in the elimination of the virus in the patient.

Introduction

Throughout the world, several million individuals are infected with hepatitis C virus (HCV). Between 50 and 70% of the individuals infected with HCV develop chronic hepatitis (Alter *et al.*, 1992). Of these chronically infected individuals, 8–46% and 11–19% will develop liver cirrhosis and hepatocarcinoma, respectively (Seeff, 1997). The pathogenic mechanism of the onset of cirrhosis and carcinoma is unknown. Between 10 and 40% of HCV hepatitis results from the transfusion of infected blood, while the rest is sporadic community-acquired or from unknown causes (Alter *et al.*, 1992). Vertical transmission of HCV infection has also been reported (Inoue *et al.*, 1991; Dienstag, 1997). Screening of blood donors reduces the number of cases of posttransfusion HCV hepatitis but does not eliminate sporadic community-acquired hepatitis C. Therefore, a vaccine to prevent HCV infection is required to eradicate hepatitis C.

Cellular immune responses, particularly those mediated by cytotoxic T lymphocytes (CTL), have a dominant role in the clearance of viruses in an infected host (Lukacher *et al.*, 1984; Riddell *et al.*, 1992; Yap *et al.*, 1978). CTL specific for HCV are also suspected to be an important component of protective immunity against HCV infection. On the other hand, there is growing evidence that CTL contribute to the development of chronic hepatitis. A previous study demonstrated the presence of hepatitis B virus (HBV)-specific CTL in the liver of patients with chronic HBV infection (Barnaba *et al.*, 1989). Similarly, HCV-specific CTL have been demonstrated in the liver of

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humans (Koziel *et al.*, 1992) and chimpanzees (Erickson *et al.*, 1993; Wong *et al.*, 1996) with chronic hepatitis C. However, it still remains unknown whether CTL cause chronic hepatitis.

CTL recognize a peptide derived from viral proteins presented by MHC class I molecules (Townsend *et al.*, 1986). MHC class I molecules bind self-peptides with an allele-specific motif and characteristic residues (Falk *et al.*, 1991; Jardetzky *et al.*, 1991). The sequences of viral peptides recognized by the specific CTL are also consistent with the motifs of self-peptides bound to MHC class I molecules (Engelhard, 1994). The identification of CTL epitopes derived from viral proteins is required to develop vaccines against viruses and to study the role of CTL in various diseases associated with virus infection. Most CTL epitopes have been identified using specific human CTL clones. However, difficulties in the generation of human CTL clones and the complexity of the mapping procedure have yielded only a very limited number of CTL epitopes of viruses, including HCV. We recently identified multiple CTL epitopes of human immunodeficiency virus type 1 (HIV-1) by a strategy using an HLA class I binding peptide motif called 'reverse immunogenetics' (Shiga *et al.*, 1996; Ikeda-Moore *et al.*, 1997). In the present study, we use this strategy to identify HCV CTL epitopes presented by HLA-B35, which is found in about 15% of Japanese and Caucasian populations. The identified epitope was analysed in detail to clarify its possible use as a vaccine candidate.

Methods

■ **Cells.** RMA-S cells expressing human β2-m (RMA-S-hβ2m), T2 cells and C1R cells were cultured in RPMI 1640 medium supplemented with 10% FCS. RMA-S-hβ2m cells expressing HLA-B*3501 (RMA-S-B*3501), T2 cells expressing HLA-B*3501 (T2-B*3501) and C1R cells expressing HLA-B*3501 (C1R-B*3501) were previously generated (Takamiya *et al.*, 1994; Takiguchi *et al.*, 1994). RMA-S-B*3501 and C1R-B*3501 cells were cultured in RPMI 1640 medium containing 10% FCS and 0·2 mg/ml hygromycin B, while T2-B*3501 cells were cultured in RPMI 1640 medium containing 10% FCS.

■ **MAbs.** The hybridomas secreting anti-HLA class I MAb, W6/32 and anti-HLA-Bw6 MAb, SFR8-B6 (Radka *et al.*, 1982) were obtained from the ATCC.

■ **Peptide synthesis.** Peptides were prepared utilizing a multiple peptide synthesizer (Shimadzu model PSSM-8) with the FMOC strategy. Synthetic peptides were examined by reverse-phase HPLC. Peptides less than 90% pure were purified by reverse-phase HPLC.

■ **Peptide binding assay.** RMA-S-B*3501 cells were cultured at 26 °C for 18–24 h. Cells [2×10^5 in 50 µl PBS supplemented with 20% FCS (PBS-FCS)] were incubated at 26 °C for 1 h with 50 µl of a solution of peptide at different concentrations and then at 37 °C for 3 h. After washing with PBS-FCS, the cells (2×10^5) were incubated for 30 min on ice with an appropriate dilution of SFR8-B6 anti-HLA-Bw6 MAb. After two washes with PBS-FCS, the cells were incubated for 30 min on ice with an appropriate dilution of FITC-conjugated sheep anti-mouse Ig antibodies (Silenus Laboratories). Cells were then washed three times with PBS-FCS and the fluorescence intensity was measured using a FACScan. RMA-S-B*3501 cells cultured at 26 °C and those incubated at 37 °C for 3 h were stained with SFR8-B6 MAb under the same experimental conditions as the controls.

■ **Patient.** An HLA-B35 positive patient with acute HCV infection was studied. Diagnosis of acute hepatitis was based on the findings of a high level of serum alanine aminotransferase, serum HCV RNA (see Fig. 3), seropositivity for HCV-specific antibody by enzyme immunoassay (Abbott Laboratories), histological examination of liver tissue and the recent onset of jaundice and other typical symptoms of acute hepatitis. The patient completely recovered from the illness with normalization of serum alanine aminotransferase and clearance of HCV RNA from the serum for 2 years since 1 month after the onset. The use of peripheral blood lymphocytes (PBL) from this patient was approved by the Review Board in Yokohama City University.

■ **Evaluation of HLA-B*3501 binding peptides.** Peptides at a concentration of 10^{-4} M that gave more than 25% of the mean fluorescence intensity (MFI) of RMA-S-B*3501 cells were evaluated as HLA-B*3501 binding peptides. The MFI of RMA-S-B*3501 cells was obtained by subtracting the MFI value of RMA-S-B*3501 cells incubated at 37 °C and stained with SFR8-B6 MAb from the MFI value of RMA-S-B*3501 cells cultured at 26 °C and stained with SFR8-B6 MAb. The peptide concentration that yielded the half-maximal levels of the MFI (the BL_{50} value) was calculated.

■ **Induction of HCV peptide-specific CTL from PBL of a patient carrying HLA-B35 with acute hepatitis C.** PBL were separated from the whole blood of a patient carrying HLA-B35 (HLA-A24/A26, B35/B62 and Cw4/Cw9) with acute hepatitis C on the eighth day after the onset of the disease. PBL (2×10^6) were cultured with each of the HLA-B*3501 binding peptides at a concentration of 10^{-6} M in RPMI 1640 medium with 10% FCS and 50 U recombinant human IL2 (r-hIL2) in a 24-well culture plate. The culture was maintained by changing half of the medium containing 50 U r-hIL2 every 2–3 days and weekly stimulation with PHA-stimulated cells pulsed with the peptides at a concentration of 10^{-6} M. After the second and fourth stimulation, CTL activities were examined by 4 h ^{51}Cr release assays.

■ **CTL assay.** T2-B*3501 and T2 cells (5×10^5) cultured at 26 °C overnight were incubated for 90 min with 100 µCi $Na_2{}^{51}CrO_4$ in PBS at 26 °C, and washed three times with RPMI 1640 medium containing 10% FCS. Medium (50 µl) containing ^{51}Cr-labelled target cells (5×10^3 per well) was added in 96-well round-bottom microtitre plates (Nunc), and then 50 µl of the medium containing various concentration of peptides (10^{-6}–10^{-10} M) was added. After 1 h incubation at 37 °C, 100 µl of the medium containing a serial dilution of effector cells was added and the mixtures were incubated for 4 h at 37 °C. The supernatants were collected and analysed with a gamma counter. The spontaneous ^{51}Cr release (c.p.m. spn) was determined by measuring the c.p.m. in the supernatant in wells containing only target cells. The maximum release (c.p.m. max) was determined by measuring the release of ^{51}Cr from target cells in the presence of 2·5% Triton X-100. Specific lysis was calculated using the following formula: % specific lysis = [(c.p.m. exp) − (c.p.m. spn)/(c.p.m. max) − (c.p.m. spn)] × 100, where c.p.m. exp is the c.p.m. in the supernatant in wells containing target and effector cells.

■ **Construction of a recombinant HCV vaccinia virus.** A recombinant HCV vaccinia virus was constructed as follows. The transfer vector, pTM345 or pTMORF, carried a type 1b HCV sequence (Kato *et al.*, 1990; Tanaka *et al.*, 1992) corresponding to amino acid residues 1027–3010 (NS3, 4A, 4B, 5A and 5B regions) or L1-3010 (full-length open reading frame) in plasmid pTM-1. The HCV constructs were under the control of the bacteriophage T7 promoter. Recombinant vaccinia viruses containing NS3–5 of HCV (WR-HCV-NS3-5) and the full-length

open reading frame (WR-HCV-ORF) were generated by homologous recombination between wild-type vaccinia virus (WR strain) and the transfer vectors, purified by three cycles of plaque cloning with LTK^- cells in the presence of 5-bromo-2′-deoxyuridine, and propagated in HeLa G cells. A recombinant vaccinia virus, vTF7-3 (WR-T7), which expresses bacteriophage T7 RNA polymerase, was kindly provided by B. Moss (Laboratory of Viral Diseases, Bethesda, USA). The expression of all HCV proteins in the cells infected with the recombinant vaccinia virus was confirmed by Western blotting analysis with the specific antibodies (data not shown).

■ **Generation of CTL clones specific for HCV peptides.** Peptide-specific CTL clones were generated from established HCV-specific bulk CTL culture by seeding at 0·8 cells per well in round-bottom 96-well microtitre plates (Nunc) with 5×10^5 irradiated allogenic PBL from a healthy donor and 5×10^4 irradiated T2-B*3501 cells prepulsed with 10^{-6} M corresponding peptide in RPMI 1640 supplemented with 10% FCS and 100 U/ml r-hIL2. Wells which were found to be positive for growth were tested for specific CTL activity after 2–4 weeks. Positive clones were maintained in RPMI 1640 medium containing 100 U/ml r-hIL2, stimulated weekly with peptide-pulsed T2-B*3501 cells.

■ **CTL assay for target cells infected with recombinant vaccinia virus.** Target cells (C1R and C1R-B*3501) were incubated with both 10 p.f.u. recombinant vaccinia virus containing HCV sequences and 10 p.f.u WR-T7 per target cell for 1 h, washed once and cultured overnight. These infected cells (5×10^5) were incubated for 60 min in 100 μCi $Na_2{}^{51}CrO_4$ in PBS, and washed three times with RPMI 1640 medium containing 10% FCS. Target cells (5×10^3 per well) were added with the CTL clones at an E:T ratio of 2:1 and the mixture incubated for 4 h at 37 °C.

■ **Cloning and sequencing of the HCV gene coding the CTL epitope.** RNA was extracted from 100 μl serum by the guanidium thiocyanate–phenol–chloroform method. The 3′ terminus of the RNA was chemically modified by periodate oxidation followed by reduction with $NaBH_4$ to inactivate endogenous priming activity in cDNA synthesis and to allow strand-specific detection. RNA from 10 μl of the serum was used for amplification of the region encoding the NS3 by RT-nested PCR. Strand-specific HCV RNA was detected by performing RT in the presence of sense (5′ CTGCAACACTGGGCTTTGGTGCTTACATGT 3′) or anti-sense (5′ TGAAGGTGGGGTCCAAGCTGAA 3′) primer. PCR was performed for 35 cycle with 2·5 U *Pfu* DNA polymerase (Statagene). The reaction cycle was composed of denaturation at 94 °C for 1 min, annealing at 45 °C for 45 s and extension at 72 °C for 2 min for the first PCR. In the second PCR, 1 μl of the first products of the reaction was amplified with internal primers (sense primer 5′ CAGAGGTGAGAATTCGTGACCGATTCGTTACAGTC 3′, and anti-sense primer 5′ CGAGCCTTGGAATTCCCATCACGTACTCCACCTA 3′) according to the same protocol, with the exception that the annealing was performed at 55 °C. PCR products were cloned into the *Eco*RI site of the pUC19 plasmid vector. The nucleotide sequences of more than 10 independent cDNA clones isolated from the specimen were determined by the dideoxynucleotide chain termination method.

Results

HLA-B*3501 binding HCV peptides

The HLA-B*3501 binding self-peptide motif has two anchor residues, at position 2 and at the C terminus (Falk *et al*., 1993, 1994; Schönbach *et al*., 1995, 1996; Shiga *et al*., 1996). Pro is the dominant residue at position 2 while five different amino acids (Tyr, Met, Phe, Leu and Ile) prevail at position 9. Tyr is also seen at position 10, suggesting that peptides longer than 9-mers containing Pro at position 2 and Tyr at the C terminus bind to HLA-B*3501 molecules. Therefore, we have identified 9-mers containing Pro at position 2 and Met, Phe, Leu and Ile at position 9 as well as 8- to 11-mers containing Pro at position 2 and Tyr at positions 8, 9, 10 and 11 from all the amino acid sequences of the JT strain of HCV. A total of 53 matched sequences were selected for peptide synthesis. The chemically synthesized peptides were tested using an HLA-B*3501 stabilization assay.

RMA-S cells which carry non-functional transporter associated with antigen processing (TAP) express very low levels of MHC class I molecules when they are cultured at 37 °C. On the other hand, they express high levels of empty MHC class I molecules when they are cultured at lower than 30 °C. The RMA-S-B*3501 cells can continue to express high levels of HLA-B*3501 at 37 °C after the cells cultured at 26 °C were pulsed with HLA-B*3501 binding peptides. The expression level of HLA-B*3501 is dependent on peptide affinity (Takamiya *et al*., 1994). Therefore, peptide affinity can be assessed by measuring the surface expression of HLA-B*3501 on RMA-S-B*3501 cells pulsed with this peptide as described previously (Takamiya *et al*., 1994).

The binding of 53 peptides to HLA-B*3501 molecules was tested at the concentrations of 10^{-4}–10^{-8} M using RMA-S-B*3501 cells. Of the 53 peptides, 28 (52·8%) were evaluated as HLA-B*3501 binding peptides according to the criterion described in Methods (Table 1). The peptides which bound could be classified into three categories according to their binding affinity (BL_{50}): $< 10^{-5}$, high affinity peptide; 10^{-5}–10^{-4}, medium affinity peptide; and $\geqslant 10^{-4}$, low affinity peptide. Of the 28 HLA-B*3501 binding peptides, eight, nine and 11 peptides were high, medium and low affinity peptides, respectively. In addition to 8-mer and 9-mer peptides, two 11-mers (HCV-B35-22 and HCV-B35-23) and five 10-mers (HCV-B35-14, HCV-B35-15, HCV-B35-17, HCV-B35-18 and HCV-B35-19) bound to HLA-B*3501 molecules.

Induction of HCV peptide-specific CTL by HLA-B*3501 binding HCV peptides in a patient with acute hepatitis C

PBL of a patient possessing HLA-A24/A26, B35/B62 and Cw4/Cw9 with acute hepatitis C were stimulated with 28 HLA-B*3501 binding HCV peptides on the eighth day after the onset of the disease. The culture cells were re-stimulated with PHA-induced auto-PBL pulsed with the peptide weekly. After the fourth stimulation, the specific CTL activity of the culture cells was tested against T2 cells and T2-B*3501 cells pulsed with the corresponding peptide. Out of 28 HLA-B*3501 binding HCV peptides, six induced the specific CTL activity (Table 1). Four out of the eight high affinity peptides induced the specific CTL, while one of nine medium affinity peptides and one of the eleven low affinity peptides induced

Table 1. Induction of CTL activity by HLA-B*3501 binding HCV peptides in PBMC of an HLA-B35 positive patient with acute hepatitis C

Peptides	Sequence	Position	Region	BL_{50} (M)	Relative CTL activity*
HCV-B35-57	TPIPAASQL	2956–2964	NS5	$1{\cdot}5 \times 10^{-6}$	−3·1
HCV-B35-38	HPNIEEVAL	1359–1367	NS3	$1{\cdot}6 \times 10^{-6}$	**80·0**
HCV-B35-46	LPKLPGVPF	2000–2008	NS4	$1{\cdot}7 \times 10^{-6}$	**58·9**
HCV-B35-23	TPCTCGSSDLY	1121–1131	NS3	$2{\cdot}9 \times 10^{-6}$	0·4
HCV-B35-47	EPEPDVAVL	2163–2171	NS5	$3{\cdot}1 \times 10^{-6}$	**80·0**
HCV-B35-19	LPQAVMGSSY	2601–2610	NS5	$3{\cdot}9 \times 10^{-6}$	−0·9
HCV-B35-12	VPGAAYALY	783–791	NS2	$8{\cdot}3 \times 10^{-6}$	2·6
HCV-B35-22	VPASQVCGPVY	497–507	E2/NS1	$9{\cdot}5 \times 10^{-6}$	**46·2**
HCV-B35-33	YPCTVNFTI	618–626	E2/NS1	$1{\cdot}0 \times 10^{-5}$	−2·5
HCV-B35-56	EPLDLPQII	2874–2882	NS5	$1.\ 2 \times 10^{-5}$	−3·6
HCV-B35-13	LPIWARPDY	2285–2293	NS5	$1{\cdot}5 \times 10^{-5}$	−0·8
HCV-B35-44	SPLTTQNTL	1799–1807	NS4	$1{\cdot}7 \times 10^{-5}$	6·0
HCV-B35-15	TPRCIVDYPY	604–613	E2/NS1	$1{\cdot}8 \times 10^{-5}$	1·7
HCV-B35-17	VPAAYAAQGY	1240–1249	NS3	$3{\cdot}0 \times 10^{-5}$	0·6
HCV-B35-41	TPAETTVRL	1537–1539	NS3	$3{\cdot}4 \times 10^{-5}$	**78·0**
HCV-B35-58	IPAASQLDL	2958–2966	NS5	$3{\cdot}7 \times 10^{-5}$	−1·2
HCV-B35-37	SPPAVPQTF	1215–1223	NS3	$5{\cdot}7 \times 10^{-5}$	2·4
HCV-B35-2	YPYRLWHY	611–618	E2/NS1	$> 10^{-4}$	−1·1
HCV-B35-30	LPGCSFSIF	1069–1077	NS3	$> 10^{-4}$	**27·0**
HCV-B35-18	VPFFSCQRGY	2006–2015	NS4/NS5	$> 10^{-4}$	−0·2
HCV-B35-28	WPLYGNEGL	83–91	C	$> 10^{-4}$	−5·0
HCV-B35-50	LPPTTGPPI	2316–2324	NS5	$> 10^{-4}$	2·0
HCV-B35-34	LPALSTGLI	682–690	E2/NS1	$> 10^{-4}$	−6·4
HCV-B35-42	APPPSWDQM	1599–1607	NS3	$> 10^{-4}$	0
HCV-B35-39	IPTSGDVVI	1426–1434	NS3	$> 10^{-4}$	1·0
HCV-B35-36	CPSGHAVGI	1171–1179	NS3	$> 10^{-4}$	−0·1
HCV-B35-14	EPRDLDQRPY	476–485	E2/NS1	$> 10^{-4}$	2·8
HCV-B35-1	TPRCIVDY	604–611	E2/NS1	$> 10^{-4}$	−4·6

* % specific lysis of T2-B*3501 cells loaded with 1 μM peptides minus % specific lysis of T2-B*3501 cells loaded without peptide. The specific lysis was tested at an E:T ratio of 40:1. Bold shows positive response (more than 20% relative CTL activity).

the specific CTL. These peptides failed to induce the specific CTL in PBL from two HLA-B35 positive healthy individuals (data not shown).

Identification of HCV CTL epitopes presented by HLA-B*3501 molecules

In order to confirm the CTL specificity for six peptides, we attempted to generate CTL clones specific for these peptides. Seventeen CTL clones specific for five peptides (HCV-B35-30, HCV-B35-38, HCV-B35-41, HCV-B35-46 and HCV-B35-47) were generated (Table 2). These CTL clones killed T2-B*3501 cells pulsed with the corresponding peptides in a concentration-dependent fashion (Fig. 1). The CTL clones specific for the HCV-B38-38 and HCV-B38-41 peptides effectively recognized these peptides at 10^{-10} M, while the sensitivity of the CTL clones for the HCV-B35-30, HCV-B35-46 and HCV-B35-47 peptides was 10–100 times lower than that of the CTL clones for HCV-B35-38 and HCV-B35-41 peptides. We examined whether these five peptides were processed and presented to T cells using the recombinant vaccinia viruses which contained the HCV sequences matching these peptides. The CTL clones specific for HCV-B35-38 killed C1R-B*3501 cells infected with WR-HCV-NS3-5 and WR-T7, while they did not kill C1R-B*3501 cells infected with WR-T7 and C1R cells infected with both WR-HCV-NS3-5 and WR-T7 (Fig. 2). In contrast, the CTL clones specific for the other four peptides failed to kill C1R-B*3501 cells infected with WR-HCV-NS3-5 or WR-HCV-ORF together with WR-T7 (data not shown). These results indicate that the HCV-B35-38 peptide is processed and presented to T cells.

Identification of the sequence of HCV-B35-38 CTL epitope in HCV isolates from the patient with acute hepatitis C

The sequence of HCV-B35-38 (HPNIEEVAL) is widely present in different HCV strains (Table 3). However, it remains

Table 2. Generation of CTL clones specific for five HCV peptides

Results shown as % specific lysis. The cytotoxicity of each CTL clone for the target cells was tested at an E:T ratio of 2:1.

CTL clones	Target cells			
	T2-B*3501 (+peptide)*	T2-B*3501 (−peptide)	T2 (+peptide)*	T2 (−peptide)
HCV-B35-30-73	91·6	3·1	8·8	9·0
HCV-B35-30-79	83·3	0·5	1·4	2·5
HCV-B35-30-32	89·2	1·2	20·6	−0·2
HCV-B35-38-20	95·4	0·9	29·2	1·9
HCV-B35-38-22	80·2	1·5	13·3	2·6
HCV-B35-38-82	89·5	1·2	20·6	−0·2
HCV-B35-41-10	92·1	−0·4	−0·5	−0·7
HCV-B35-41-11	83·1	1·3	9·8	1·3
HCV-B35-41-19	84·6	−0·5	0·5	3·9
HCV-B35-41-22	80·6	0·0	−0·2	−0·1
HCV-B35-46-20	77·5	1·3	3·1	4·2
HCV-B35-46-22	89·9	0·6	0·3	0·4
HCV-B35-46-27	80·3	4·3	6·6	7·7
HCV-B35-46-39	89·4	0·7	0·3	−0·2
HCV-B35-47-1	82·3	3·8	3·9	4·0
HCV-B35-47-7	85·7	6·7	7·5	8·8
HCV-B35-47-101	92·9	6·5	10·7	9·3

* T2-B*3501 cells or T2 cells loaded with 1 μM of the peptide were used as target cells.

unknown whether this epitope is found in the virus from the patient with acute hepatitis C. We investigated the sequence of the epitope of the viruses from this patient. The sequences of seven isolates were identical to that of HCV-B35-38, while one isolate had Val at position 8 (data not shown). Thus, since this patient was infected with HCV carrying the HCV-B35-38 epitope, it is likely that this epitope was presented to T cells in this patient.

Strong CTL activity specific for the HCV-B35-38 epitope in the acute phase PBL of the patient with acute hepatitis C

CTL specific for the HCV-B35-38 epitope was detected in PBL of the patient after the fourth stimulation with the peptide (Table 1). There remained the possibility that, in spite of the weak epitope, the specific CTL were expanded from a small number of precursor T cells after four stimulations with peptides. To clarify whether this epitope was effectively recognized by T cells in the patient, we examined the CTL activity in the PBL of the patient after one stimulation with the peptide. The specific CTL were detected in the PBL during the acute phase, whereas they were not observed in the PBL during the recovery phase (Fig. 3). On the other hand, the HCV-B35-38 peptide failed to induce specific CTL in the PBL of two HCV-seronegative normal individuals after the fourth stimulation (data not shown). Together these results suggest that the CTL specific for this epitope can be induced after HCV infection and that they may play an important role in the elimination of HCV from the patient.

Failure of induction of the specific CTL in PBL of HLA-B35 positive patients with chronic hepatitis C

We investigated whether the HCV-B35-38 peptide could induce the specific CTL in PBL of HLA-B35 positive patients with chronic hepatitis C. PBL from seven HLA-B35 positive patients with chronic hepatitis C were stimulated weekly with the HCV-B35-38 peptide for 4 weeks and then CTL specific activity for this peptide was examined. Specific CTL activity was not found in PBL stimulated with the peptide from seven patients with chronic hepatitis C (data not shown). Moreover, 27 other HLA-B*3501 binding peptides failed to induce the specific CTL in PBL from two patients with chronic hepatitis (data not shown). Previous studies of HBV-specific CTL showed that there is only a small number of precursor CTL specific for the virus in PBL of patients with chronic hepatitis (Nayersina *et al.*, 1993; Penna *et al.*, 1991). Therefore it is likely that a small number of precursor CTL specific for this epitope were in PBL of patients with chronic hepatitis C. However, it still remains possible that this epitope is neither presented nor recognized in patients with chronic hepatitis due to mutation in it.

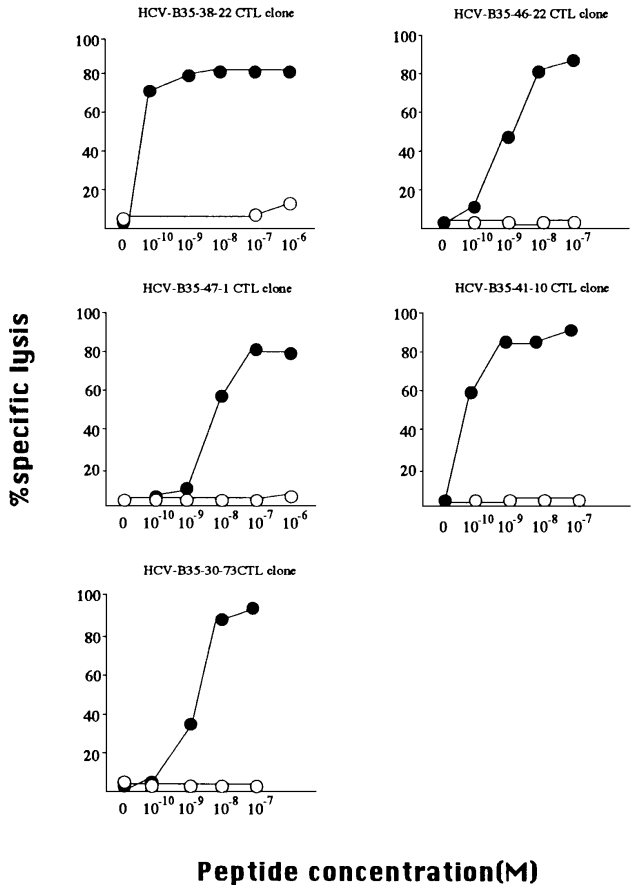

Fig. 1. Recognition of HLA-B35-restricted HCV-specific CTL clones. The cytotoxicity of T2 cells (○) and T2-B*3501 cells (●) loaded with various concentration of the peptide by the CTL clones specific for the HCV peptide were tested at an effector:target (E:T) ratio of 2:1.

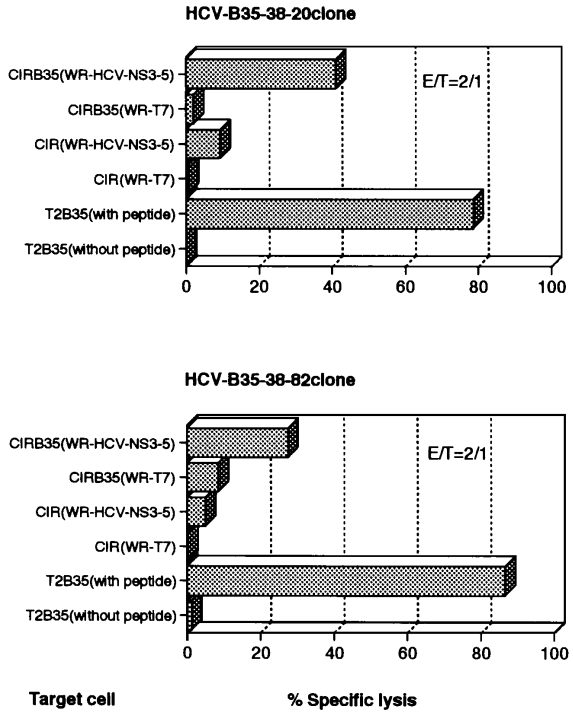

Fig. 2. Killing of target cells infected with vaccinia recombinant virus by CTL clones specific for the HCV-B35-38 CTL peptide. The cytotoxicity of target cells infected with both the vaccinia recombinant virus containing NS3–5 of HCV (WR-HCV-NS3-5) and T7 RNA polymerase (WR-T7) by HCV-B35-38 clones was examined at an E:T ratio of 2:1. T2-B*3501 cells pulsed with 1 μM HCV-B35-38 peptide or without the peptide were used as positive and negative controls.

Recognition of HCV-B35-38 variant epitopes by the CTL clone specific for HCV-B35-38

HCV-B35-38 derived from the JT strain is the consensus among HCV isolates, while several mutations are found in some strains (Table 3). Mutations in the HCV-B35-38 epitopes were observed in nine out of 18 isolates. Interestingly, seven viruses showed three mutations (Ala, Leu or Ser for Pro) at position 2. Since Leu at position 2 is not an anchor residue for HLA-B*3501 molecules (Falk *et al.*, 1993), it is thought that this mutant epitope does not bind to HLA-B*3501 molecules. In contrast, it is known that Ala and Ser at position 2 are weak anchor residues for HLA-B*3501 molecules (Falk *et al.*, 1993; Schönbach *et al.*, 1996). Therefore, we generated four mutant peptides (HANIEEVAL, HSNIEEVAL, HPNIEEIAL and HPNIEEIGL) to investigate the recognition of these mutant epitopes by the CTL clones specific for the HCV-B35-38 epitope.

The mutation of Ile for Val at position 7 did not affect the recognition of the HCV-B35-38-82 CTL clone, while other mutations reduced the recognition of the CTL clone (Fig. 4). Approximately 5–10 times higher concentrations of the

Table 3. Mutation of an HCV-B35-38 epitope in sequenced HCV strains

Genotype	HCV strain	HCV-B35-38
I/1a	HCV-1	HPNIEEVAL
	HCV-H	*********
	HCV-J1	*A*******
II/1b	HCV-J	*********
	HCV-BK	*********
	HCV-T	******I**
	HCV-J4/91	******IG*
	HCV-JK1	*********
	HCV-JT	*********
	HCV-N	*********
	HCV-L1	*L*******
	HCV-L2	*********
	HC-C2	*S*******
	HC-G9	*S*******
III/2a	HC-J6	*********
IV/2b	HC-J8	*S*******
V/3a	NZL1	*S*******
	NCV-K3a/650	*S*******

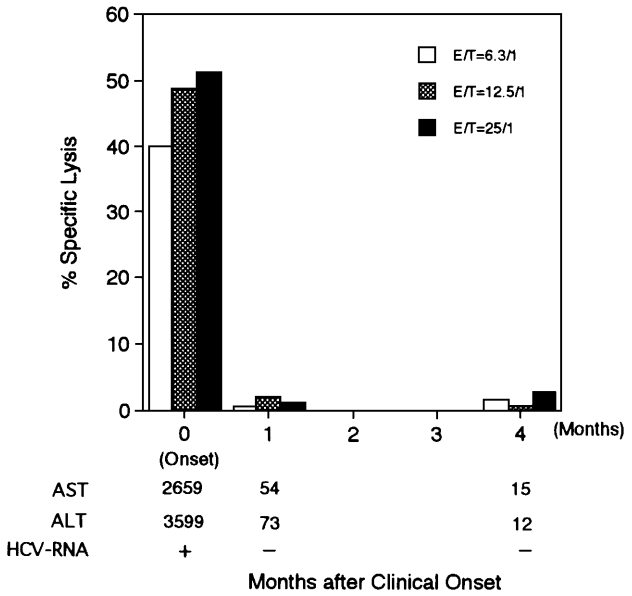

Fig. 3. CTL activity for HCV-B35-38 epitope in the PBL of a patient during the clinical course of acute hepatitis C. CTL activity for HCV-B35-38 epitope in the PBL of the patient with acute hepatitis C was examined during the clinical course. PBL on the eighth day, the first month and the third month after the onset of disease were stimulated once with HCV-B35-38 peptide. After 7 days in culture the CTL activity was tested using Epstein–Barr virus-transformed B cell line PT carrying HLA-B35 (Yamamoto *et al.*, 1990) as target cells pulsed with the peptides at various E:T ratios.

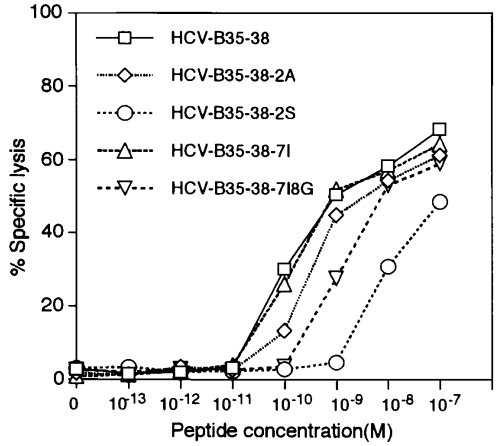

Fig. 4. Recognition of HCV-B35-38 mutant epitopes by the CTL clones specific for the HCV-B35-38 epitope. Recognition of the HCV-B35-38-specific CTL clone for the mutant epitopes was examined using T2-B*3501 target cells pulsed with the peptides at an E:T ratio of 2:1.

mutant peptides HANIEEVAL and HPNIEEIGL were required to provide the same killing activity as the CTL clone for the target cells pulsed with HCV-B35-38 peptide. Binding of these mutant peptides to HLA-B*3501 molecules was approximately three- to fivefold lower than that of HCV-B35-38 peptides (Fig. 5). Together these results showed that reduced recognition of the CTL clones mostly results from diminished affinity of these mutant peptides to HLA-B*3501 molecules. Similarly, the killing activity of the CTL clone for target cells pulsed with HSNIEEVAL was 100 times lower than that for target cells pulsed with HCV-B35-38 peptide (Fig. 4). This can also be explained by the reduction of the peptide affinity to HLA-B*3501 molecules (Fig. 5).

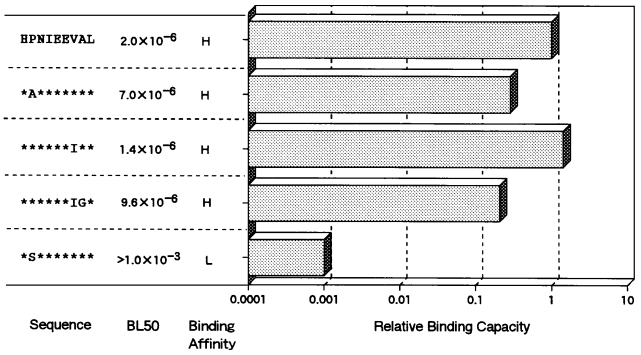

Fig. 5. Binding of HCV-B35-38 mutant peptides to HLA-B*3501 molecules. Binding of HCV-B35-38 mutant peptides was tested using an HLA-B*3501 stabilization assay. The affinity of these peptides was compared to that of the HCV-B35-38 peptide.

Discussion

The strategy using an MHC class I binding peptide motif, called reverse immunogenetics, was introduced for the first time by Pamer *et al.* (1991), who demonstrated one CTL epitope of *Listeria monocytogenes* presented by H-2K^b molecules from 11 candidate peptides. Hill *et al.* (1992) extended this approach to identify three CTL epitopes of *Plasmodium falciparum* in humans. Furthermore, several studies have employed this strategy to identify HBV epitopes (Nayersina *et al.*, 1993), HCV epitopes (Cerny *et al.*, 1995), melanoma antigen epitopes (Battegay *et al.*, 1995) presented by HLA-A2, and HIV-1 epitopes presented by HLA-B35 (Shiga *et al.*, 1996). These studies have shown that HLA class I-restricted CTL epitopes are effectively identified by this strategy. In the present study, we employed this technique to identify HCV CTL epitopes presented by HLA-B*3501 molecules. Of the 53 HCV peptides, 28 (52·8%) were evaluated as HLA-B*3501 binding peptides. The percentage of HLA-B*3501 binding HCV peptides is almost the same as that in the analysis of 320 8–11-mer peptides possessing HLA-B*3501 anchor residues (53·1%) (Schönbach *et al.*, 1995, 1996). Moreover, the number which induced the peptide-specific CTL (six out of the 28 HLA-B*3501 binding HCV peptides) is comparable to that of HIV-1 peptide-induced specific CTL (nine and four out of the 27 HLA-B*3501 binding HIV-1 peptides in two HIV-1 infected individuals) (Shiga *et al.*, 1996). However, only one HCV peptide (HCV-B35-38) was confirmed as an HCV CTL epitope, while four peptides were excluded. This is in contrast to previous studies where ten out of 11 HIV-1 peptides which induced HLA-B*3501-restricted, HIV-1 peptide-specific CTL were confirmed as HIV-1 CTL epitopes (Shiga *et al.*, 1996) and

four out of nine HCV peptides which induced HLA-A2-restricted CTL were demonstrated as HCV CTL epitopes in chronic active hepatitis (Cerny *et al.*, 1995). This may be explained as follows. In the present study, we used the PBL from a patient with acute hepatitis C. Presentation of a small amount of HCV peptides in the HCV infected hepatocytes might induce the specific CTL in acute hepatitis, although the experiment using the specific CTL clones and the recombinant vaccinia virus failed to detect the presentation of these peptides by HLA-B35. Another possibility is that the sequences corresponding to these peptides and their flanking regions in the HCV of the patient might be different from those in the recombinant vaccinia virus. For example, mutation in the flanking region of HCV sequences used in the recombinant vaccinia may prevent the processing of the epitopes (Eisenlohr *et al.*, 1992). Furthermore, we can speculate that a very restricted number of CTL epitopes might be recognized by CTL in the acute phase of acute hepatitis C. Indeed, a recent study has shown that only the CTL response in HIV-1 primary infection is focused on the one epitope (Borrow *et al.*, 1997). Further analysis of these peptides will be required to clarify the antigen processing of HCV to T cells.

So far, 28 HCV CTL epitopes presented by HLA class I molecules have been reported. These epitopes were presented by 13 HLA class I molecules; 13 by HLA-A2 (Cerny *et al.*, 1995; Battegay *et al.*, 1995; Kozier *et al.*, 1995; Rehermann *et al.*, 1996) and three by -A11 (Koziel *et al.*, 1993, 1995; Wong *et al.*, 1996), two by HLA-A3 (Koziel *et al.*, 1995), with one each by HLA-A29, -B35 (Koziel *et al.*, 1992), -A23, -B7, -B8, -B53 (Koziel *et al.*, 1995), -B50, -B51 (Koziel *et al.*, 1993), -B44 (Kita *et al.*, 1993) and -B60 (Kaneko *et al.*, 1996). All these CTL epitopes were identified using PBL and liver-infiltrating lymphocytes from patients with chronic hepatitis. In the present study, we selected a patient carrying HLA-B35 with acute hepatitis C to induce the specific CTL by HLA-B*3501 binding peptides because the precursor number of HBV-specific CTL in PBL of patients with acute hepatitis B is more than that of patients with chronic hepatitis B (Nayersina *et al.*, 1993; Penna *et al.*, 1991). The identified epitope induced strong specific CTL in the acute phase of acute hepatitis C while it failed to induce the specific CTL in seven patients with chronic hepatitis C. These findings imply that this epitope may be dominantly presented to CTL in the acute hepatitis C. The HCV-specific CTL response may be able to exert some degree of control over virus load in chronically infected patients (Rehermann *et al.*, 1996). A strong CTL response specific for the HCV-B35-38 epitope was observed in the acute phase PBL of the patient with acute hepatitis C, while it was not detected 1 month after the onset of the disease. This suggests that HCV-specific CTL disappeared as soon as they eliminated the virus from the body. The role of this epitope and the identification of other epitopes in chronic hepatitis C is now under investigation using infiltrating T cells in the liver of patients with chronic hepatitis C.

HCV isolates from the patient investigated in the present study carried a sequence identical to HCV-B35-38, indicating that this epitope is presented by HLA-B35 in this patient. Since the sequence of HCV-B35-38 is the consensus in HCV strains, it is thought that this epitope is recognized by CTL in most HCV infected individuals carrying HLA-B35. One mutant carries Leu at position 2, which is not an anchor residue for HLA-B*3501 molecules, suggesting that this mutation critically affects the binding of the peptide to HLA-B*3501 molecules. Two mutations (Ser and Ala for Pro) at position 2 affected the binding of peptides to HLA-B*3501 molecules and reduced the recognition of T cells. Since the mutation of Ser for Pro at position 2 markedly reduced the recognition of the CTL clone specific for the HCV-B35-38 epitope, it is assumed that the CTL fails to kill target cells infected with the virus carrying this mutant. Reduction of CTL recognition for the mutant carrying Ser at position 2 results from very low binding of this mutant to HLA-B*3501 molecules. This implies that HLA-B*3501-restricted CTL specific for this mutant are not induced in patients who are infected with HCV carrying this mutant epitope.

In the present study, we identified the CTL epitope in acute hepatitis. The mechanisms of virus clearance in acute hepatitis C and the onset of chronic hepatitis C are still unknown. HCV-specific CTL are strongly suggested to be involved in these mechanisms, and analysis of HCV CTL epitopes will contribute to the understanding of these mechanisms in both acute and chronic hepatitis C.

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