Visual Demonstration of Hepatitis C Virus–Specific Memory CD8$^+$ T-Cell Expansion in Patients With Acute Hepatitis C

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Hepatitis C virus (HCV)-specific CD8$^+$ T cells in peripheral blood mononuclear cells (PBMCs) from patients infected with HCV were quantitatively analyzed by flow cytometry using an HLA-B*3501-HCV epitope tetrameric complex. In chronic hepatitis C, tetramer$^+$CD8$^+$ T cells were detected at frequencies ranging from 0.05% to 0.12% of total CD8$^+$ T cells. The number of tetramer$^+$CD8$^+$ T cells in acute phase PBMCs from patients with acute hepatitis C was about 3 to 5 times higher than in recovery phase PBMCs from the same patients and in PBMCs from patients with chronic hepatitis C. Expanding tetramer$^+$CD8$^+$ T cells in PBMCs from patients with acute hepatitis C express a CD28$^+$CD45RA$^-$ memory T-cell phenotype. In contrast, tetramer$^+$CD8$^+$ T cells in PBMCs from patients with chronic hepatitis C did not predominantly express this phenotype. These tetramer$^+$CD8$^+$ T cells did not have perforin in their cytoplasma. The present study visually showed that a high number of circulating HCV-specific CD8$^+$ T cells in acute phase PBMCs from patients with acute hepatitis C are mostly memory T cells. (Hepatology 2001;33:287-294.)

It is believed that cellular immune responses involving cytotoxic T lymphocytes (CTLs), helper T lymphocytes, and natural killer cells are involved in both the pathogenesis of viral hepatitis and clearance of the virus.[1,2] Hepatitis C virus (HCV)-specific CTLs are found in peripheral blood and liver tissue from individuals infected with hepatitis C.[3-11] Several studies suggest that vigorous CTL responses to HCV are essential for viral clearance. For example, patients with acute hepatitis C who can spontaneously clear HCV produced a stronger HCV-specific CTL response than patients with chronic hepatitis C.[5] In addition, several studies have shown an inverse correlation between HCV-specific CTL response of peripheral blood lymphocytes and HCV viral titer.[12,13] Between 50% and 90% of individuals with acute hepatitis C develop chronic hepatitis.[14-17] The mechanism by which acute hepatitis shifts to chronic hepatitis is unknown, but HCV-specific CTLs are also implicated in the pathogenesis of chronic hepatitis. For example, the lymphocytes that infiltrate into hepatic lobules are mainly CD8$^+$ T cells, and HCV-specific CTL clones can be established from these liver-infiltrating lymphocytes.[2,8-9]

Limiting dilution assays showed that the number of HCV-specific CTLs in peripheral blood mononuclear cells (PBMCs) from patients with chronic hepatitis C is low.[18] Therefore, quantitative analysis of HCV-specific CTL is difficult in these patients. In fact, *in vitro* expansion by peptide stimulation for several weeks is required to induce HCV-specific CTLs from PBMCs of such patients.[3-7,11,13] However, new methodology using MHC-peptide tetrameric complexes has improved the detection of antigen-specific T cells[19] and has enabled direct detection of HCV-specific CD8$^+$ T cells in PBMCs from patients with chronic hepatitis C.[20]

Because HCV-specific CTLs play an important role in both acute and chronic hepatitis C, precise analysis of HCV-specific CTLs is required to clarify the role of these CTLs in viral clearance and in the immunopathogenesis of chronic hepatitis C. In a previous study we showed that stimulation with HLA-B*3501-restricted epitope HCV NS3 1359-1367 (HPNIEEVAL) induced strong peptide-specific CTL activity in PBMCs from a patient with acute hepatitis C but failed to induce specific CTLs in PBMCs from patients with chronic hepatitis C.[5] This result implies that HCV-specific CTLs play an important role in viral clearance in patients with acute hepatitis C. However, approaches requiring *in vitro* T-cell expansion may underestimate CTL frequency.

In the present study, we quantitatively analyzed HCV-specific CTLs in PBMCs from patients with acute and chronic hepatitis C using HLA-B*3501- HCV NS3 1359-1367 epitope tetramers. Expanded HCV-specific T cells were further analyzed by four-color flow cytometric analysis to clarify their immunologic phenotype. This is a study that visually shows expanding HCV-specific CD8$^+$ T cells in patients with acute hepatitis C and analyzes their immunologic phenotype in detail.

PATIENTS AND METHODS

Patients. Two HLA-B35-positive patients with acute hepatitis C and eight HLA-B35-positive patients with chronic hepatitis C were investigated. Blood samples were obtained from Yokohama City University Hospital, Fujisawa City Hospital and Tokyo University Hospital. All blood samples were collected with oral informed consent. Research protocols were approved by the institutional review boards at Yokohama City University Hospital, Fujisawa City Hospital, and

Abbreviations: CTL, cytotoxic T lymphocyte; HCV, hepatitis C virus; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; PBS, phosphate-buffered saline; FCS, fetal calf serum; HIV, human immunodeficiency virus; EBV, Epstein-Barr virus.

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Tokyo University Hospital. Acute HCV infection was diagnosed by high levels of serum transaminase, presence of HCV RNA in the serum, histologic examination of liver tissue, seroconversion from negative to positive for an HCV-specific antibody as assessed by enzyme immunoassay, and the recent onset of jaundice and other symptoms typical of acute hepatitis. Patients with acute hepatitis C completely recovered from the illness with clearance of HCV RNA from the serum for 6 months. All patients with chronic HCV infection displayed elevated serum transaminase and seropositivity for an HCV-specific antibody and HCV RNA.

Control blood samples were obtained from 7 HCV seronegative healthy individuals with HLA-B35.

Peptides. The peptide HCV-NS3 1359-1367 was synthesized using an automated peptide synthesizer (model PSSM-8, Shimazu Co., Kyoto, Japan), with F-moc strategy followed by cleavage. Peptide purity was determined by mass spectrometry and high pressure liquid chromatography.

Production of HLA-Class I Peptide Tetrameric Complexes. HLA-B*3501-HCV peptide tetrameric complexes were synthesized as previously described.[19] Briefly, recombinant HLA-B*3501 and human β_2 microglobulin (β_2m) were purified from *Escherichia coli* cells transformed with the relevant expression plasmids. The heavy chain was modified by deletion of the transmembrane cytosolic tail and COOH-terminal addition of a sequence containing the BirA enzymatic biotinylation site. HLA-B*3501 complexes were folded *in vitro* with the HLA-B*3501-restricted HCV epitope peptide, HCV-NS3 1359-1367. The resulting 45-kd complexes were purified by gel filtration using a superdex G75 column (Amersham Pharmacia Biotech, Uppsala, Sweden). Purified complexes were biotinylated enzymatically with BirA enzyme (Avidity, Denver, CO). The biotinylated complexes were purified by gel filtration using a Superdex G75 column followed by further purification using a Mono Q column (Amersham Pharmacia Biotech). HLA-B*3501-peptide tetrameric complexes (HLA-B*3501 tetramers) were generated by mixing the monomer complexes with phycoerythrin-labeled ExtrAvidin (Pharmingen, San Diego, CA) or APC-labeled Streptavidin (Sigma Chemical Co., Irvine, CA) at a molar ratio of 4:1.

Monoclonal Antibodies. Anti-CD8-PerCp and anti-CD8-APC monoclonal antibodies (mAbs) were obtained from Becton Dickinson (San Jose, CA) and Immunotech (Marseilles, France), respectively. Anti-Perforin, anti-CD28-FITC and anti-CD45RA-Cy-chrome mAbs were obtained from Pharmingen International.

CTL Clones. CTL clones specific for the HCV-NS3 1359-1367 peptide were generated previously.[5] The clones were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 100 U/mL human recombinant interleukin 2 and were stimulated weekly with peptide-pulsed C1R-B*3501 cells.

Flow Cytometric Analysis. A total of 0.5 to 1.0 $\times$ 10^6 cryopreserved PBMCs were mixed with 3 μL HLA-B*3501 tetramers at a concentration of 0.11 ~ 0.45 mg/mL and incubated for 30 minutes at 4°C. mAbs were then added and the mixture was incubated for 20 minutes at 4°C. Cells were washed 3 times with phosphate-buffered saline (PBS) supplemented with 10% FCS, and then resuspended in PBS containing 2% paraformaldehyde. Samples were analyzed using a FACS Calibur with Cell Quest software (Becton Dickinson).

Intracellular Perforin Staining. After cell surface staining with anti-CD8-PerCp, anti-CD28-FITC, and APC-labeled HLA-B*3501 tetramers as described above, cells were fixed with PBS containing 4% paraformaldehyde at 4°C for 15 minutes. Fixed cells were then permeabilized with PBS supplemented with 0.1% saponin and 10% FCS at 4°C for 10 minutes, followed by intracellular staining with anti-Perforin-PE or PE-conjugated mouse IgG2b control mAb at 4°C for 30 minutes. Cells were washed 3 times and then resuspended in PBS containing 2% paraformaldehyde.

In Vitro *Peptide Stimulation of PBMCs From Patients With Acute Hepatitis C.* A total of 10^6 acute phase PBMCs from patients with acute hepatitis C were cultured in RPMI 1640 medium supplemented with 10% FCS and 100 U/mL human recombinant interleukin 2 after stimulation with 1 μmol/L HCV-NS3 1359-1367 peptide. The phenotype of the cultured cells was analyzed at day 7, day 11, and day 15 by flow cytometry.

RESULTS

*Identification of HCV-Specific CTLs in PBMCs From Patients With Acute and Chronic Hepatitis C Using an HLA-B*3501-HCV Peptide Tetrameric Complex.* We previously showed strong CTL activity for an HLA-B*3501-restricted HCV epitope, HCV-NS3 1359-1367, in PBMCs from a patient with acute hepatitis C but not in patients with chronic hepatitis C.[5] This suggested that PBMCs from patients with acute hepatitis C contains a high number of precursor CTLs specific for HCV. To quantitatively analyze HCV epitope-specific $CD8^+$ T cells in PBMCs from patients with hepatitis C, we synthesized an HLA-B*3501-HCV-NS3 1359-1367 peptide tetrameric complex (HLA-B*3501 tetramer). Specificity of the HLA-B*3501 tetramer was confirmed using specific CTL clones: the tetramer effectively bound to a CTL clone specific for this epitope (Fig. 1A) but not to a $CD8^+$ CTL clone with a different specificity (Fig. 1B).

We tested PBMCs from 7 HCV-seronegative individuals carrying HLA-B35 and acute phase PBMCs from 7 HLA-B35–negative patients with acute hepatitis C to determine nonspecific binding levels of the HLA-B*3501 tetramer. The tetramer bound to 0.02 $\pm$ 0.01% of total $CD8^+$ T cells from 7 HCV-seronegative individuals carrying HLA-B35 and 0.01 $\pm$ 0.01%

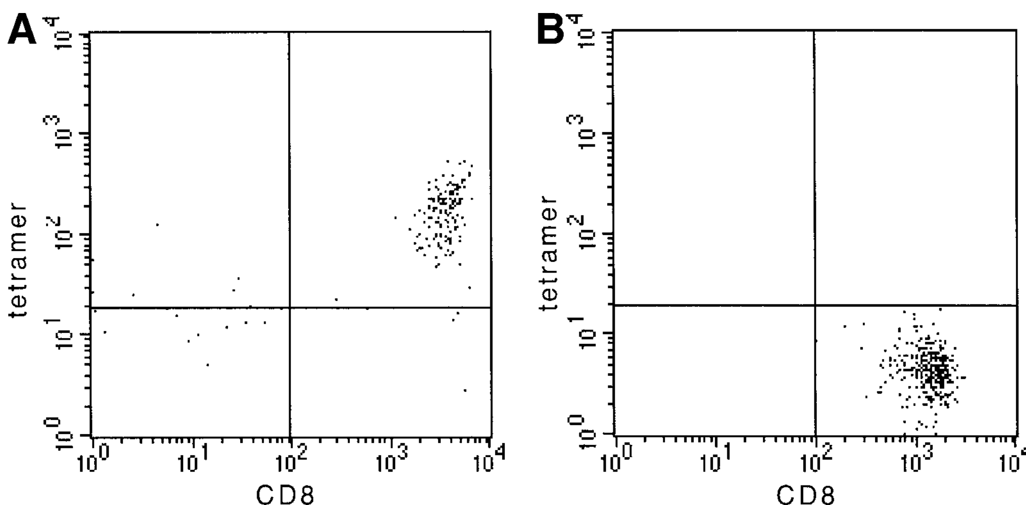

FIG. 1. Specific binding of the HLA-B*3501 tetramer to a CTL clone specific for HCV-NS3 1359-1367. The HCV-NS3 1359-1367–specific CTL clone, HCV-B35-38-20 (A), and the HIV-1 Env-specific CTL clone, SF2-33-115 (B), were stained with an anti-CD8 mAb and the HLA-B*3501 tetramer and then analyzed by flow cytometry.

TABLE 1. Percentage of HLA-B*3501 Tetramer Binding $CD8^+$ T Cells in PBMCs From Patients With Acute and Chronic Hepatitis

Patients	ALT (U/mL)	Histology	$Tetramer^+CD8^+$ Cells
Acute hepatitis			
A1	1,201	Acute hepatitis	**0.30***
A2	732	Acute hepatitis	**0.76**
Chronic hepatitis			
C1	524	Chronic active hepatitis	**0.07**
C2	311	Chronic active hepatitis	**0.11**
C3	61	Cirrhosis	**0.12**
C4	87	Chronic active hepatitis	**0.06**
C5	53	Chronic active hepatitis	**0.11**
C6	143	Chronic active hepatitis	**0.05**
C7	79	Chronic active hepatitis	**0.05**
C8	47	Chronic persistent hepatitis	**0.11**
Acute hepatitis C without HLA-B35 (n = 7)			0.01 ± 0.01
HCV seronegative individuals with HLA-B35 (n = 7)			0.02 ± 0.01

NOTE. Boldface indicates positive binding.
* Percentage of tetramer-positive cells in $CD8^+$ T cells.

of total $CD8^+$ T cells from 7 HLA-B35–negative patients with acute hepatitis C (Table 1). Therefore, we evaluated less than 0.04% of total $CD8^+$ T cells as having nonspecific binding in our assay. Thus, although it has been shown that T-cell staining with tetramers at 4°C increased nonspecific binding,[21] this tetramer showed low level of nonspecific binding. We used the HLA-B*3501 tetramer to detect HCV-specific $CD8^+$ T cells in PBMCs from HLA-B35–positive individuals with hepatitis C. PBMCs from 2 HLA-B35–positive patients infected with acute hepatitis C (A1 and A2) at 1 week after clinical onset were stained with the HLA-B*3501 tetramer and anti-CD8 mAb, and then were analyzed by flow cytometry. The tetramer and CD8-positive ($tetramer^+CD8^+$) T-cell population could be clearly discriminated from other populations (Fig. 2). Of total $CD8^+$ T cells, the percentage of $tetramer^+CD8^+$ cells was 0.30% and 0.76% in acute phase PBMCs from the patients A1 and A2, respectively, whereas in 8 patients with chronic hepatitis C the percentage of $tetramer^+CD8^+$ cells ranged between 0.05 and 0.12% (Table 1). Thus, although HCV-specific $CD8^+$ T cells were detected in all patients with acute and chronic hepatitis C, the number of HCV-specific $CD8^+$ T cells was much lower in PBMCs from patients with chronic hepatitis C compared with those in the acute phase of acute hepatitis C.

Quantitative Analysis of HCV-Specific $CD8^+$ T Cells Through the Clinical Course of Patients With Acute Hepatitis C. Our previous study showed that *in vitro* stimulation with the HCV-NS3 1359-1367 peptide induced strong peptide-specific CTL activity in acute phase PBMCs (1 week after clinical onset of acute hepatitis C) but not in recovery phase PBMCs from the same patient.[5] This finding suggested that acute phase PBMCs from patients with acute hepatitis C contain a high number of memory and/or effector CTLs specific for HCV and that the number of these memory and/or effector CTLs markedly decreased in recovery phase PBMCs. We quantitatively analyzed the number of HCV-NS3 1359-1367-specific CTLs in PBMCs from 2 patients with acute hepatitis C by the HLA-B*3501 tetramer (Fig. 3). In patient A1, the percentage of $tetramer^+CD8^+$ T cells decreased from 0.30% (acute phase) to 0.06% (18 weeks after clinical onset). Similarly, in patient A2 the percentage of $tetramer^+CD8^+$ T cells decreased from 0.76% (acute phase) to 0.11% (18 weeks after clinical onset). These results are consistent with our previous results of CTL activity in PBMCs from patient A1.[5]

Expansion of Memory $CD8^+$ T Cells in Acute Phase PBMCs of Acute Hepatitis C Patients. Naive, memory, and effector $CD8^+$ T cells can be distinguished by analysis of the surface expression of CD28 and CD45RA molecules: naive cells are $CD28^+CD45RA^+$, memory cells are $CD28^+CD45RA^-$ cells, while effector cells are $CD28^-CD45RA^+$ and $CD28^-CD45RA^-$.[22] This identification was confirmed by 4-color flow cytometric analysis of PBMCs from HCV-seronegative individuals (Fig. 4). This analysis showed that as expected, $CD28^+CD45RA^+CD8^+$ T cells had no perforin and $CD28^+CD45RA^-CD8^+$ T cells had a low level of perforin, whereas $CD28^-CD45RA^+CD8^+$ and $CD28^-CD45RA^-CD8^+$ T cells had a high level of perforin in their cytoplasm. We next examined the surface expression of CD28 and CD45RA on HCV-specific CTL clones. These CTL clones expressed neither CD28 nor CD45RA (Fig. 5A). HIV-1–specific CTL clones

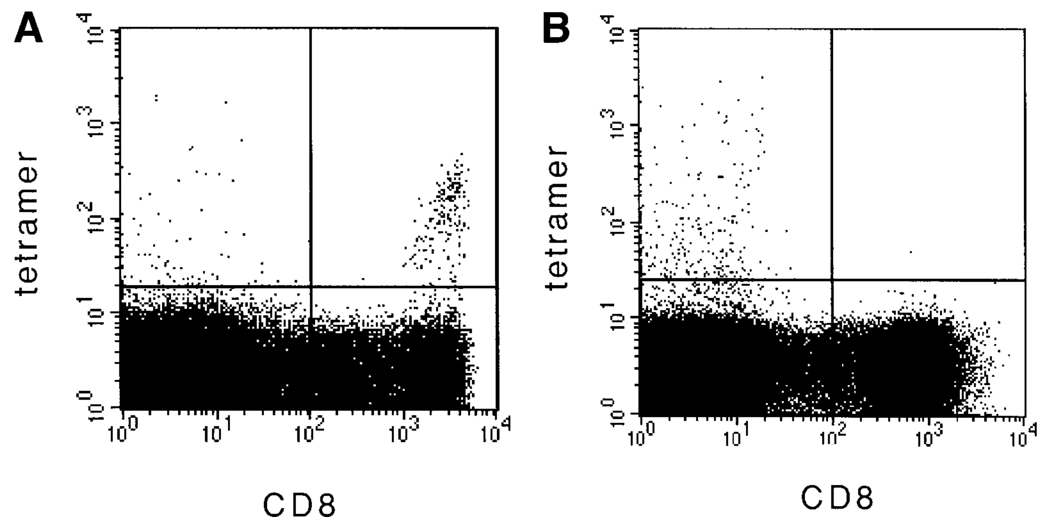

FIG. 2. Detection of HLA-B*3501-restricted, HCV-NS3 1359-1367–specific CTLs in PBMCs from a patient with acute hepatitis C. PBMCs from patient A2 at 1 week after clinical onset of acute hepatitis C (A) and an HCV seronegative individual with HLA-B35 (B) were stained with the HLA-B*3501 tetramer and an anti-CD8 mAb, and were then analyzed by flow cytometry. The tetramer bound to 0.76% of total $CD8^+$ T cells in patient A2.

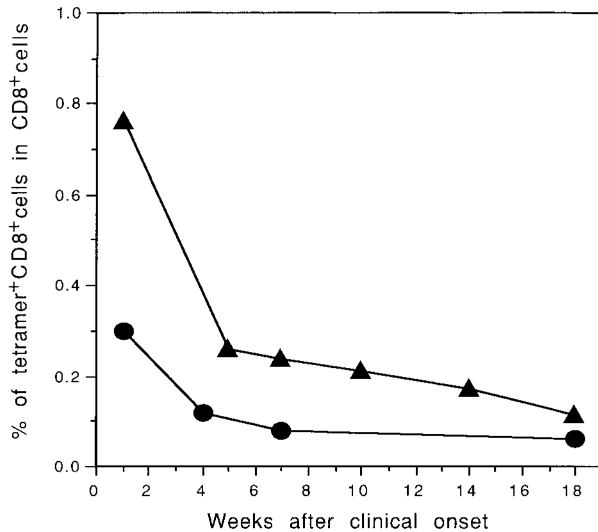

FIG. 3. The percentage of tetramer$^+$CD8$^+$ cells in total CD8$^+$ cells changes through the clinical course of acute hepatitis C. PBMCs from two patients (A1 [●] and A2 [▲]) with acute hepatitis C were taken at various times through the clinical course of the infection (1 week to 18 weeks after clinical onset). The PBMCs were stained with the HLA-B*3501 tetramer and an anti-CD8 mAb and then analyzed by flow cytometry. In patients A1 and A2, serum HCV-RNA changed negative from positive in 5 and 8 weeks after clinical onset, respectively.

also express neither CD28 nor CD45RA (our unpublished observation). Thus it is likely that these CD28$^-$CD45RA$^-$ CD8$^+$ T cells are effector CTLs.

We further examined the CD28CD45RA phenotype of tetramer$^+$CD8$^+$ T cells in acute phase PBMCs from 2 patients with acute hepatitis C. Most tetramer$^+$CD8$^+$ T cells (A1: 85.3%, A2: 75.2%) had a CD28$^+$CD45RA$^-$ memory cell phenotype (Fig. 5B). The tetramer$^+$CD8$^+$ T cells with this phenotype did not have perforin in their cytoplasm (Fig. 6). The predominant expansion of tetramer$^+$CD8$^+$ T cells expressing this phenotype was observed by 7 weeks (A1) and 14 weeks (A2) after clinical onset of hepatitis C (Fig. 7). Thus, HCV-specific memory CD8$^+$ T cells expand in the acute phase of acute hepatitis C.

To investigate the *in vitro* induction of effector CD8$^+$ T cells from HCV-specific memory CD8$^+$ T cells, we stimulated acute phase PBMCs from patient A2 with the HCV-NS3 1359-1367 peptide. Cells were cultured for 7 to 15 days and the CD28CD45RA phenotype of the tetramer$^+$CD8$^+$ T cells was analyzed by flow cytometry. The percentage of cells with a CD28$^+$CD45RA$^-$ memory phenotype decreased whereas that with a CD28$^-$CD45RA$^-$ effector phenotype increased during the culture period (Fig. 8). The expression of perforin was much higher in cultured tetramer$^+$CD8$^+$ T cells than those in PBMCs before culture (Fig. 8). These results suggest that the induced effector T cells are derived from CD28$^+$CD45RA$^-$ memory T cells.

CD28CD45RA phenotype of tetramer$^+$CD8$^+$ T cells in PBMCs from patients with chronic hepatitis C was also examined. There was no significant increase of any particular CD28CD45RA phenotype (Fig. 9). This is in contrast to the predominant expansion of CD28$^+$CD45RA$^-$ phenotype in the acute phase of acute hepatitis C.

DISCUSSION

Recent analyses using MHC-peptide tetrameric complexes directly showed virus-specific CD8$^+$ T cells in PBMCs from patients infected with human immunodeficiency virus 1 (HIV-1),[19,23] Epstein-Barr virus (EBV),[24] and HTLV-1.[25] Only two studies have used such tetramers to identify hepatitis C virus–specific CD8$^+$ T cells, one in patients with chronic hepatitis C[20] and the other in both acute and chronic hepatitis C.[26] Two HLA-A2-HCV NS3 peptide tetramers detected epitope-specific CD8$^+$ T cells in PBMCs from 53% and 50% of patients with chronic hepatitis C, whereas 75% of patients had CD8$^+$ T cells specific for one or both epitopes.[20] In contrast, the

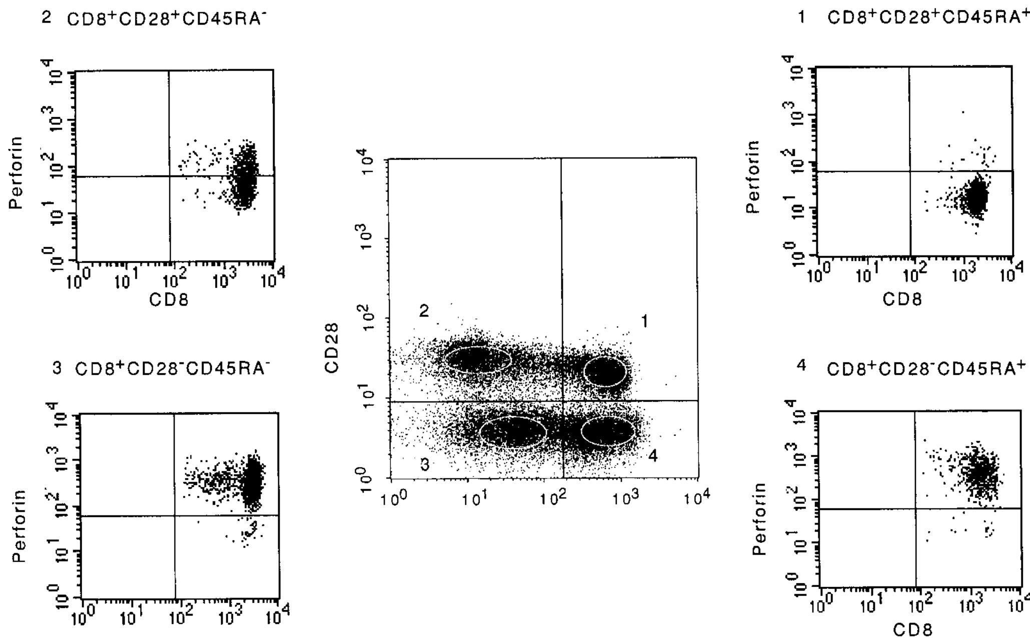

FIG. 4. Perforin expression in CD8$^+$ T cells expressing different CD28 CD45RA phenotypes. PBMCs from an HCV seronegative individual were stained with anti-CD8, anti-CD28, anti-CD45RA, and anti-perforin mAbs.

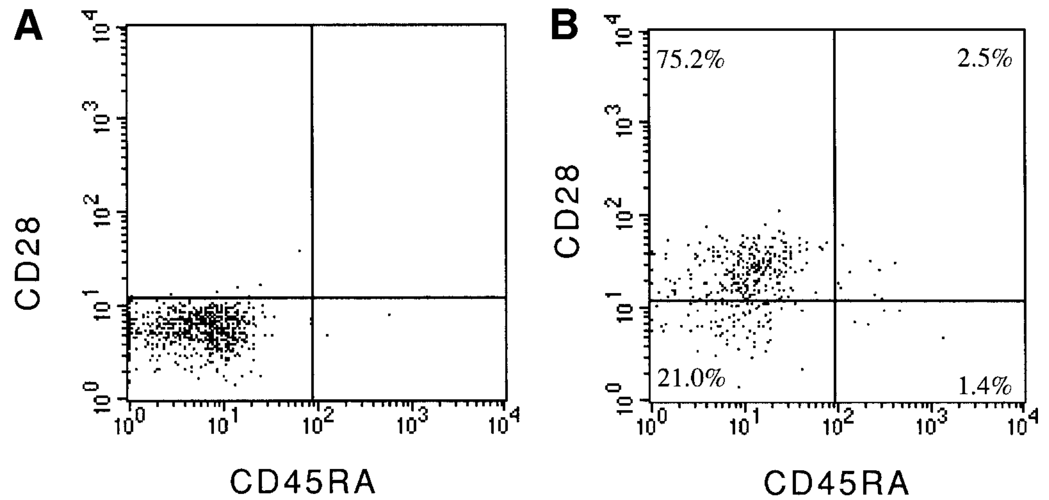

FIG. 5. Analysis of CD28 and CD45RA expression on tetramer^{+} CD8^{+} T cells. An HCV-NS3 1359-1367–specific CTL clone (A) and PBMCs from patient A2 (1 week after clinical onset; B) were simultaneously stained with the HLA-B*3501 tetramer, anti-CD8 mAb, anti-CD28 mAb, and anti-CD45RA mAb.

present study showed that CD8^{+} T cells specific for HCV NS3 1359-1367 were detectable in PBMCs from 100% of HLA-B35–positive patients with chronic hepatitis C. This suggests that the HCV NS3 1359-1367 is immunodominant epitope and is more commonly recognized than the HLA-A2–restricted NS3 epitopes in patients with chronic hepatitis C. Chronic hepatitis C patients have only a low number of HCV-specific CTLs in their PBMCs. It is therefore difficult to identify these CTLs in PBMCs that have been stimulated with epitope peptides by the conventional ^{51}Cr releasing assay. Indeed, using this assay we failed to induce HCV-specific CTLs in PBMCs from 7 patients with chronic hepatitis C after stimulation with the HCV NS3 1359-1367 peptide. In contrast, the use of the HLA-B*3501-tetramers in the present study detected HCV NS3 1359-1367-specific CD8^{+} T cells in PBMCs from all 8 chronic hepatitis C patients examined. Thus, flow cytometric analysis using HLA class I tetramers is a useful method for identification of rare antigen-specific CD8^{+} T cells in PBMCs.

Two studies used conventional ^{51}Cr releasing assay to investigate HCV-specific CTLs in patients with acute hepatitis C.[4,5] In one study, no significant difference was observed in the magnitude of the CTL response to HLA-A3–restricted epitopes, or in the total number of HLA-A3–restricted epitopes recognized between patients with acute and chronic hepatitis C.[4] However, our previous study showed that the CTL response to an HLA-B*3501–restricted HCV epitope in patients with acute hepatitis C is much stronger than in those with chronic hepatitis C.[5] This was confirmed by the present study using HLA-B*3501 tetramers. In addition, a recent study using HLA-A*0201 tetramers showed that the frequencies of tetramer^{+}CD8^{+} T cells in patients with acute hepatitis C is higher than those with chronic hepatitis C.[26] Taken together these studies indicate that strong CTL activity specific for HCV-epitope peptides is induced in acute phase PBMCs from patients with acute hepatitis C.

Effector CTLs can be discriminated from naive or memory T cells by examining the expression of CD45RA, CD28, and/or CD27.[22] Naive CD8^{+} T cells express CD45RA, CD28, and CD27 and memory CD8^{+} T cells express CD28 and CD27 but lose CD45RA, while effector CD8^{+} T cells are CD28^{-}CD27^{-}CD45RA^{-} or CD28^{-}CD27^{-}CD45RA^{+}. The analysis of perforin expression in CD8^{+} T cells with each phenotype supported this classification (Fig. 4). Expansion of CD28^{-}CD27^{-}CD45RA^{-}CD8^{+} and CD28^{+}CD27^{+}CD45RA^{-}CD8^{+} T cells occurs during viral infection. The observation that these expanding cells are enriched for perforin-containing T cells[22] suggests that both phenotypes are effector CD8^{+} T cells. Our HCV-specific CD8^{+} CTL clones express neither CD28 nor CD45RA (Fig. 5). Furthermore, we showed that HCV-specific CD28^{+}CD45RA^{-} CD8^{+} T cells did not have perforin in their cytoplasm, which supports previous studies suggesting that CD28^{+}CD45RA^{-}CD8^{+} T cells are memory

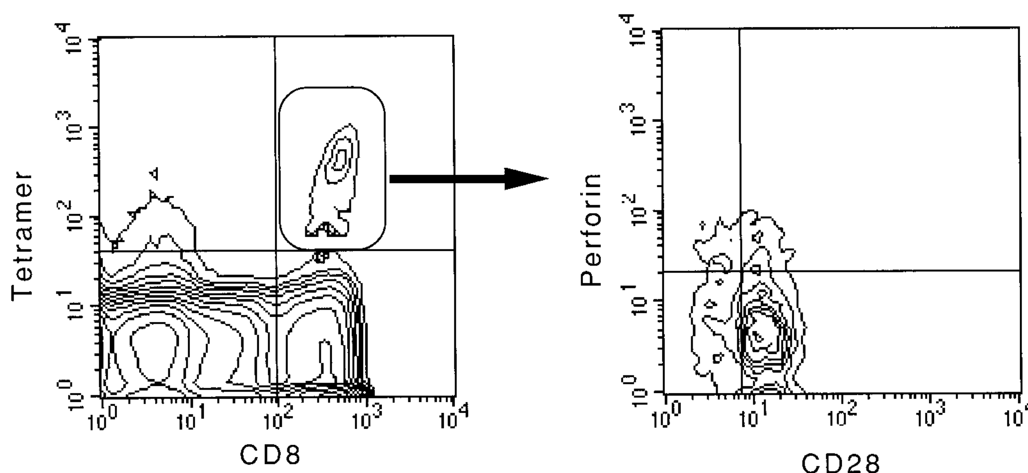

FIG. 6. Perforin expression in tetramer^{+}CD8^{+} T cells. PBMCs from patient A2 (1 week after clinical onset) were stained simultaneously with the HLA-B*3501 tetramer, anti-CD8 mAb, anti-CD28 mAb, and anti-perforin mAb. Eighty-three percent of tetramer^{+}CD8^{+} T cells did not express perforin whereas the remaining 17% expressed a low level of perforin.

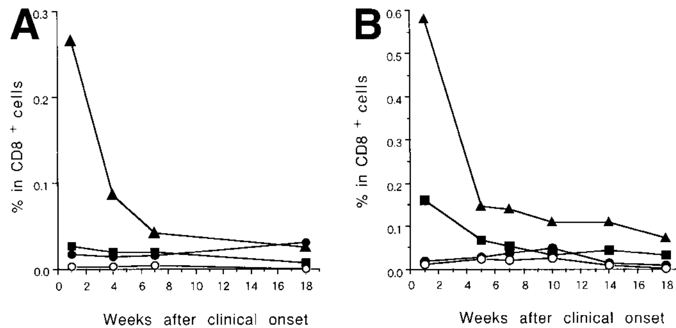

FIG. 7. Changes in the CD45RA and CD28 phenotypes of tetramer$^+$CD8$^+$ T cells during the clinical course of acute HCV hepatitis. PBMCs taken from 2 patients with acute hepatitis C (A, patient A1; B, patient A2) at various times through the clinical course of their infection were stained simultaneously with a mixture of the HLA-B*3501 tetramer, anti-CD8 mAb, anti-CD28 mAb, and anti-CD45RA mAb. The percentage of CD28$^+$CD45RA$^-$ (▲), CD28$^+$CD45RA$^+$ (●) CD28$^-$CD45RA$^-$ (■), and CD28$^-$CD45RA$^+$ (○) cells in total CD8$^+$ T cells was calculated from the total number of tetramer$^+$CD8$^+$ T cells and each CD28 CD45RA phenotype.

T cells. After culture for 7 days with *in vitro* peptide stimulation, perforin expression in HCV-specific CD28$^+$CD45RA$^-$CD8$^+$ T cells increased. This strongly suggests that HCV-specific effector CTLs are induced from HCV-specific CD28$^+$CD45RA$^-$ CD8$^+$ memory T cells. On the other hand, HCV-specific CD28$^-$CD45RA$^-$CD8$^+$ CTL clone did not change its phenotype to CD28$^+$CD45RA$^-$ after culture for 2 weeks without *in vitro* peptide stimulation (data not shown), implying that differentiated effector CD8$^+$ T cells hardly switch to memory CD8$^+$ T cells.

Several studies have investigated the phenotype of expanding viral-antigen–specific CD8$^+$ T cells using MHC-peptide tetrameric complexes. In individuals with acute EBV infection, EBV-specific CD8$^+$ T cells generally lost CD45RA expression whereas CD28 expression was highly variable.[24] Similarly, in chronic HIV-1 infection, a large population of HIV-1–specific CD8$^+$ T cells lost CD45RA expression whereas CD28 expression was variable.[27] However in contrast, our recent study showed expansion of HIV-1–specific CD45RA$^-$CD28$^-$ CD8$^+$ T cells in these patients.[28] Furthermore, in chronic HCV infection, the expression of both CD45RA and CD27 were variable in patients.[20] There is thus controversy over the phenotype of expanding antigen-specific

FIG. 8. The CD28 and CD45RA phenotype of tetramer$^+$CD8$^+$ T cells in PBMCs after *in vitro* peptide stimulation. Acute phase PBMCs from patient A2 stimulated with the HCV-NS3 1359-1367 peptide were cultured and then CD28CD45RA phenotype on tetramer$^+$CD8$^+$ cells were analyzed by flow cytometry with a mixture of the HLA-B*3501 tetramer, anti-CD8 mAb, anti-CD28 mAb, and anti-CD45RA mAb and with that of the tetramer, anti-CD8 mAb, and anti-perforin mAb. Perforin expression on tetramer$^+$CD8$^+$ T cells increased after culture. Mean fluorescence intensity of the cells stained with anti-perforin is as follows: before culture, 6.7; 7 days after culture, 37.3; 15 days after culture, 67.9. Background levels obtained by PE-conjugated mouse IgG2b mAb were between 2 and 3.

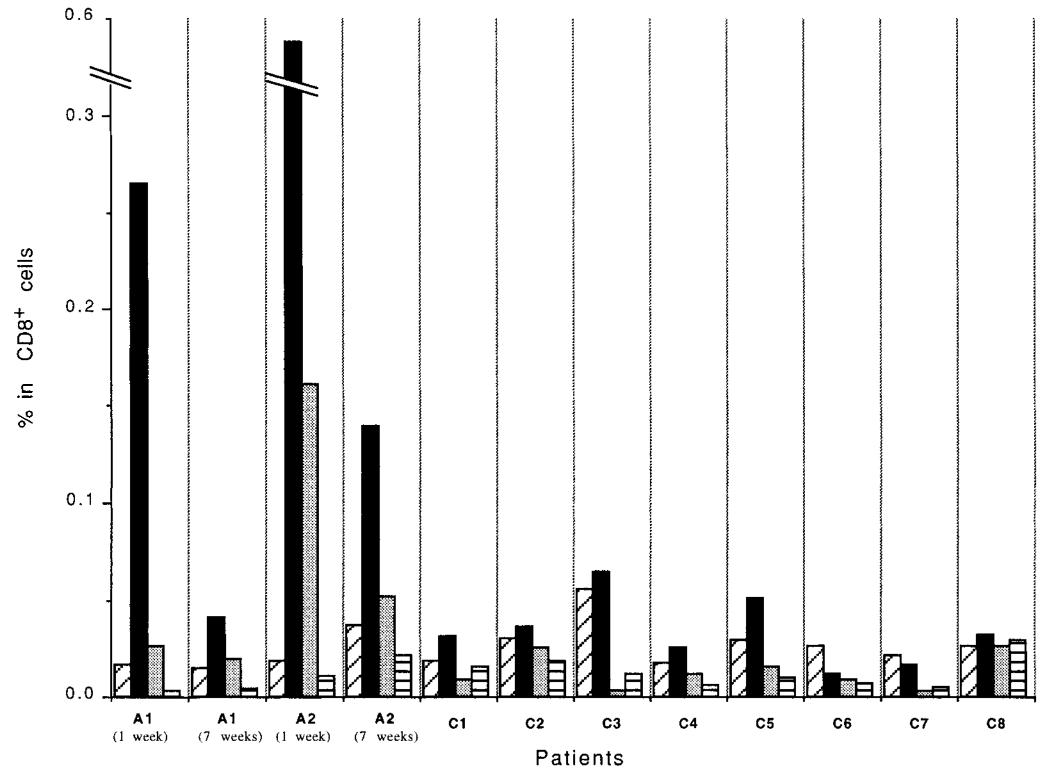

FIG. 9. Comparison of CD28 CD45RA phenotype of tetramer$^+$ CD8$^+$ T cells between patients with acute and chronic hepatitis. The CD28CD45RA phenotype of tetramer$^+$ CD8$^+$ T cells from 2 patients with acute hepatitis C and 8 patients with chronic hepatitis C was analyzed using the HLA-B*3501 tetramer, anti-CD8 mAb, anti-CD28 mAb, and anti-CD45RA Ab. More than 1.0×10^5 CD8$^+$ T cells were nalyzed in PBMCs from the patients. CD28$^+$CD45RA$^+$ (▨); CD28$^+$ CD45RA$^-$ (■); CD28$^-$ CD45RA$^-$ (▩); CD28$^-$CD45RA$^+$ (▤).

CD8$^+$ T cells in PBMCs from patients with acute and chronic viral infection. Using 4-color analysis, the present study clearly showed expansion of HCV-specific CD28$^+$ CD45RA$^-$CD8$^+$ memory T cells in acute phase PBMCs from patients with acute hepatitis C. Phenotypic analysis of HCV-specific CD8$^+$ T cells in liver from acute hepatitis patients is expected to clarify whether CD28$^+$CD45RA$^-$CD8$^+$ T cells migrate from the liver. If CD8$^+$ T cells with memory phenotype exist in the liver, it is likely that these memory cells selectively migrated from the liver. After these memory cells return to the liver, they might differentiate into effector CTLs after recognizing HCV epitopes on hepatocytes because these cells can differentiate to effector CTLs. Further analysis of HCV-specific CD8$^+$ T cells in both liver and PBMCs from patients with acute and chronic hepatitis C will clarify immunopathogenesis of hepatitis C.

In the present study, we directly showed HCV-specific CD8$^+$ T cells in PBMCs from patients with acute and chronic hepatitis C, and that expanding HCV-specific CD8$^+$ T cells in acute phase PBMCs from patients with acute hepatitis C have a memory T cell phenotype. These findings suggest an important role for HCV-specific CTL in clearance of HCV in acute hepatitis C.

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