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A Peptide Derived From Hepatitis C Virus (HCV) Core Protein Inducing Cellular Responses in Patients With HCV With Various HLA Class IA Alleles

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C35-44 peptide is a well known HLA-A2-restricted CTL epitope originating from hepatitis C virus (HCV) core protein. It was reported that the majority of HCV positive patients had significant levels of serum IgG specific to this peptide. This study addressed whether C35-44 peptide could induce CTL activity restricted to various HLA class IA alleles or could not. This peptide demonstrated binding activity to HLA-A*2402, -A*2601, -A*3101, and -A*3303 molecules, but not to HLA-A*1101 by means of stabilization assay. This peptide also induced CTL activity restricted to each of them, except HLA-A11$^+$ peripheral blood mononuclear cells from HCV 1b$^+$ patients by means of ^{51}Cr-release assay. With regard to HLA-A2 subtypes, this peptide demonstrated binding activity to HLA-A*0201 and -A*0206, but not to -A*0207 molecules. Furthermore, this peptide induced CTL activity from both the patients and healthy donors with all the HLA class IA molecules mentioned above by means of interferon-γ production assay. These results may provide new insights for the development of a novel peptide vaccine against HCV compatible with various HLA class IA types. ***J. Med. Virol. 81:1232–1240, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: hepatitis C Virus; peptide vaccine; HLA

INTRODUCTION

Hepatitis C virus (HCV) infection is a serious worldwide health problem; approximately 180 million people in the world are infected persistently with HCV and have a high risk of liver cirrhosis or hepatocellular carcinoma later. Among the 11 known genotypes, HCV genotype 1b is a dominant type in Japanese patients, and more than 60% of patients infected with HCV 1b$^+$ are resistant to interferon (INF)-based treatments. Therefore, there is an urgent need to develop new treatments [Lauer and Walker, 2001]. One type of treatment could be a peptide-based specific immunotherapy since CD8$^+$ cytotoxic T lymphocytes (CTLs) are known to play an important role in the elimination of HCV virus [Rehermann and Chisari, 2000]. A relatively large number of peptides derived from HCV capable of inducing HLA class IA-restricted CTLs have been reported in the past decade, and several clinical trials using those peptides have been conducted [Schlaphoff et al., 2007; Yutani et al., 2007; Klade et al., 2008]. However, CTL-directed peptide-based immunotherapy has at least two disadvantages as compared to whole-molecule-based immunotherapy. One disadvantage is the need to prepare different peptides matched to each HLA class IA allele since each allele has its individual pocket to which peptides present in the groove adhere [Falk et al., 1991; Rudensky et al., 1991]. The other is the inability to activate CD4$^+$ T helper cells to direct B cells to produce an antibody against HCV.

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It has been reported that the majority of HCV-positive patients with different HLA class IA alleles had significant levels of IgG specific to the C35-44 peptide [Takao et al., 2004], which originates from the core region of the HCV protein and is well known as a peptide capable of inducing HLA-A2 restricted CTLs [Cerny et al., 1995]. This observation led us to investigate whether or not the C35-44 peptide could induce CTLs from patients infected with HCV and healthy donors with different HLA types.

MATERIALS AND METHODS

Patients and Samples

The Institutional Ethical Review Board of Kurume University has approved the study protocol conforming to the ethical guidelines of the 1975 Declaration of Helsinki, and informed written consent was obtained from all the subjects whose peripheral blood mononuclear cells (PBMCs) were taken and used for this study. None of the participants was infected with human immunodeficiency virus (HIV) or hepatitis B virus (HBV). PBMCs were prepared from blood of patients with HCV 1b infection and blood of HCV-negative healthy donors by Ficoll–Conray centrifugation. HCV infection was confirmed as positive for anti-HCV antibodies by HCV core-antibody immunoradiometric assay, and was also confirmed as positive for HCV-RNA in a clinical laboratory (SRL, Inc., Tokyo, Japan) using quantitative RT-PCR assay. HLA types of the participants were examined as following: briefly, 50–100 μl of blood was incubated with monoclonal antibody to HLA-A2, -A3, -A11, -A24, -A26, -A31, or -A33, respectively, at 4°C for 30 min. Subsequently, red blood cells were lysed by addition of lysing reagent (150 mM NH_4Cl, 0.1 mM EDTA-Na, 10 mM $KHCO_3$, pH 7.3). Cells were washed with PBS once and then incubated with FITC-conjugated secondary antibodies at 4°C for another 30 min. Finally, cells were washed with PBS once and fixed with 1% formalin. Expression of HLA-A2, -A24, -A11, -A31, -A33, or -A26 molecules in PBMCs was analyzed by flow cytometry analysis with EPICS-XL (Beckman Dickinson, Mountain View, CA).

Antibodies

The following antibodies were used in this study: anti-HLA-A2 monoclonal antibody (mAb) was prepared from a hybridoma (clone BB7.2) that was purchased from ATCC (Rockville, MD). Anti-HLA-A24 mAb (Cat. No. 0041HA), anti-HLA-A11 mAb (Cat. No. 0284HA), anti-HLA-A26 mAb (Cat. No. 0514AHA), anti-HLA-A31 mAb (Cat. No. 0273HA), and anti-HLA-A33 mAb (Cat. No. 0612HA) were purchased from One Lambda (Canoga, CA). Anti-MHC class I (W6/32) (Cat. No. M0736) was from Dako (Glostrup, Denmark). Fluorescein-conjugated goat IgG to mouse IgG (Cat. No. 55493) was purchased from Cappel (Aurora, Ohio) and FITC labeled goat anti-mouse IgM was purchased from Bioscource (Camarillo, CA). Anti-CD4 (Nu-Th/I, IgG1), -CD8 (Nu-Ts/c, IgG2a), -CD14 (JML-H14, IgG2a), and -MHC class II (H-DR-1, IgG2a) antibodies were purified from ascites of mice immunized with corresponding hybridomas.

Cell Lines

RMA-S (a transporter associated with antigen processing [TAP]-deficient mouse lymphoma cell line) [Ljunggren et al., 1990], C1R cells (human B lymphoblastoid cell line), and T2 cells (T-B lymphoblast hybrid) were maintained in RPMI 1640 supplemented with 10% FCS. RMA-S-A*2402/K^b cells (kindly provided by Dr. H. Takasu, Research Institute of Sumitomo Pharmaceutical, Osaka, Japan) were maintained in RPMI 1640 medium supplemented with 10% FBS and 0.75 mg/ml of Geneticin (Calbiochem, Darmstadt, Germany). C1R-A*2402 cells (kindly provided by Dr. Takiguchi, Kumamoto University, Japan) were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.5 mg/ml Hygromycin (Wako, Osaka, Japan).

Establishment of Stable Transfectant Cell Lines

In order to evaluate the binding affinities of C35-44 peptide to various HLA molecules, RMA-S stable transfectant cells expressing HLA-A*0201, -A*0206, -A*0207, -A*1101, -A*3101, -A*3303, or -A*2601 were established: Each HLA gene was cloned into pCR3.1 vector (Invitrogen, Carlsbad, CA) as reported previously [Takedatsu et al., 2004]. The recombinant plasmid DNA was individually transfected into RMA-S cells by using Microporator MP-100 (DigitalBio, Seoul, Korea). Geneticin (0.75 mg/ml)-resistant cells were isolated and single cell cloning was done to establish a stable transfectant cell line expressing each kind of HLA molecule.

In cases of HLA-A*3101, -A*3303, and -A*2601, HLA expressions were rarely detected since mouse beta-2-microglobulin (b2m) cannot recognize HLA-A*3101, -A*3303, or -A*2601 [Rein et al., 1987]. To overcome this problem, human b2m (hb2m) cDNA (Origene, Rockville, MD) was inserted into pIRESpuro3 vector (Clontech, Moutain View, CA) at the *Not* I restriction enzyme site, and then transfected into RMA-S-A*3101, -A*3303, and -A*2601, respectively. Geneticin (0.75 mg/ml)/puromycin (2 μg/ml)-resistant cells were selected and single cell clone with highest HLA expression level was amplified. Expression of each kind of HLA protein was confirmed by flow cytometry analysis as shown in Figure 1A.

C1R stable transfectant cells expressing -A*0201, -A*0206, -A*0207, -A*1101, -A*3101, -A*3303, or -A*2601 were established in a similar way as described above. They were used as target cells for IFN-γ production assay and ^{51}Cr-release assay. Protein expression levels of each HLA on cell surface are shown in Figure 1B.

Peptides

The C35-44 peptide at good manufacturing practice (GMP) level with a purity >99.5% was purchased from

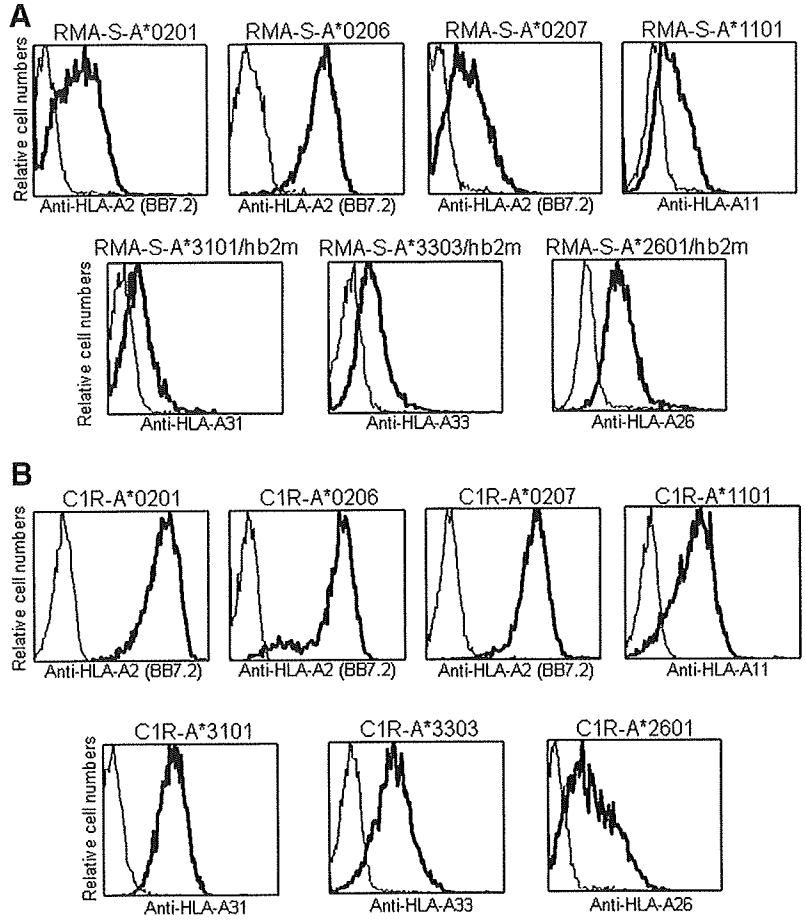

Fig. 1. HLA expression in RMA-S and C1R stable transfectant cells. After construction of RMA-S (**A**) and C1R (**B**) stable transfectant cell lines expressing HLA molecules alone or together with hb2m, HLA protein expression was confirmed by flow cytometry analysis. Cells were stained with monoclonal antibody to each HLA, followed by reaction with FITC-conjugated secondary antibody. Finally expression levels were analyzed with EPICS XL. Dotted line indicates negative control without first mAb, while bold line indicates the expression profiles of HLA molecules.

Multiple Peptide Systems (MPS, San Diego, CA). All other peptides were purchased from Thermo Fisher Scientific (Waltham, MA) and were guaranteed to have a purity >90%. All peptides were dissolved in DMSO to a final concentration of 10 mg/ml and stored at −80°C prior to use. The details are shown in Table I.

Stabilization Assay

Binding activities of the C35-44 peptide to HLA-A*0201, -A*0206, -A*0207, -A*2402, -A*1101, -A*3101, -A*3303, and -A*2601 were examined using a stabilization assay as previously reported [Yamada et al., 1999; Imai et al., 2001]. Peptide loaded cells were analyzed with EPICS XL (Beckman Dickinson). The affinity of peptide to HLA molecules was evaluated by the percent mean fluorescence intensity (MFI %) increase of the HLA molecules detected by staining with specific monoclonal antibody: MFI increase % = [(MFI induced by the C35-44 peptide − MFI induced by DMSO)/(MFI induced by DMSO)] × 100%.

IFN-γ Production Assay

Peptide-specific CTLs from HCV1b$^+$ patients were detected previously as reported [Matsueda et al., 2005]. Briefly, PBMCs (1×10^5 cells/well) were incubated with 10 μM of each peptide in a U-bottom-type 96 well microculture plate in 200 μl of culture medium (45% RPMI 1640, 45% AIM-V medium, 10% FBS, 100 U/ml of IL-2, 0.1 mM MEM nonessential amino acid solution). Every 3–4 days, half of the culture medium was replaced with new medium containing corresponding peptide (20 μM). After peptide pulse for five times, cultured cells were separated into four wells. Two wells were used for the culture with the corresponding peptide-pulsed target cells, and the other two were used for the culture with HIV-peptide pulsed target cells. After 18-hr incubation, supernatant was collected and IFN-γ release was determined by ELISA.

Besides the C35-44 peptide, EBV and Flu derived peptides were used as positive controls, while HIV

TABLE I. List of Peptides Used in This Study

Peptides	Sequence	Binding HLA	Applications	References
C35-44	YLLPRRGPRL	A2	Target peptide	Takao et al. [2004]
HNRPL 501–510	NYLHFFNAPL	A2	PC in Figure 2A; NC in Figure 2E	Ito et al. [2001]
HIV-A2	SLYNTVATL	A2	PC in Figure 2B	Takao et al. [2004]
PSMA 624–632	TYSVSFDSL	A24	NC in Figure 2A,B	Horiguchi et al. [2002]; Kobayashi et al., 2003
EBV-A2	GLCTLVAML	A2	PC in Figure 2C	Takao et al. [2004]
SART3 109–118	VYDYNCHVDL	A24	PC in Figure 2D	Miyagi et al. [2001]
C30-39	IVGGVYLLPR	A11/A31/A33	NC in Figure 2C,D,H	Matsueda et al. [2007]
NS2 918–926	LIRACMLVR	A11/A31/A33	PC in Figure 2F	Matsueda et al. [2007]
C36-44	LLPRRGPRL	A24	NC in Figure 2F,G	Mashiba et al. [2007]
NS3 1582–1590	ENLPYLVAY	A26	PC in Figure 2H	Neumann-Haefelin et al. [2007]
EBV-A24	TYGPVFMCL	A24	PC in Table II	Inoue et al. [2001]
Flu-A2	GILGFVFTL	A2	PC in Table II	Bocchia et al. [1996]
Flu-A24	PFYIQMCYEL	A24	PC in Table II	Inoue et al. [2001]
Flu-A3	NVKNLYEKVK	A11/A31/A33	PC in Table II	Matsueda et al. [2005]
EB-A3	IVTDFSVIK	A11/A31/A33	PC in Table II	Matsueda et al. [2005]
HIV-A3	RLRDLLLIVTR	A11/A31/A33	PC in Figure 2E,G; NC in Table II, Figure 4B,C	Matsueda et al. [2005]
HIV-A24	RVLRQQLLGI	A24	NC in Figure 3A	Inoue et al. [2001]
HIV-A26	EVIPMFSAL	A26	NC in Figure 4D	Yamada et al. [1999]

PC, positive control; NC, negative control.

derived peptide as negative control for the induction of specific T cells. As target cells, peptide-pulsed T2, C1R-A*0206, -A*0207, -A*2402, -A*1101, -A*3101, -A*3303, or -A*2601 cells were used in each experiment. The successful induction of peptide-specific CTLs was judged to be positive only when the significant value of $P < 0.05$ was reached by a two-tailed Student's t-test and when the difference in IFN-γ production compared with the HIV peptide exceeded 50 pg/ml.

^{51}Cr-Release Assay

$CD8^+$ T cells were positively isolated from peptide-stimulated PBMCs using a CD8-positive isolation kit (Dynal, Oslo, Norway). Its cytotoxicity against C35-44 or HIV peptide pulsed C1R-A*2402, -A*1101, -A*3101, -A*3303, or -A*2601, cells was examined by a standard 6-hr ^{51}Cr-release assay [Matsueda et al., 2007]. The specificity of the cytotoxicity led by peptide-stimulated PBMCs was confirmed by blocking inhibition assay. Anti-HLA class I (W6/32), anti-HLA class II, anti-CD4, anti-CD8, or anti-CD14 antibodies were, respectively, added at a final concentration of 20 μg/ml. After incubation at 37°C for 1 hr, specific ^{51}Cr-release was measured at an E/T ratio of 40.

RESULTS

Binding Activity of the C35-44 Peptide to Various HLA Class IA Molecules

RMA-S stable transfectant cells expressing various HLA molecules were established (Fig. 1A) and were used for stabilization assay to evaluate the binding affinities of C35-44 peptide to HLA molecules. Initially, HLA stabilization assay was performed by using RMA-S cells expressing HLA-A*0201 molecules. Surface expression of HLA-A*0201 molecules was stabilized in a dose-dependent manner when they were cultured with either a positive control or the C35-44 peptide (MFI increase at 1, 10, and 100 μM was 36%, 67%, and 137%, respectively), whereas the expression was not stabilized when cultured with either DMSO alone or PSMA 624-632 peptide (negative control) [Horiguchi et al., 2002; Kobayashi et al., 2003] (Fig. 2A). Similar results were obtained with HLA-A*0206 although the expression levels were modest (MFI increase at 1, 10, and 100 μM was 9%, 25%, and 42%, respectively) (Fig. 2B). In contrast, C35-44 peptide did not show binding activity with HLA-A*0207 (Fig. 2C).

Next, the binding activity of the C35-44 peptide to HLA-A*2402 molecules was tested. As a result, HLA-A*2402 molecules on RMA-S-A*2402 cells were only stabilized slightly, and MFI increase at 1, 10, and 100 μM of the C35-44 peptide was 5%, 8%, and 18%, respectively (Fig. 2D).

Binding activity of the C35-44 peptide with HLA-A*1101 (Fig. 2E), -A*3101 (Fig. 2F), and -A*3303 (Fig. 2G) molecules was further tested, which belong to the HLA-A3 supertype. HLA-A*3101 molecules on RMA-S-A*3101/hb2m cells were stabilized, and MFI increases at 1, 10, and 100 μM of the C35-44 peptide were 20%, 22%, and 73%, respectively; stabilized HLA-A*3303 molecules gave an increase of 0%, 42%, and 53% at 1, 10, and 100 μM, respectively. In contrast, the binding activity to HLA-A*1101 was not detected, and MFI increase at 1, 10, and 100 μM of the C35-44 peptide was 7%, 3%, and 9%, respectively.

Finally, the binding activity of the C35-44 peptide with HLA-A*2601 was examined. As a result, HLA-A*2601

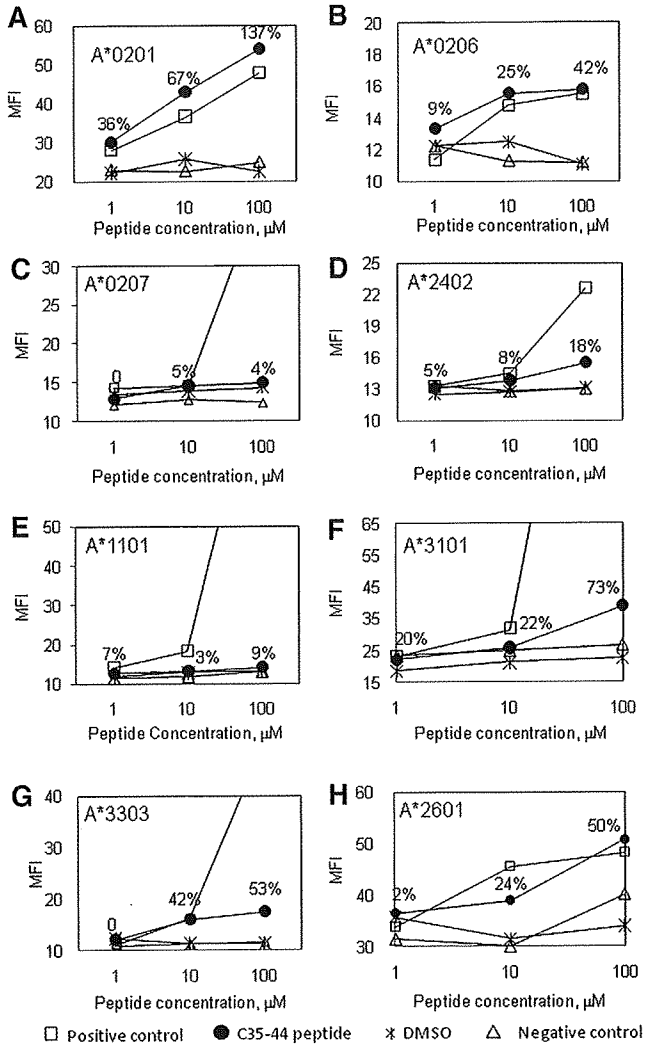

Fig. 2. Stabilization assay of the C35-44 peptide to various HLA molecules. The binding activities of the C35-44 peptide to HLA-A*0201 (**A**), -A*0206 (**B**), -A*0207 (**C**), -A*2402 (**D**), -A*1101 (**E**), -A*3101 (**F**), -A*3303 (**G**), and -A*2601 (**H**) were examined by using RMA-S cells stably expressing corresponding HLA molecules. Positive control peptide, negative control peptide, and DMSO were included in each experiment. The positive control peptides used were: HNRPL 501–510 (A), HIV-A2 (B), EBV-A2 (C), SART3 109–118 (D), HIV-A3 (E,G), NS2 918–926 (F), and NS3 1582–1560 (H), respectively. Negative control peptides used for each HLA were: PSMA 624–632 (A,B), C30-39 (C,D,H), HNRPL 501–510 (E), and C36-44 (F, G), respectively. MFI was recorded at 1, 10, and 100 μM of the peptide or DMSO. The MFI increase induced by the C35-44 peptide compared with DMSO was calculated and is shown in each figure. Representative results from at least three separate experiments are shown.

molecules on RMA-S-A*2601/hb2m cells were stabilized, and MFI increase at 1, 10, and 100 μM of the C35-44 peptide was 2%, 24%, and 50%, respectively (Fig. 2H).

Induction of Peptide-Specific CTL Activity From HCV$^+$ Patients by IFN-γ Assay

Next it was determined whether the C35-44 peptide could induce peptide-specific CTLs from the PBMCs of HLA-A2$^+$, -A11$^+$, -A24$^+$, -A31$^+$, or -A33$^+$ patients by means of IFN-γ production assay (Table II). Peptide-pulsed C1R cells expressing various HLA molecules were used as target cells (Fig. 1B). The C35-44 peptide induced peptide-reactive CTL activity from the PBMCs of 3 of 4 HLA-A*0206$^+$ patients, 1 of 1 HLA-A*0207$^+$ patients, 3 of 4 HLA-A*2402$^+$ patients, 2 of 3 HLA-A*1101$^+$ patients, 1 of 2 HLA-A*3101$^+$ patients, and 2 of 3 HLA-A*3303$^+$ patients. Due to insufficient numbers of PBMCs obtained from HLA-26$^+$ patients, only cytotoxicity assay was performed while IFN-γ production assay was not done.

EBV and Flu peptides, taken as positive control peptides, also induced peptide-specific CTL activity in certain cases of HLA-A2$^+$, -A24$^+$, -A11$^+$, -A31$^+$, and -A33$^+$ patients tested. The positive rates of successful CTL induction specific to the C35-44 peptide were not much different from those specific to the EBV or Flu peptides, indicating that CTL precursor frequencies for each of three viral antigens are not so different from each other.

Induction of CTLs by the ^{51}Cr-Release Assay

A standard 6-hr-^{51}Cr-release assay was performed to determine whether or not the peptide-stimulated CTLs could exhibit cytotoxicity against C1R cells pulsed with the C35-44 peptide. PBMCs from HLA-A24$^+$ patients, stimulated in vitro with the C35-44 peptide, exhibited significantly higher levels of cytotoxicity against C35-44 peptide-pulsed C1R-A*2402 cells than that against C1R-A*2402 cells pulsed by negative control peptide (Fig. 3A). PBMCs induced from HLA-A31$^+$ or HLA-A33$^+$ patients also exhibited higher levels of cytotoxicity against C35-44 peptide-pulsed C1R-A*3101 or C1R-A*3303 cells than negative control (Fig. 3B,C). The same phenomenon was observed with C35-44 peptide-stimulated HLA-A26$^+$ PBMCs by using C1R-A*2601 as target cells (Fig. 3D). In contrast, PBMCs from two HLA-A11$^+$ patients, which were stimulated in vitro with C35-44 peptide, failed to exhibit cytotoxicity against C1R-A*1101 cells (data not shown).

The cytotoxicity of C35-44 peptide pulsed HLA-A24$^+$ (Fig. 4A), -A31$^+$ (Fig. 4B), -A33$^+$ (Fig. 4C), and -A26$^+$ (Fig. 4D) CTLs was suppressed by incubation with anti-MHC class I or anti-CD8 antibodies, but not by incubation with anti-MHC class II, anti-CD14, or anti-CD4 antibodies. These results indicate that the cytotoxicity of C35-44 peptide pulsed CTLs was largely mediated by CD8$^+$ CTLs in an HLA class I-restricted manner.

Induction of Peptide-Specific CTLs From Healthy Donors

Finally, it was investigated whether the HCV C35-44 peptide could induce CTL activity from HCV-uninfected healthy volunteers from the standpoint of developing a prophylactic peptide vaccine for HCV. PBMCs of different HLA types were stimulated with the C35-44 peptide for seven times and then incubated for an additional 5–7 days without peptide stimulation. IFN-γ produc-

TABLE II. CTL Induction From Patients Infected With HCV 1b

	Patients						Net IFN-γ (pg/ml) production		
	HLA alleles	Age	Gender	AST (IU/mL)	ALT (IU/mL)	Target cells	C35-44	Flu*	EBV*
HLA-A*0206									
Pt.1	A*0206/A*2402	51	M	41	47	C1R-A*0206	163	—	—
Pt.2	A*0206/A*3303	49	F	142	129	C1R-A*0206	182	nt	nt
Pt.3	A*0206/A*3303	38	M	208	358	C1R-A*0206	155	87	—
Pt.4	A*0206/A*2402	61	F	94	53	C1R-A*0206	—	359	—
HLA-A*0207									
Pt.5	A*0207/A*2402	71	M	84	114	C1R-A*0207	74	nt	nt
HLA-A*2402									
Pt.6	A*2402/A*2402	51	F	34	43	C1R-A*2402	1723	440	222
Pt.7	A*2402/A*2402	50	M	105	180	C1R-A*2402	—	1014	—
Pt.8	A*2402/A*2402	71	M	50	75	C1R-A*2402	217	—	531
Pt.9	A*2402/A*0201	62	F	28	29	C1R-A*2402	714	—	661
HLA-A*1101									
Pt.10	A*1101/A*3303	69	F	35	32	C1R-A*1101	—	—	—
Pt.11	A*1101/A*2402	63	F	60	73	C1R-A*1101	395	nt	263
Pt.12	A*1101/A*2402	63	F	57	52	C1R-A*1101	134	nt	—
HLA-A*3101									
Pt.13	A*3101/A*1101	61	F	73	98	C1R-A*3101	76	—	70
Pt.14	A*3101/A*2601	51	M	51	52	C1R-A*3101	114	238	—
Pt.15	A*3101/A*2402	70	M	122	136	C1R-A*3101	87	—	—
HLA-A*3303									
Pt.10	A*3303/A*1101	69	F	35	32	C1R-A*3303	—	179	—
Pt.16	A*3303/A*1101	60	M	27	16	C1R-A*3303	91	—	157
HLA-A*2601									
Pt.17	A*2601/A*0201	58	F	72	66	nt	nt	nt	nt

Characteristics of HCV1b-positive patients (chronic hepatitis or liver cirrhosis), including liver function (AST and ALT), were given. The PBMCs from these patients were stimulated in vitro with each of the indicated peptides, and peptide-specific reactivity was examined. Only the positive results are shown, and they are statistically different from negative control with a P-value < 0.05 by the two-tailed Student's t-test. Net IFN-γ production shown above was calculated by subtracting IFN-γ values of negative control from those of peptides (C35-44, Flu, and EBV).
Asterisk indicates peptides used as positive control. Flu-A2 and EBV-A2 were used for HLA-A2; Flu-A3 and EB-A3 were used for HLA-A11, -A31, and -A33 positive patients; Flu-A24 and EB-A24 were used for HLA-A24 positive patients.
nt: not tested.

tion against peptide-pulsed cells (T2 for HLA-A2, C1R-A*2402 for HLA-A24, C1R-A*1101 for HLA-A11, C1R-A*3101 for HLA-A31, and C1R-A*3303 for HLA-A33 positive PBMCs) was measured by ELISA. Part of the representative results is shown in Figure 5. As a result, the peptide-stimulated PBMCs released significant levels of IFN-γ in response to the C35-44 pulsed C1R cells expressing relevant HLA-A molecules (homozygous as well as heterozygous) in the donors with all the HLA class IA molecules as far as tested, including HLA-A2, -A11, -A24, -A31, and -A33. These PBMCs, however, failed to induce IFN-γ production in response to those with irrelevant HLA class IA molecules.

DISCUSSION

This study demonstrated that C35-44 peptide had binding activity to HLA-A*0201, -A*0206, -A*2402, -A*3101, -A*3303, and -A*2601, but not to HLA-A*0207 and -A*1101. The results are consistent with its ability to induce CTLs from HCV1b$^+$ patients when ^{51}Cr-release assay was employed. However, there was a discrepancy between the results of binding assay and IFN-γ production assay with regard to HLA-A*0207 and -A*1101. The results of stabilization assay are not only related to peptide-HLA binding affinities but also related to the HLA expression levels on cell surface, and thus the assay itself may not always reflect the real binding activity of peptide to certain HLA molecules. Alternatively, IFN-γ production assay could be feasible to measure HLA class IA-restricted CTL activity induced by a peptide with lower binding activity to corresponding HLA molecules since it took overnight culture instead of 6-hr culture for ^{51}Cr-release assay. CD4$^+$ T helper cells might be involved in this phenomenon. Further studies, such as employing the other binding assays, are needed to solve this discrepancy.

Since CTLs induction by peptides was not detected in all patients, it was analyzed whether there was correlation between immune response to C35-44 peptide and patients' disease status, such as HCV viral load and serum level of ALT (alanine amino transferase). But for the moment no direct relation was found yet from the analysis results of around 30 patients.

There are several HLA class IA alleles that are relatively dominant in the areas other than Japan, such as HLA-A1 and -A3. In this study the ability of C35-44 peptide to induce CTL restricted to those alleles could not be tested because of inability to obtain corresponding samples. CTL might be induced in PBMCs from those alleles, since the majority of HCV$^+$ patients had IgG antibody specific to this peptide [Takao et al., 2004], and

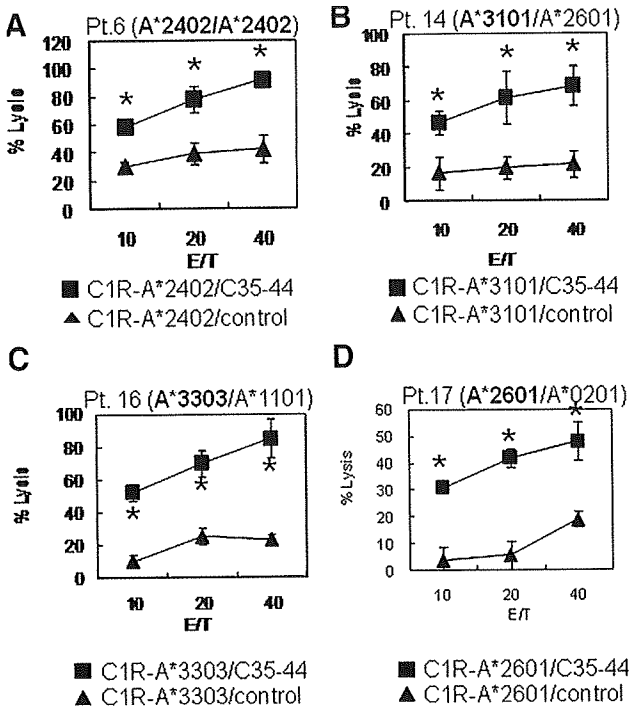

Fig. 3. Cytotoxicity of C35-44 peptide-specific CTLs generated in vitro from patients infected HCV 1b. $CD8^+$ T cells purified from the peptide-stimulated PBMCs of HCV $1b^+$ patients with different HLA alleles were tested for their cytotoxicity at E/T ratio of 10, 20, and 40 by ^{51}Cr-release assay against C1R-A*2402 (**A**), C1R-A*3101 (**B**), C1R-A*3303 (**C**), or C1R-A*2601 (**D**) cells, which were prepulsed with either C35-44 or HIV peptide (negative control). Experiments were done in triplicate and repeated at least twice. Representative results were shown here. Statistical analysis was performed by a two-tailed Student's *t*-test (* $P < 0.05$). E/T, ratio of effector/target cells number.

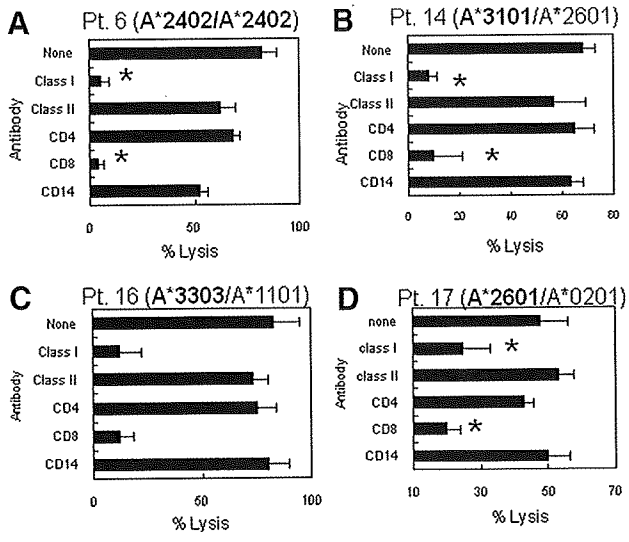

Fig. 4. Specificity of C35-44 peptide induced CTLs cytotoxicities from HCV $1b^+$ patients C35-44 peptide-pulsed CTLs and corresponding C1R stable transfectant cells were treated with anti-CD4, -CD8, -CD14, -MHC class I, or -MHC class II antibody (20 μg/ml) at 37°C for 1 hr, and then the cytotoxicity at an E/T ratio of 40 was measured in triplicate as described before. Representative results were shown for each. Statistical analysis was performed by a two-tailed Student's *t*-test (* $P < 0.05$).

also it induced CTL from PBMC restricted to each allele being tested so far.

Peptides used as therapeutic vaccines usually consist of nine to ten amino acids capable of binding to a particular MHC molecules and having the ability to activate CTLs reactive to cancer cells or virus infection in a particular MHC-restricted manner. In spite that peptide-MHC binding was previously thought to be very specific, recent studies have shown that it is neither as narrowly specific nor as unique as originally believed. Sidney and his colleagues have pointed out that, among 945 different HLA-A and -B alleles examined to date, over 80% alleles can be assigned to one of the nine subtypes; but the remained alleles might be associated with repertoires that overlap multiple supertypes [Sidney et al., 2008]. This indicates the possibility that one peptide can bind to several different HLA alleles. In fact, this phenomenon has been reported by many researchers [Tanigaki et al., 1994; del Guercio et al., 1995; Sidney et al., 1996; Torikai et al., 2007]. In this study, it is also discovered that C35-44 peptide could bind to multiple HLA molecules include HLA-A*0201, -A*0206, -A*2402, -A*3101, -A*3303, and -A*2601, which are the dominant HLA types in Japanese population.

HLA-A24 binding peptides are characterized by the presence of Y or F residues at amino acid position 2 and L, F, I, or W residues at their C terminus [Maier et al., 1994]. The optimal COOH-terminal amino acid of HLA-A31 or -A33-binding peptides is arginine [Rammensee et al., 1995; Takiguchi et al., 2000]. In addition, it has been demonstrated that acidic amino acids at P1 and five hydrophobic residues (Val, Thr, Ile, Leu, and Phe) at P2 are anchor residues for HLA-A26 [Yamada et al., 1999]. These findings also suggest the possibility that the C35-44 peptide (YLLPRRGPRL) can bind to HLA-A*2402, -A*3101, -A*3303, and -A*2601 molecules.

In order to explore the possibility of developing a prophylactic vaccine, C35-44 peptide-pulsed CTLs from healthy donors were examined for their ability to release IFN-γ against C1R cells pulsed with corresponding peptides. Compared with those from patients, naïve T cells from healthy donors are more difficult to induce. Therefore, instead of five times peptide stimulation for PBMCs from patients, seven times peptide stimulation was done for PBMCs from healthy donors. In addition, after peptide stimulation, PBMCs were further incubated without the peptide for another 5–7 days. This might explain why previously C35-44 peptide was not detected of its ability to induce IFN-γ production in healthy donors [Takao et al., 2004].

Although the C35-44 peptide has binding activity to all the HLA molecules tested, the binding affinities varied widely, from the lowest, with HLA-A*2402, to the highest, with HLA-A*0201. These differences, however, did not largely influence in vitro CTL activity toward C1R cells expressing relevant HLA molecules. Namely, CTL activity was induced equally from PBMCs with different HLA class IA molecules. Due to the failure in

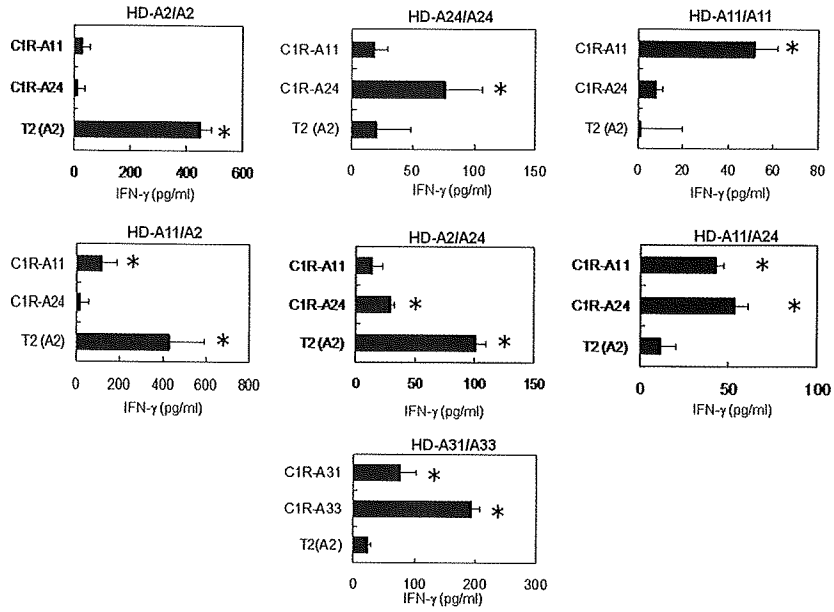

Fig. 5. Induction of C35-44 peptide-specific CTLs from PBMCs of healthy donors PBMCs from HCV-negative healthy donors of different HLA types were stimulated with C35-44 or HIV peptides for seven times and then incubated without peptide for another 5–7 days. PBMCs were incubated with C35-44 peptide-pulsed T2 (HLA-A2$^+$), C1R-A*2402, C1R-A*1101, C1R-A*3101, or C1R-A*3303 cells, respectively, or irrelevant C1R stable transfectant cell lines as negative controls. The level of IFN-γ secretion in the supernatant was determined by ELISA. Induction of peptide-specific CTLs was judged to be successful when the *P*-value was less than 0.05 and when the difference in IFN-γ production compared to that of the control HIV peptide exceeded 50 pg/ml (*). Representative results from two separate experiments are shown.

establishment of HCV-infected cell lines, C1R cells pulsed with exogenous peptides were used as target cells for ^{51}Cr-release assay in this study, In order to confirm the above findings, it is necessary to explore a kind of cell line that can process naturally and present C35-44 peptides as target cells for cytotoxicity assay. In the future, an optional protocol will be used for this purpose by transfection of HCV core region to C1R cells expressing different HLA molecules.

HCV is known as a highly variable virus, but the amino acid sequence of the C35-44 peptide is well conserved in the entire HCV genotype [Hitomi et al., 1995]. HLA-A2, -A11, -A24, -A26, -A31, and -A33 types constitute 98% of the Asian population, 74% of Caucasians, 72% of Spaniards, 76% of Indians, and 59% of Blacks. Thus, this peptide could be useful as a prophylactic and therapeutic HCV vaccine for the majority of people in the world.

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