with 10 cycles for the matching primer. On the other hand, when 10^8 copies of the CTG (codon c91) template were amplified using the primer with a base mismatch, approximately 23 cycles were required before the crossing threshold was reached. This compares with 10 cycles for the matching primer (Figure 1C and D).

The detection limits of these methods were at least 10 copies, 10 copies, 1000 copies, and 10 copies of c70-wild primer sets (HCV-70W and HCV-c-reverse), c70-mutant primer sets (HCV-70M and HCV-c-reverse), c91-wild primer sets (HCV-c-sense and HCV-91W), and c91-mutant primer sets (HCV-c-sense and HCV-91M), respectively (Figure 1).

Selectivity of ARMS assay

Using the plasmid mixture containing the wild-type (p-core-W) and the mutant-type (p-core-M) as a template, real-time ARMS PCR was performed to establish the concentration at which the c70-wild primer sets (HCV-70W and HCV-c-reverse) would detect the wild-type DNA (codon c70). In Table IIIA we present the results of these primer sets showing that, when the wild DNA was 10^9 copies/tube, from 10^5 to 10^9 copies of mutant templates did not affect the results. When the mutant DNA was 10^9 copies/tube, from 10^9 to 10^7 copies of the wild templates could be detected. Similarly, each primer could clearly distinguish the difference between p-core-W and p-core-M at the same copy numbers. Concerning substitution 70, the ratios 100:1, 10:1, 1:1, 1:10, and 1:100 of p-core-W versus p-core-M could be distinguished (Table IIIA and B). However, for substitution 91, the ratios 100:1, 10:1, 1:1, 1:10, 1:100, and 1:1000 could be distinguished, confirming the sensitivity and specificity of the assay [16] (Table IIIC and D).

Hepatitis C core substitutions in serum by real-time ARMS RT-PCR

Quantitative ARMS assays were carried out in parallel reactions, one with a primer matching the variant at the 3′ end, and the other with the primer matching the wild-type variant. We measured the HCV core substitutions at residues c70 and c91 in two patients who did not respond to combination peginterferon and ribavirin therapy after 12 weeks and finally did not become SVRs (Table IV). In patient no. 1, we could detect the minority, wild-type at c70 (4% at 4 weeks). This became diminished at 12 weeks after treatment. In both patients, we could not detect any wild-type template at 12 weeks after treatment.

Comparison of real-time ARMS RT-PCR and conventional sequencing

The real-time ARMS RT-PCR method was compared to direct sequencing in patients treated with peginterferon and ribavirin. In patient no. 1, the minority, wild-type at c70 at 4 weeks could not be detected by direct sequencing (Table IV). In patient no. 2, there were some discrepancies between the results of direct sequencing and those of real-time ARMS RT-PCR (Table IV).

Table III. A mixture of the dilution series of mutants with fixed concentration of wild-type DNA or mutant-type DNA was assayed with each primer to establish the concentration at which the primers would detect each DNA by real-time ARMS PCR. Copy number: copies/tube; template W: p-core-W; template M: p-core-M.

Copy number of template (W:M)	Ct (cycle number)
A. c70-wild primer sets (HCV-70W and HCV-c-reverse).	
10^5:10^9	12.66±0.050
10^6:10^9	12.47±0.099
10^7:10^9	11.46±0.036
10^8:10^9	8.87±0.279
10^9:10^9	5.29±0.018
10^9:10^8	5.24±0.075
10^9:10^7	5.24±0.070
10^9:10^6	5.15±0.091
10^9:10^5	5.13±0.014
B. c70-mutant primer sets (HCV-70M and HCV-c-reverse).	
10^9:10^5	14.44±0.026
10^9:10^6	14.18±0.017
10^9:10^7	12.66±0.044
10^9:10^8	9.68±0.041
10^9:10^9	6.00±0.126
10^8:10^9	5.72±0.10
10^7:10^9	5.57±0.028
10^6:10^9	5.90±0.072
10^5:10^9	5.77±0.063
C. c91-wild primer sets (HCV-c-sense and HCV-91W).	
10^5:10^9	22.77±0.197
10^6:10^9	20.99±0.182
10^7:10^9	17.46±0.0457
10^8:10^9	13.36±0.10
10^9:10^9	9.30±0.053
10^9:10^8	9.29±0.12
10^9:10^7	9.19±0.043
10^9:10^6	9.14±0.060
10^9:10^5	9.23±0.0011
D. c91-mutant primer sets (HCV-c-sense and HCV-91M).	
10^9:10^5	20.89±0.056
10^9:10^6	18.52±0.351
10^9:10^7	14.89±0.016
10^9:10^8	11.53±0.033
10^9:10^9	7.99±0.023
10^8:10^9	7.82±0.0040
10^7:10^9	7.80±0.0098
10^6:10^9	7.86±0.044
10^5:10^9	7.82±0.0025

Table IV. HCV core substitutions at residues c70 and c91 detected by real-time ARMS RT-PCR and direct sequencing.

Patients No.	Study Week	ALT (IU/L)	HCV-RNA (log copies/ml)	c70 W:M	c91 W:M	Direct sequencing c-70/c-91
1.	0	31	6.6	0:100	0:100	M/M
	4	26	6.3	4:96	0:100	M/M
	12	24	5.8	0:100	0:100	M/M
2.	0	53	6.3	0:100	ND	Mix/M
	4	25	6.0	0:100	0:100	M/M
	12	14	5.3	0:100	0:100	M/M

Abbreviations: ARMS = amplification refractory mutation system; ALT = alanine aminotransferase; W = wild-type; M = mutant-type; Mix = mixed-type; ND = not determined.
"Study Week" = weeks after administration of peginterferon and ribavirin.

Discussion

In this article we describe a rapid and sensitive method for the quantitative detection and monitoring of the core amino acid substitutions of HCV genotype 1b. SyBr Green real-time PCR and specific ARMS primers were used to quantify viral RNAs carrying particular sequences, HCV amino acid substitutions 70 and 91 in the core coding region. The specificity of the ARMS primers results in large differences in PCR crossing thresholds being observed between matching and mismatched targets.

For the current standard treatment with peginterferon alpha and ribavirin in patients with chronic hepatitis C, infection with HCV genotypes 2 and 3, lower baseline viral load, Asian and Caucasian ethnicity, younger age, low γ-GTP levels, absence of advanced fibrosis/cirrhosis, and absence of steatosis in the liver have been identified as independent pretreatment predictors of SVR [19]. Early virological response (EVR), defined as a ≥2-log reduction in HCV-RNA or undetectable HCV-RNA at 12 weeks, is associated with a favorable virological response. EVR is reached in only ~70% of patients infected with genotype 1 treated with combination therapy [20,21].

Recently, it was reported that core residues Arg70 and Leu91 were associated with response therapy in Japanese genotype 1b patients [11,13]. Donlin et al. [12] reported a similar association of Arg70 with a marked response for genotype 1b but not 1a; however, Met91 was highly dominant in both the marked- and poor-responder sequences, but few other studies have examined the role of diversity in the core in the outcome of therapy. Concerning hepatocarcinogenesis associated with HCV genotype 1b, Akuta et al. [22] reported that cumulative hepatocarcinogenesis rates in double wild-type (Arg70 and Leu91) of the HCV core region were significantly lower than those in non-double wild-type. Direct sequencing [11,13] and nested-RT-PCR using ARMS primers with gel electrophoresis [12,22] were performed in these studies. Higher sensitivity assays may be more useful for predicting the outcomes of therapy and hepatocarcinogenesis [23]. The real-time ARMS RT-PCR described here does not require restriction enzyme digestion, gel-electrophoresis or sequence analysis of PCR products, and it can quantify the core substitution proportions more quickly.

Hepatitis C core substitutions in serum detected by real-time ARMS RT-PCR showed mutant c70 and mutant c91 at 12 weeks in two non-EVRs (Table IV). Most non-SVR rates result from non-EVR. It was reported that the 72-week regimen significantly improved the SVR rates in non-EVRs with Arg70 and/or Leu91 of core [24]. Peginterferon plus ribavirin treatment is costly and has several side effects, possibly reducing its attractiveness for patients. If we were able to identify these HCV core substitutions at 12 weeks, we would know whether to stop or continue treating patients. This could prevent patients from serious side effects or bring about a better treatment outcome by the resulting shorter regimens. Moreover, if direct viral enzyme inhibitors such as protease inhibitor and polymerase inhibitor, which potently suppress viral replication, could be used, the predictability of outcome would be even more important. Recently, it was also reported that maintenance or prolonged peginterferon did not reduce the incidence of HCC in advanced chronic hepatitis C patients [1,25]. We are now focusing on a larger study, and real-time ARMS RT-PCR is expected to be useful for the important prediction of peginterferon plus ribavirin treatment outcomes or that of hepatocarcinogenesis in hepatitis C patients.

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Phase I clinical study of a peptide vaccination for hepatitis C virus-infected patients with different human leukocyte antigen-class I-A alleles

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Hepatitis C virus (HCV) infection has a high risk of liver cirrhosis and hepatocellular carcinoma at later stages. We recently identified a peptide derived from the HCV core protein capable of inducing both cellular and humoral responses to nearly all HCV-positive patients in Japan with different human leukocyte antigen (HLA)-class I-A alleles. To assess the safety and immune responses to this novel peptide, we conducted a phase I dose-escalation study of the vaccination for 26 HCV-positive patients who were either non-responders to the interferon-based therapy ($n = 23$) or refused it ($n = 3$). The regimen was well tolerated, with no severe vaccine-related toxicity. Twenty-five and 22 patients completed the first and second cycle vaccination (6 and 12 vaccine injections), respectively. After a series of six vaccine injections, peptide-specific CTL activity was augmented in peripheral blood mononuclear cells from 15 of 25 patient samples, with an expected optimal dose of 1 mg peptide. After 12 vaccine injections, peptide-specific IgG production was augmented in plasma from the majority of patients (15 of 22 patients) tested, but not in a dose-dependent fashion. There were two HCV RNA responders with >1 log declines. Among patients whose pre-vaccination levels of alanine aminotransferase and alpha feto-protein exceeded the normal ranges, a <30% decrease was found in 7 of 24 and three of six patients, respectively. Because of its tolerability and higher rate of immune boosting, this protocol is recommended for a phase II study to investigate its clinical efficacy. (*Cancer Sci* 2009; 100: 1935–1942)

Hepatitis C virus (HCV) is prevalent worldwide, with nearly 180 million individuals infected.[1,2] Interferon (IFN)-based therapies, although effective in 80% of patients infected with the HCV2 and HCV3 genotypes and also 50% of patients with the HCV1b genotype, have several limitations, including medical or physical contraindications, adverse events, and high cost.[1–4] HCV1b is the most frequently observed genotype in Japan (70%) and is also frequently observed in the USA.[3,4] HCV-infected patients tend to develop liver cirrhosis (LC) and ultimately hepatocellular carcinoma (HCC). From this point of view, therapeutic HCV vaccines are also prophylactic cancer vaccines for HCV-related HCC. Thus, there have been a variety of efforts to develop a HCV vaccine, including clinical trials of HCV vaccines with peptides capable of inducing human leukocyte antigen (HLA)-A2- or HLA-A24-restricted CTL responses.[5–7] However, no promising clinical outcome in those trials have been reported at the present time from the view of sustained viral responses. This might be in part due to insufficient activity to boost CTL activity. Alternatively, this might be in part due to an inability to induce humoral responses.

We recently identified a peptide derived from the HCV core protein capable of inducing both cellular and humoral responses to nearly all HCV-positive patients in Japan with various HLA-class I-A alleles (Niu Y, Komatsu N, Komohara Y, Matsueda S, Yutani S, Ishihara Y, Itou M, Yamada A, Itoh K, Shichijo S unpubl. data). This peptide is well known as a HLA-A2-restricted CTL epitope,[8,9] and its sequence is shared in the HCV1a, HCV1b, HCV2a, and HCV3a major genotypes found worldwide. In the present paper, we have reported the results of a phase I dose-escalation study of this peptide vaccination.

Materials and Methods

Patient eligibility. This was a phase I dose-escalation study. All laboratory tests required in order to assess eligibility had to be completed within 7 days prior to the start of treatment. The following inclusion criteria were mandatory:

(1) Patients were required to have persistent HCV infection confirmed by HCV RNA test using serum.
(2) All patients were diagnosed with chronic hepatitis (CH) or LC by laboratory tests (hepatic enzymes and platelet count) and ultrasonography.
(3) Patients were either non-responders to the previously conducted IFN-based treatments ($n = 23$) or refused to receive them ($n = 3$).
(4) Patients had no detectable levels of HCC at the time of entry.
(5) Patients were required to be positive for one of the following alleles: HLA-A2, HLA-A11, HLA-A24, HLA-A26, HLA-A31, or HLA-A33.
(6) Patients were required to have an Eastern Cooperative Oncology Group performance status of 0–1, age between 20 and 75 years, and adequate hematological (white blood cell count ≥2400/μL, hemoglobin level ≥8.0 g/dL, platelet counts ≥50 000/μL), renal (serum creatinine ≤1.4 g/dL), and hepatic (total bilirubin <2.5 mg/dL) functions.
(7) Patients were required to be negative for hepatitis B antigens.
(8) Patients were required to have had at least 4 weeks of recovery from the toxic effects of any previous therapy before trial entry.

Pregnant patients and patients with an autoimmune disease, an active infection, cancer, or hepatic encephalopathy were excluded. Patients with ascites were also excluded. The study protocol was approved by the Ethical Committee of Kurume University, and complete written informed consent was obtained from all patients at the time of enrollment. A total of 26 patients who were seen at our institution between October 2004 and June 2008 were included in this study.

Peptide and vaccination. The vaccinated peptide was originated from HCV core protein at positions 35–44 (C35-44,

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YLLPRRGPRL), a well-known HLA-A2-restricted CTL epitope[8,9] that is conserved in various HCV genotypes (3, 4, 8, and 9). Our manuscript currently under submission elsewhere can be summarized as follows. This peptide demonstrated binding activity to HLA-A*2402, HLA-A*2601, HLA-A*3101, and HLA-A*3303 molecules, but showed no binding to HLA-A*1101. With regard to HLA-A2 subtypes, the peptide demonstrated binding activity to HLA-A*0201 and HLA-A*0206 molecules, but not to HLA-A*0207 molecules. The peptide induced CTL activity in both patients and healthy donors with all the HLA-class I-A molecules mentioned above including HLA-A*1101 and HLA-A*0207, as far as tested by the IFN-γ production assay.

The peptide was prepared under the conditions of Good Manufacturing Practices by the American Peptide Company (San Diego, CA, USA), and was dissolved and stored at –80°C. Stock solutions were diluted with saline just before use. For injection of 0.3, 1, and 3 mg of peptide (levels 1, 2, and 3), 1 mL of the peptide, which was supplied in vials containing 1, 2, or 4 mg/mL sterile solution, was mixed with 1 mL of incomplete Freund's adjuvant (Montanide ISA51VG; Seppic, Paris, France) and emulsified in 5-mL sterilized syringes followed by 0.6, 1, and 1.5 mL injection, respectively. The peptide emulsion was injected biweekly into the subcutaneous region of the side abdomen. All patients were treated in an outpatient clinic. Blood for immunological studies was obtained before vaccine injections, after the sixth and 12th injections. The first cycle consisted of six vaccinations, and second or later cycles of six vaccinations were conducted when the patients agreed and in the absence of severe toxicity. Twenty-five and 22 patients completed the first and second cycle vaccination (six and 12 vaccine injections), respectively.

Cellular and humoral responses to peptides. Thirty milliliters of peripheral blood was obtained before and after vaccinations for the measurement of CTL precursors in peripheral blood mononuclear cells (PBMC) and IgG specific to C35-44 peptide in plasma, according to previously reported methods.[10–13] The positive control peptides used in this study were Epstein–Bar virus (EBV)- and influenza virus (Flu)-derived peptides capable of inducing CTL activity restricted to HLA-A2, HLA-A3 supertype (A11, A31, A33), and HLA-A24 alleles, as reported previously.[10,11,14] The negative control peptides were human immunodeficiency virus (HIV)-derived peptides capable of inducing CTL activity restricted to HLA-A2, HLA-A3 supertype, and HLA-A24 alleles as reported previously,[10,11,14] whereas the sequence of the peptide to the HLA-A26 allele is EVIPMFSAL.[15] In brief, for the CTL precursor assay, the peptide-stimulated PBMC were harvested and tested for their ability to produce IFN-γ in response to T2 cells for HLA-A2$^+$ (A0201, A0206, A0207) cases or CIR cells expressing HLA-A1101, HLA-A2401, HLA-A2601, HLA-A3101, or HLA-A3303 molecules for the corresponding cases. These cells were preloaded with either a corresponding peptide or with a HIV peptide as a negative control. The level of IFN-γ was determined by an ELISA carried out in quadruplicate. A two-tailed Student's *t*-test was used for the statistical analysis. A well was considered positive when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($P < 0.05$) than that in response to a HIV peptide, and when the amount of IFN-γ produced in response to the peptide was more than 50 ng/mL greater than the amount produced in response to a HIV peptide.

The plasma levels of peptide-specific IgG were measured by an ELISA system, and the results were shown as optical density as reported previously.[12] An increment of peptide-specific IgG was judged to have occurred if the IgG amount showed a more than 1.5-fold increase.

The peptide-specific antibody levels were also measured by microsuspension array technique as reported previously.[7] Briefly, diluted plasma samples were incubated with peptide-coated microspheres. After washing, the microspheres were incubated with antihuman Ig isotype-specific antibodies. After washing, the microspheres bound with each antibody were reacted with the biotin-labeled detection antibody and R-phycoerythrin corresponding antibody, and the antibody levels were detected using a Luminex system, FLEXMAP3D, Luminex Corp., Austin, TX. As a control, IgG to HCV core protein was also measured by radioimmunoassay at the commercial level (SRL, Tokyo, Japan), and the levels were shown in international units (IU). All of the pre- and post-vaccination samples were measured simultaneously to avoid any possible biases associated with *in vitro* assays.

Adverse events. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (http://ctep.cancer.gov).

Clinical laboratory data. Clinical laboratory values, such as serum levels of alanine aminotransferase (ALT), alpha fetoprotein (AFP), and blood platelet numbers, were measured by the clinical laboratory division of the Kurume University Hospital. Quantitation of HCV RNA was carried out by a clinical laboratory company (SRL).

Results

Patient demographics. Seven, seven, and 14 patients were initially planned for level 1 (0.3 mg per peptide), level 2 (1 mg per peptide), and level 3 (3 mg per peptide), respectively, in this phase I dose-escalation study. As a result, a total of 26 patients infected with HCV (25 HCV1b and 1 HCV2a: patient 5) were enrolled (Table 1). Among them, three patients under level 1 setting (patients 2, 3, and 6) received at first 0.3 mg per peptide followed by 1 mg per peptide because of both no severe toxicity and stable disease with a new informed consent. Therefore, only five patients were invited for level 2 setting. One patient (patient 14) dropped out of the study after the second vaccination with vaccine-unrelated death. Twenty-five and 22 patients completed the first and second cycle vaccination (six and 12 vaccine injections), respectively, and thus were evaluable for toxicity and immunological evaluations. The patients' characteristics are shown in Table 1. Twenty-one patients were diagnosed with CH, and the remaining five were diagnosed with LC. Of the five LC patients, three had HCC that had been removed prior to their enrollment in the study. Twenty-three patients were non-responders to the IFN-based treatments, whereas the remaining three had no history of IFN therapy.

Toxicity. One LC patient (patient 14) had acute intestinal infection and pneumococcal infection with acidosis, disseminated intravascular coagulation, and renal insufficiency 11 days after the second vaccination, and died of sepsis 21 days after the vaccination. Any vaccination-related symptomatic alterations were not observed after the last vaccination. The institutional safety evaluation committee concluded that the death of patient 14 was not a vaccine-related event. It should be noted that patient 14 had a history of splenectomy 11 months earlier, which might have affected host immunity against the infection. Except for this case, no severe toxicity was observed during the study. Grade 1 or 2 local inflammation at the injection site was observed in 13 or 11 patients during the vaccinations, respectively. The local events disappeared within 1 week after the vaccination in most cases. Grade 1 fatigue and headache were observed in 11 and four patients, whereas grade 1 or 2 flu-like symptoms were observed in three or one patients, respectively. No correlation was observed between the inoculated doses of vaccine peptides and the onset or duration of symptoms. These results indicated that this protocol was well tolerated.

Cellular immune responses. Peptide (C35-44)-specific cellular immune activities were measured using the PBMC before vaccination and after the sixth and 12th vaccinations (Table 2). Each of the two HLA allele-restricted CTL activities was independently measured in the patients with heterozygous HLA-class I-A alleles. Augmentation of peptide-specific CTL responses was judged to have occurred either if the number of positive wells increased or

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Table 1. Patient's characteristics

Patient	HLA type†	Disease‡	Age/Sex	Previous treatment	Peptide dose (mg)	Total vaccination (times)
1	A*3303	CH	50/F	IFN + RBV	0.3	15
2	A*1101/3101	CH	61/F	IFN, IFN + RBV	0.3, 1	33
3	A*2602/A3101	CH	51/M	IFN	0.3, 1	19
4	A*0201/A3303	LC	50/M	IFN + RBV	0.3	38
5	A*0207/A2402	CH	71/M	IFN + RBV	0.3	64
6	A*0206/A2402	CH	55/M	IFN	0.3, 1	57
7	A*0206/A2402	CH	62/M	IFN + RBV	0.3	8
8	A*0206/A3303	CH	49/F	IFNβ, IFN + RBV	1	26
9	A*0206/A3303	CH	38/M	IFN (3 times), p-IFN	1	39
10	A*0201	LC	61/M	IFN + RBV	1	10
11	A*3101	CH	58/M	–	1	13
12	A*0207/A2402	CH	60M	IFN + RBV, p-IFN	1	6
13	A*0206/A2402	LC	61/F	IFN + RBV, IFN	3	22
14	A*0206	LC	69/F	IFN + RBV, p-IFN	3	2
15	A*0201/A3303	LC	58/M	IFN, p-IFN	3	27
16	A*1101/A3101	CH	70/M	IFN + RBV	3	33
17	A*0207/A2402	CH	68/M	IFNβ, IFN + RBV	3	15
18	A*2402	CH	64/M	IFNβ, IFN + RBV	3	19
19	A*2603/A3303	CH	70/M	IFNα, IFNβ	3	29
20	A*2402	CH	62/F	–	3	25
21	A*0201/A3303	CH	53/F	IFN	3	22
22	A*1101/A2402	CH	63/F	–	3	26
23	A*0201/A2603	CH	58/F	IFN + RBV	3	25
24	A*1101/A2402	CH	63/F	IFN + RBV (2 times)	3	24
25	A*2402	CH	57/F	IFN + RBV	3	17
26	A*0201/A2402	CH	58/F	IFN	3	17

†HLA, human leukocyte antigen. ‡CH, chronic hepatitis; IFN, interferon; LC, liver cirrhosis; p-IFN, peg interferon; RBV, ribavirin.

if the amounts of INF-γ increased more than twofold in the case of equal numbers of positive wells in the quadruplicate assays. Under these circumstances, the peptide-specific CTL responses at least by one of the HLA-I-A alleles in PBMC after the sixth vaccination were augmented in one of seven, five of five, and 9 of 13 of patients at levels 1, 2, and 3, respectively. The augmentation also occurred in PBMC after the 12th vaccination. CTL augmentation in more than two wells among four wells occurred mostly in the post-vaccination samples from patients with levels 2 and 3. These results indicated that level 1 (0.3 mg peptide per injection) is not sufficient to induce peptide-specific CTL activity, and level 2 (1 mg peptide) seemed to be sufficient to induce CTL activity under this biweekly protocol.

We also measured HLA-restricted CTL activity to EBV-derived peptide in 15 patients and Flu-derived peptide in nine patients, taken as positive peptides, to determine whether or not the vaccination of C35-44 peptide influences cellular responses to the other viruses. Pre-injection, post-sixth injection, and post-12th injection PBMC from 15 patients were incubated with each of C35-44, EBV, or Flu peptide relevant to the patients' HLA alleles, after which their CTL activity was measured in quadruplicate assays. CTL precursors were considered to be present when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($P < 0.05$) than that in response to a HIV peptide, and if the amount of IFN-γ produced in response to the peptide was more than 50 ng/mL greater than the amount produced in response to a HIV peptide. Under these circumstances, the CTL precursors for C35-44, EBV, and Flu were detectable in the pre-vaccination PBMC from 6 of 15, 2 of 15, and one of nine patients tested; in the post-sixth vaccination, PBMC from 9 of 15, 4 of 14, and six of nine patients; and in the post-12th vaccination PBMC from 11 of 13, 4 of 13, and six of nine patients, respectively (data not shown). Representative results of four cases are shown in Figure 1. These results indicate that the C35-44 peptide vaccination did not suppress, but rather had a trend to facilitate CTL activity to both EBV- and Flu-derived peptides.

Humoral immune responses. Peptide (C35-44)-specific IgG responses were measured using plasma collected before vaccination and after the sixth and 12th vaccinations (Table 2). The increment was rarely observed in the samples collected after the sixth injections (5 of 26 patients, 19%), but was observed in the majority (15 of 22 patients, 68%) of the post-12th vaccination samples without a dose-dependent manner.

We further measured all the other Ig isotypes and all IgG subclasses reactive to the vaccinated peptide to examine a dominant type of vaccine-induced immune reaction (Th1- or Th2-type reactions), if any. IgG against HCV core protein was also measured as a control. As a result, augmentation in all the other Ig isotypes and all IgG subclasses (IgG1–IgG4) was observed in most patients whose plasma showed the increased IgG response shown in Table 2. Detailed results are given in Tables 3 and 4. The results suggest that both Th1 and Th2 cells are involved in the vaccination-induced humoral responses. The vaccination, however, did not augment IgG reactive to a recombinant HCV core protein (Table 3).

Clinical evaluation. Clinical evaluation was not the objective of this phase 1 study. However, the available information, though very limited, might be important for developing further clinical studies of peptide-based vaccination to HCV-infected patients. During the vaccination period for up to the 12th vaccination, no patient received any treatment other than the vaccination; notably, none of them received injection of glycyrrhizin, a standard drug for patients unresponsive to IFN-based therapies. Detailed results of the clinical evaluation are given in Table 5. A more than one log difference in HCV RNA was considered significant, whereas a more than 30% difference with a consistent trend throughout the vaccination period (1st to 12th) in ALT, platelet counts, and AFP was considered significant. Under these circumstances, no patient showed an increase in HCV RNA, whereas two patients showed a decrease. Two patients at level 1 showed an increase in ALT after the vaccination, whereas seven patients showed a significant decrease. No patient showed either a significant decrease or increase in platelet count. Three patients showed a significant decrease in

Table 2. Immune responses during vaccination†

Patient	CTL response (IFN-γ, pg/mL)				IgG response (OD)§		
	HLA-restriction‡	Pre	Post-sixth	Post-12th	Pre	Post-6th	Post-12th
1	A*3303	0	0	61/66¶	0.403	0.441	1.238
2	A*1101	0	0	0	0.637	0.743	1.624
	A*3101	0	0	0			
3	A*2602	0	179	50	0.526	1.786	2.335
	A*3101	0	56	0			
4	A*0201	0	0	111	0.24	0.24	0.326
	A*3303	0	0	0			
5	A*0207	61	0	130	0.8	2	2
	A*2402	0	0	0			
6	A*0206	0	0	0	0.805	0.878	0.959
	A*2402	0	0	0			
7	A*0206	0	0	NA	0.94	1	2.639
	A*2402	0	0	616			
8	A*0206	182	60	94	0.512	0.65	2.735
	A*3303	0	55	0			
9	A*0206	0	0	82	0.867	0.912	2.82
	A*3303	0	272	0			
10	A*0201	0	52/107/116	NA	0.556	0.456	NA
11	A*3101	82	168/213/571	NA	0.677	0.488	NA
12	A*0207	0	94	NA	0.853	0.631	NA
	A*2402	0	467				
13	A*0206	0	0	405/1094/1534	1.588	1.595	2.568
	A*2402	0	50	0			
15	A*0201	340	277/278	0	0.764	0.66	1.164
	A*3303	0	0	0			
16	A*1101	0	0	0	0.1	0.2	0.4
	A*3101	0	0	0			
17	A*0207	0	0	0	1.296	1	1.631
	A*2402	0	153/2339	780			
18	A*2402	0	0	0	0.5	0.5	0.8
19	A*2603	0	0	152/1147	2	2	2.5
	A*3303	0	66	0			
20	A*2402	0	69/343	276	2.538	2.33	2.761
21	A*0201	169	1670	290/380/1520	0.514	0.922	2.703
	A*3303	161	92/643	163/650/678/5288			
22	A*1101	86/332/395	0	0	0.824	0.685	0.985
	A*2402	151	513	88			
23	A*0201	0	199	0	1.128	1.317	1.768
	A*2603	76	104	50			
24	A*1101	134	0	0	1.087	2.084	2.757
	A*2402	90	0	111/250			
25	A*2402	0	0	0	0.886	0.556	0.597
26	A*0201	0	74/171	54/56/79	1.296	0.801	2.368
	A*2402	0	0	166			

†CTL activity was measured by interferon (IFN)-γ production assay, whereas IgG response was measured by ELISA; ‡HLA alle-restricted CTL responses were measured; §optical density at 450 nm; ¶IFN-γ production levels in positive wells of quadruplicate culture are shown. Background levels of values (<50 pg/mL) are indicated as 0. NA, not available.

AFP, a biomarker for liver cancer, among six patients whose pre-vaccination AFP levels exceeded the normal range (>8.7 ng/mL). In addition, one patient at level 1 showed a sharp but transient decline in AFP after the sixth vaccination.

Discussion

Liver damage may be induced by the boosted CTL-directed destruction of HCV-infected liver cells. However, there was no such liver damage throughout the vaccination period despite the fact that peptide vaccination induced both cellular and humoral responses in the majority (>60%) of patients. Rather, decreases in ALT and AFP were seen in a substantial numbers of patients. The other concern was the difficulty of inducing immune responses by the peptide vaccination for non-responders to IFN-based therapy, primarily because of the heterogeneity of HCV, and also because the immune system was suppressed in HCV-positive patients.[16,17] However, this concern also did not arise, and the vaccination successfully induced immune responses in the post-vaccination samples of the majority (>60%) of patients in both the CTL and IgG assays. Our present results, along with the recent increased demand for development of a HCV vaccine,[18] keep alive our hope of developing a clinically effective HCV vaccine.

Level 1 (0.3 mg per peptide) was considered too low to induce CTL activity. Level 2 (1 mg per peptide) was considered an optimal dose to induce CTL activity under this biweekly injection protocol, although level 3 (3 mg per peptide) was also recommended

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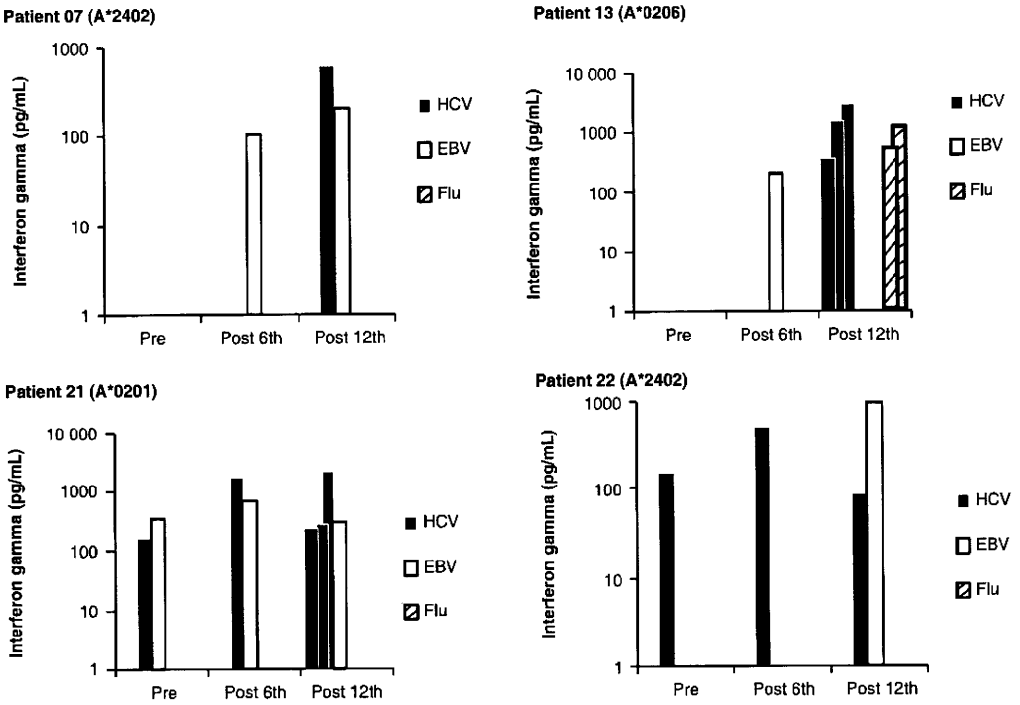

Fig. 1. CTL activity to C35-44, Epstein–Barr virus (EBV), and influenza virus (Flu) peptides. Peripheral blood mononuclear cells from pre-, post-6th, and post-12th vaccination were incubated with each of C35-44, EBV, or Flu peptide relevant to the patients' human leukocyte antigen (HLA) alleles, after which their CTL activity was measured in quadruplicate assays. Representative results of four cases (patients 7, 13, 21, and 22) are shown. Each bar indicates the interferon-γ value of positive wells of quadruplicate culture.

Table 3. Isotypes of antipeptide Ig during vaccination[†]

Patient	Anti-C35 peptide antibody								Anti-HCV core IgG	
	IgG		IgA		IgM		IgE			
	Pre	Post-12th	Pre	Post-12th	Pre	Post-12th	Pre	Post-12th	Pre	Post-12th
1	1721	2379	387	590	144	338	48	62	130	130
2	823	1071	174	227	88	103	16	17	210	180
5	13 803	12 571	6064	5479	393	276	651	596	350	330
6	432	507	85	123	94	75	10	15	47	49
8	4781	12 158	1668	5732	154	286	158	560	120	130
9	10 531	11 807	4541	4693	32	83	375	398	220	220
10	514	645	191	220	354	412	13	20	460	440
11	13 023	NA[†]	417	NA	70	NA	536	NA	NA	NA
12	2054	NA	417	NA	37	NA	58	NA	NA	NA
13	740	5070	378	1067	<5	<5	121	151	340	420
15	635	1052	33	426	127	916	19	39	86	100
16	407	1115	117	378	<5	24	8	29	120	120
17	14 537	13 154	6502	6196	70	68	874	822	260	190
18	2357	2615	610	833	50	202	60	61	260	240
19	2449	6720	677	2244	<5	626	43	175	230	250
20	8107	13 318	2990	6058	75	321	339	690	260	330
21	271	5743	68	2224	<5	383	8	154	46	77
22	2987	2134	768	534	177	88	90	68	170	79
23	3445	2925	1148	966	82	72	99	88	170	130
24	477	5404	160	2489	415	441	15	185	49	32
25	13 490	11 111	5865	4168	96	242	474	369	430	330
26	1370	2943	417	1238	169	233	31	100	130	99

[†]Isotypes of antipeptide (C35-44) Ig in the pre- and post-vaccination (12th) plasma were measured using a Luminex system, and the levels were shown by fluorescence intensity units (FIU). As a control, IgG to hepatitis C virus (HCV) core protein was also measured by radioimmunoassay, and the levels were shown by international units (IU). NA, not available.

Table 4. Subclasses of antipeptide IgG during vaccination[†]

Patient	Anti-C35 IgG subclass							
	IgG1		IgG2		IgG3		IgG4	
	Pre	Post-12th	Pre	Post-12th	Pre	Post-12th	Pre	Post-12th
1	1002	1735	462	641	309	487	596	783
2	301	410	241	294	265	209	335	404
5	17 950	17 095	9868	8498	6214	5432	8038	7302
6	291	372	117	124	57	73	119	166
8	4994	15 594	2681	9217	1341	5395	2228	7098
9	10 401	13 220	6351	7872	3821	4672	5303	5927
10	803	916	237	250	122	185	386	422
11	17 640	NA	6552	NA	5887	NA	5490	NA
12	2853	NA	495	NA	484	NA	363	NA
13	5541	6750	1927	2269	1550	1635	1881	2148
15	567	1123	148	373	57	227	184	604
16	557	1268	131	501	89	559	172	395
17	17 578	16 423	9124	8877	6977	6276	7796	7797
18	2151	3192	899	1104	600	760	928	1160
19	557	1268	131	501	611	2057	820	2761
20	9753	16 953	4718	9452	2915	6610	4423	8552
21	196	5939	122	3071	57	2240	66	2546
22	2249	1660	878	528	594	334	938	603
23	3795	3297	1368	1166	843	680	1436	1320
24	583	7089	131	3553	102	2323	98	2218
25	14 979	11 654	4428	2760	5166	3928	4647	3396
26	1979	3278	366	1182	403	2318	305	940

[†]Subclasses of antipeptide (C35-44) IgG in the pre- and post-vaccination (12th) plasma were measured using a Luminex system, and the levels were shown by fluorescence intensity units (FIU). NA, not available.

Table 5. Data for clinical outcomes after vaccination

Patient	HCV RNA (kIU/mL)			ALT (IU/L)			Plt. ($\times 10^4$/mL)			AFP (μg/mL)		
	Pre	Post-6th	Post-12th	Pre	Post-6th	Post-12th	Pre	Post-6th	Post-12th	Pre	Post-6th	Post-12th
1	1730	1830	2120	62	59	83	13.7	13.4	12.4	10.1	11.9	12.6
2	91	27	NA	77	51	NA	11.4	11.7	NA	32.6	51.1	NA
3	3420	4140	3710	41	51	64	22.4	25.4	24.0	3.2	3.5	3.7
4	500	96	6	206	157	105	11.5	8.7	10.6	74.3	32.2	67.5
5	114	102	328	114	154	186	9.3	10.2	8.7	NA	7.8	10.0
6	892	648	840	60	29	48	13.8	14.4	15.0	5.4	6.1	6.2
7	2100	1680	NA	95	100	NA	7.8	8.0	NA	7.0	NA	NA
8	2480	2660	2290	129	112	108	15.8	17.3	15.2	44.4	29.2	28.2
9	2870	2950	2330	358	233	245	5.8	7.4	6.8	28.7	19.5	19.7
10	32 000	16 000	NA	104	95	NA	11.2	10.3	NA	26.9	24.2	NA
11	20	NA	NA	83	NA	NA	13.9	NA	NA	5.6	NA	NA
12	25 000	25 000	NA	144	155	NA	14.9	14.6	NA	5.6	8.1	NA
13	1670	2130	2660	53	47	49	9.4	7.9	7.8	131	86.9	74.0
15	2130	2810	2600	45	34	25	8.0	8.4	9.2	6.8	7.6	7.9
16	2470	3510	2440	47	52	45	25.2	26.0	24.0	4.7	NA	5.0
17	2260	2140	1540	37	31	32	17.3	19.7	19.1	4	3.4	NA
18	3630	3300	2972	60	50	63	13.2	15.0	16.3	22.7	24.4	27.5
19	591	489	443	51	52	53	23.7	24.4	23.8	4.2	NA	3.3
20	583	591	346	24	22	22	19.2	17.3	20.4	4.9	NA	NA
21	4370	3575	3940	34	37	33	23.1	21.2	23.1	NA	NA	3.6
22	59	<5	5	73	25	40	12.4	12.1	12.1	3.1	NA	1.5
23	3045	2380	2730	66	64	65	10.1	10.0	11.1	8.2	9.4	7.6
24	2230	2095	4510	52	36	27	11.7	12.2	11.4	2.9	NA	NA
25	2340	3160	5000	55	55	49	11.1	10.2	12.0	8.5	NA	NA
26	2150	3025	NA	80	58	56	12.4	14.4	14.9	6.8	NA	NA

AFP, α-fetroprotein; ALT, alanine aminotransferase; NA, not assessed; Plt., platelet number.

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because of the higher rate of CTL induction. In contrast, the boosting of IgG production specific to this peptide seemed not to be dependent on the dose, and even level 1 was associated with an increase (>1.5 fold) in IgG in the post-vaccination (12th) plasma from five of seven patients.

We previously reported that a personalized peptide vaccination protocol is superior to a designated protocol from the standpoints of immune responses and clinical responses in patients with advanced stages of cancer.[19–21] This superiority might be due in part to the fact that the pre-designated peptide vaccination stimulates naive or resting T cells and suppresses memory or activated T cells in certain cases, whereas the personalized peptide vaccination stimulates the latter types of T cells.[19–21] In the present study, significant levels of peptide-specific IgG were detected in pre-vaccination plasma of all 26 patients, indicating that memory B cells at least exist in all 26 patients. The vaccination did not suppress CTL activity against Flu or EBV, but rather had a trend to facilitate CTL activity to EVB- and Flu-derived peptides. Based on these results, we considered that this peptide stimulated memory or activated T cells in those HCV-infected patients, which in turn resulted in the higher rates of immune boosting without suppression of the CTL activity to other viruses in HCV-infected patients who failed the IFN-based therapies.

This peptide boosted the CTL activity restricted to each of the HLA-A2 (A0201, A0206, A0207), HLA-A24 (A2402), HLA-A26 (A2602, A2603), HLA-A31 (A3101), and HLA-A33 (A3303) molecules in the post-vaccination samples as far as tested. This is consistent with the results of an *in vitro* analysis (Niu Y, Komatsu N, Komohara Y, Matsueda S, Yutani S, Ishihara Y, Itou M, Yamada A, Itoh K, Shichijo S unpubl. data). Although the CTL boosting was observed in none of the four HLA-A1101$^+$ patients, further studies with additional cases should be conducted to determine whether or not this peptide can boost CTL activity by *in vivo* vaccination for HLA-A1101$^+$ patients.

The sequence of this peptide is well conserved in the different genotypes of HCV, and thus could be applicable not only to HCV1b patients but also to HCV2a patients. Indeed, this peptide boosted both CTL and IgG responses in one HCV2a patient (patient 05) who received 0.3 mg vaccination, although further studies with additional HCV2a cases should be conducted to confirm this possibility.

Although the clinical benefit of this peptide vaccination will be addressed in a future phase II clinical study, it is of note that a decrease in ALT was observed in 7 of 24 patients (29%) during the vaccination. Th1-type immune responses are suggested to be involved in liver damage at chronic phase.[22,23] Therefore, the vaccination-induced CTL boosting is expected to be associated with increased ALT. Indeed, Klade *et al.* reported two such cases who had a transient decrease in HCV RNA concomitant with an ALT increase during the HCV peptide vaccination.[6] In contrast, our two cases showed declines of both HCV RNA and ALT. Although a reason for this discrepancy is unclear at the present time, the different protocols would be at least responsible for this discrepancy. We used only one CTL epitope (C35-44) emulsified with ISA51 adjuvant, whereas they used five peptides containing four CTL epitopes and three helper epitopes with poly-L-arginine as an adjuvant. Augmentation of CTL or IgG responses was observed in six of seven patients showing a decline in ALT in this trial. Nelson *et al.* reported that interleukin-10 treatment results in normalization of ALT in the majority of CH patients who are non-responders to IFN-based treatments.[24] Interleukin-10 promotes the production of IgA, IgG1, and IgG2.[25,26] We showed that all three of these Ig were increased in the post-vaccination samples, suggesting that Th2-type immune responses are at least boosted by the vaccination. Therefore, an increment in Th2-type reactions might be in part responsible for the decline of ALT in these seven patients, although the biological function of these antibodies specific to C35-44 peptide is unknown at the present time.

In addition to ALT, a decrease in AFP level was also observed in three of six evaluable patients. It is well known that HCV core protein plays a pivotal role in the development of HCC.[27,28] Therefore, the vaccination-induced CTL boosting might eliminate precancerous liver cells expressing the HLA-class I-A–C35-44 peptide complex, which may in turn result in decreased AFP. If this is the case, this vaccine may be effective as a cancer prophylactic in CH or LC patients who are resistant to IFN-based therapies and have a high risk of HCC. One LC patient (patient 4) who had a history of HCC showed a decrease in HCV RNA by the vaccination alone. This patient had received IFN-based therapy combined with the vaccination with the result of sustained viral responses. These results suggest the possible benefit of vaccination and IFN therapy for certain HCV patients who fail the standard IFN-based treatment.

HCV is known as a highly variable virus, but the amino acid sequence of this peptide is well conserved in the entire HCV genotype. HLA-A2, HLA-A11, HLA-A24, HLA-A26, HLA-A31, and HLA-A33 types constitute >99% of Japanese, 98% of Asian, 74% of Caucasian, and 50–67% of Black people.[29] This peptide can induce both cellular and humoral responses in patients with these HLA-class I-A alleles. Therefore, this peptide might be useful as a therapeutic HCV vaccine, as well as a prophylactic cancer vaccine for HCV-related HCC, for the majority of people in the world.

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Disclosure Statement

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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.***

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

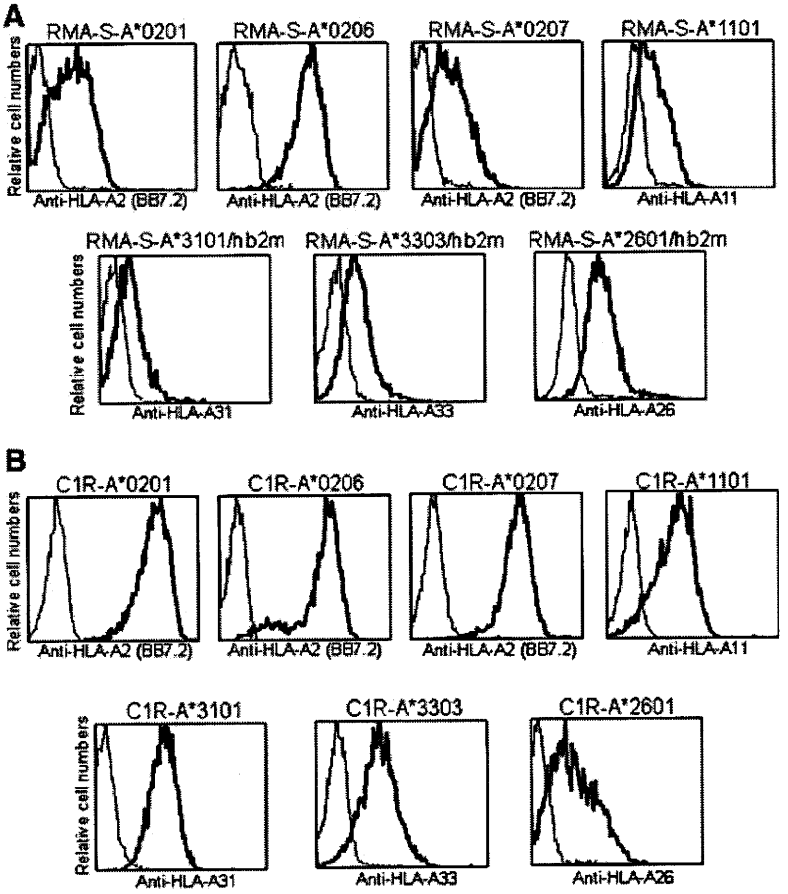

Fig. 1. HLA expression in RMA-S and C1R stable transfectant cells. After construction of RMA-S (**A**) and C1R (**B**) stable transfectant cell lines expressing HLA molecules alone or together with hb2m, HLA protein expression was confirmed by flow cytometry analysis. Cells were stained with monoclonal 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

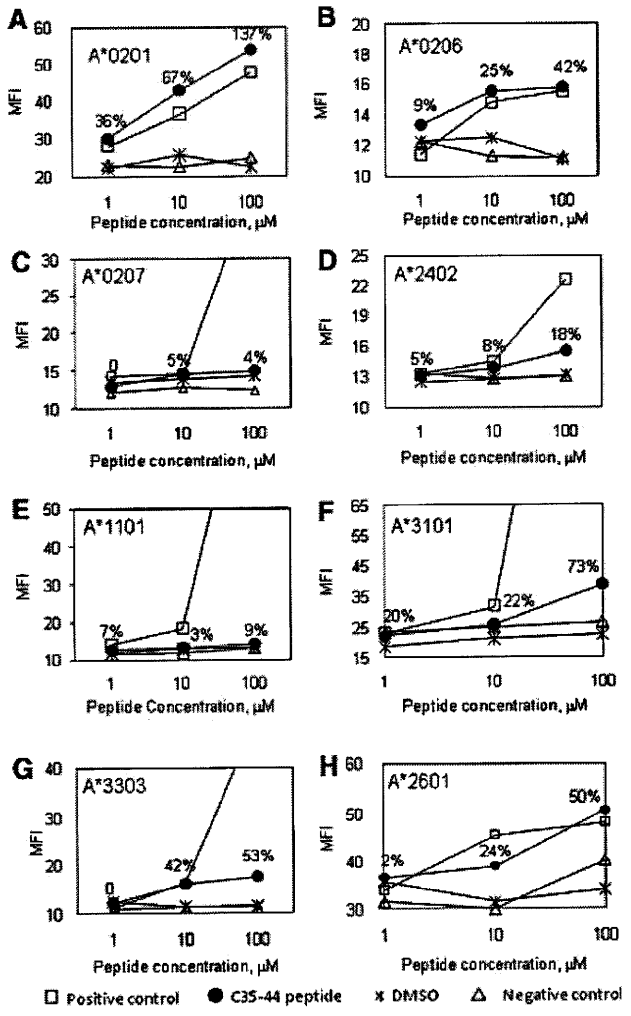

Fig. 2. Stabilization assay of the C35-44 peptide to various HLA molecules. The binding activities of the C35-44 peptide to HLA-A*0201 (**A**), -A*0206 (**B**), -A*0207 (**C**), -A*2402 (**D**), -A*1101 (**E**), -A*3101 (**F**), -A*3303 (**G**), and -A*2601 (**H**) were examined by using RMA-S cells 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

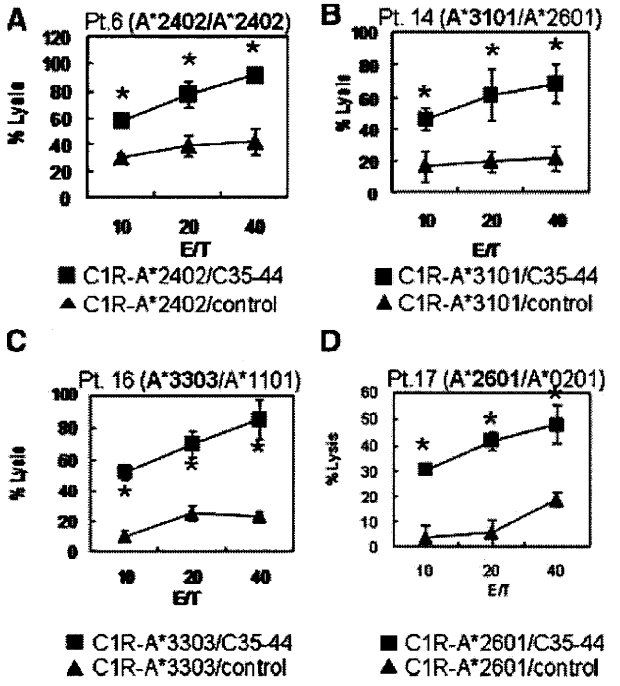

Fig. 3. Cytotoxicity of C35-44 peptide-specific CTLs generated in vitro from patients infected HCV 1b. $CD8^+$ T cells purified from the peptide-stimulated PBMCs of HCV $1b^+$ patients with different HLA alleles were tested for their cytotoxicity at E/T ratio of 10, 20, and 40 by ^{51}Cr-release assay 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.

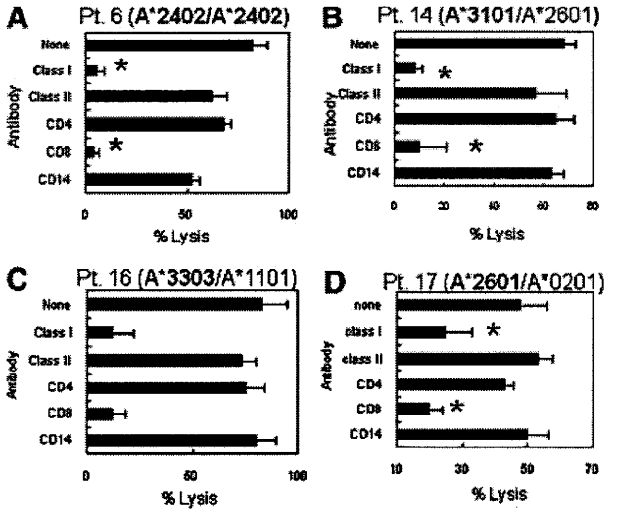

Fig. 4. Specificity of C35-44 peptide induced CTLs cytotoxicities from HCV $1b^+$ patients C35-44 peptide-pulsed CTLs and corresponding C1R stable transfectant cells were treated with anti-CD4, -CD8, -CD14, -MHC class I, or -MHC class II antibody (20 μg/ml) at 37°C for 1 hr, and then the cytotoxicity 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

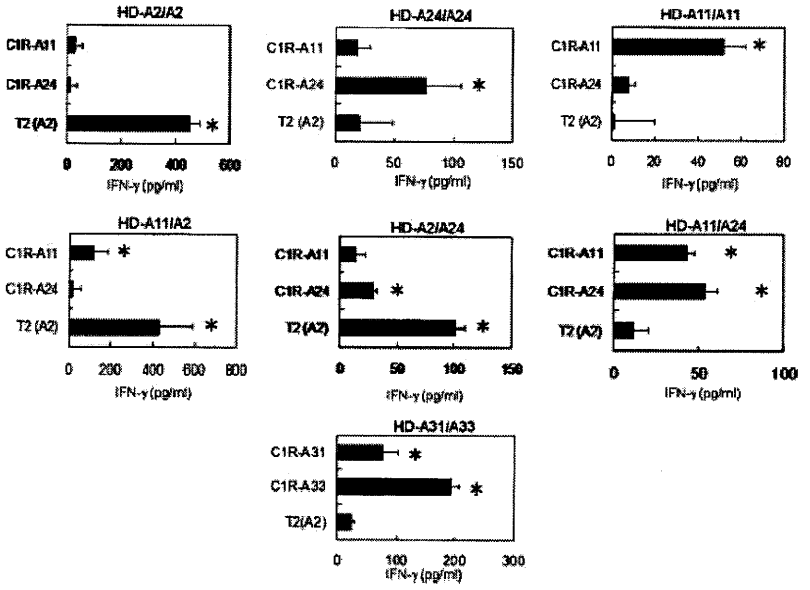

Fig. 5. Induction of C35-44 peptide-specific CTLs from PBMCs of healthy donors PBMCs from HCV-negative healthy donors of different HLA types were stimulated with C35-44 or HIV peptides for seven times and then incubated without peptide for another 5–7 days. PBMCs were incubated with C35-44 peptide-pulsed 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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