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

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	HLA-restriction[‡]	CTL response (IFN-γ, pg/mL)			IgG response (OD)[§]		
		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
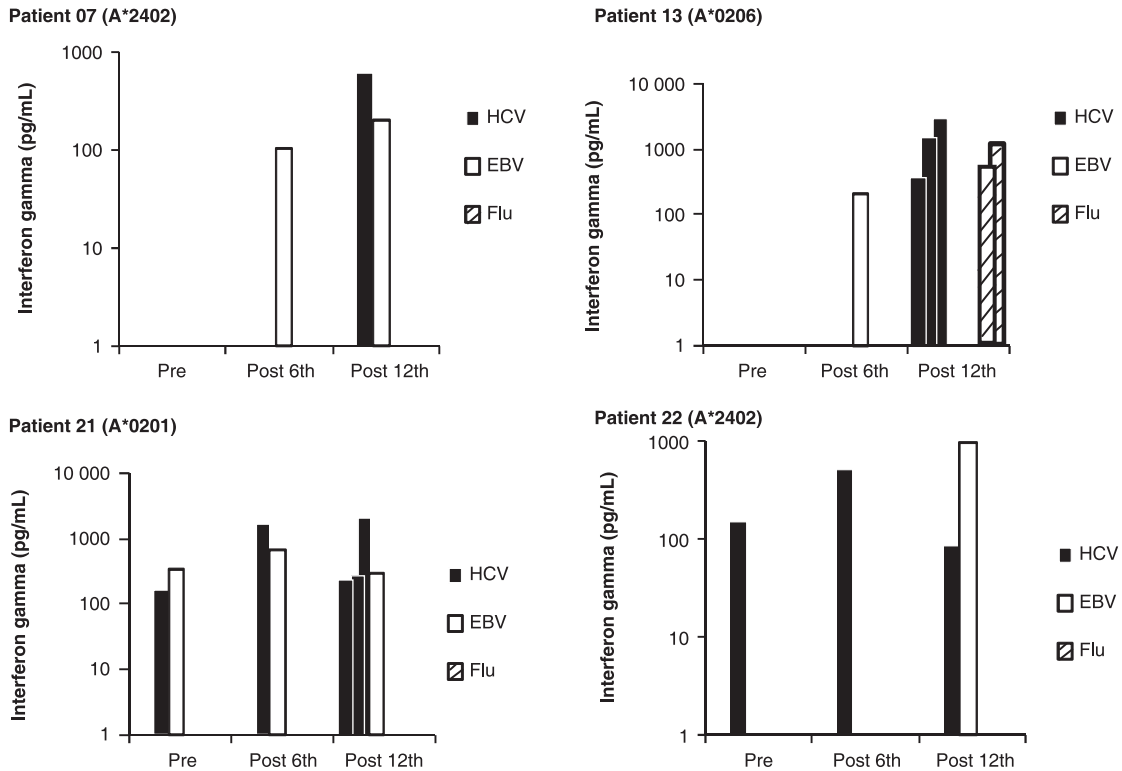
Fig. 1. CTL activity to C35-44, Epstein–Barr virus (EBV), and influenza virus (Flu) peptides. Peripheral blood mononuclear cells from pre-, post-6th, and post-12th vaccination were incubated with each of C35-44, EBV, or Flu peptide relevant to the patients' human leukocyte antigen (HLA) alleles, after 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.

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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