

Antibody Reactive to a Hepatitis C Virus (HCV)-Derived Peptide Capable of Inducing HLA-A2 Restricted Cytotoxic T Lymphocytes Is Detectable in a Majority of HCV-Infected Individuals without HLA-A2 Restriction

Yukari Takao^{1,2,3}, Akira Yamada^{*,3}, Shigeru Yutani¹, Michio Sata², and Kyogo Itoh^{1,3}

Departments of ¹Immunology and ²Internal Medicine, Kurume University School of Medicine, ³Kurume University Research Center for Innovative Cancer Therapy, and Center of the 21st Century COE Program for Medical Science, Kurume University, Kurume, Fukuoka 830–0011, Japan

Received January 6, 2004; in revised form, April 8, 2004. Accepted April 16, 2004

Abstract: Hepatitis C virus (HCV) is a single-strand RNA virus. Approximately 170 million people around the world are persistently infected and are at risk of liver cirrhosis or cancer. There is an urgent need to develop both therapeutic and diagnostic modalities of HCV. One approach to achieve these goals would be to determine highly immunodominant HCV peptides which are recognized by both cellular and humoral immunities. This study reports one such peptide, HCV-core protein at positions 35–44, having HLA-A2 binding motifs. IgG specific to this CTL-epitope peptide is consistently detectable in a majority of the patients with HCV infection regardless of the different HLA types, different disease conditions, and different HCV-genotypes tested. The sequence LPRR at positions 37–40 is considered to be the fine epitope recognized by the IgG. These results may provide new insights for the development of both therapeutic and diagnostic modalities of HCV at lower costs.

Key words: HCV, Peptide, Cytotoxic T-lymphocytes, Antibody

Extensive studies have been carried out in the past-decade in order to find immunodominant HCV-peptides, and there are many peptides capable of inducing cellular immune responses (4, 15, 19, 23, 25). None of them, however, are clinically effective. Subsequently, there is no immunization yet available, neither prophylactic nor therapeutic (7, 12). This could be, in part, due to either the weak immunogenicity of these previously reported peptides or insufficient studies regarding them. Although interferon and other reagents are effective for eliminating HCV in certain patients, they are too expensive for the majority of HCV⁺ patients in most countries. Subsequently, there is an urgent need to determine immunodominant peptides useful for the development of effective and low-priced vaccines. In addition, there is also a need to develop a simple and low-priced diagnostic tool for HCV since the currently

available kit is also expensive for the majority of people who are not living in developed countries. We previously reported that some CTL-directed peptides derived from non-mutated self-antigens were recognized by humoral immune response in both healthy subjects and cancer patients without apparent HLA-restriction (10, 16, 17, 21). Elevated levels of IgG to those CTL-epitope peptides by peptide vaccination showed a close correlation with long-term survival in vaccinated cancer patients (16, 21), whereas the class of IgG to those peptides was either lacking or unbalanced in atopic dermatitis patients (14), suggesting their positive role in host defense. In addition, there is strong evidence that

*Address correspondence to Dr. Akira Yamada, Cancer Vaccine Development Division, Kurume University Research Center for Innovative Cancer Therapy, 67 Asahi-machi, Kurume, Fukuoka 830–0011, Japan. Fax: +81–942–7745. E-mail: akiymd@med.kurume-u.ac.jp

Abbreviations: Ab, antibody; CH, chronic hepatitis; CTL, cytotoxic T lymphocyte; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HD, healthy donor; HIV-1, human immunodeficiency virus-1; HLA, human leukocyte antigen; HTLV-I, human T-lymphotropic virus type I; LC, liver cirrhosis; OD, optical density; PBMC, peripheral blood mononuclear cells; Pt., patient; RIA, radio immunoassay.

both cellular and humoral immune responses play a major role in the control of HCV infection (9, 12, 25). We then hypothesized that HCV-peptides recognized by both cellular and humoral immunities were more immunogenic than those recognized by either immunity alone, and thus are applicable for the development of both new treatment and diagnosis modalities for HCV infection. This study reports one such peptide, an HCV core peptide at positions 35–44, to which high levels of IgG are detectable in a majority of HCV-infected individuals without apparent HLA-A restriction. These results may provide a new insight useful in the development of both therapeutic and diagnostic modalities for HCV infection.

Materials and Methods

Subjects. The Institutional Ethical Review Board of Kurume University approved this study protocol, and informed written consent was obtained from all the subjects from whom sera and peripheral blood mononuclear cells (PBMCs) were taken in this study. Twelve HCV⁺ patients were seropositive for anti-HCV antibodies (Abs) as determined by second- or third-generation immunoassay test. Diagnoses of these patients deter-

mined by biochemical and histological findings, ultrasound-sonography, and computed tomography were chronic hepatitis (CH, $n=5$), liver cirrhosis (LC, $n=4$), and hepatocellular carcinoma (HCC, $n=3$) at the first time of serum samplings. As negative controls, sera from 10 HDs with no history of either viral hepatitis or vaccination of HBV, and normal liver function were tested in this study.

Peptide and measurement of peptide-reactive Ab. We used 18 different kinds of synthetic peptides having HLA-A2 binding motifs (20) from well-conserved regions of HCV-genotype 1b proteins (Table 1). An HIV derived-peptide with HLA-A2 binding motif as the negative control (11) and 15 10-mer peptides possessing overlap sequences spanning HCV core 27–50 (Fig. 3B) were also used. These peptides were purchased from BIO SYNTHESIS (Lewisville, Tex., U.S.A.) or SynPep (Dublin, Calif., U.S.A.). The desalted grade of the materials was used for the screening of peptides reactive to serum IgG or >90% purified materials for further studies as assessed by reverse-phase high-pressure liquid chromatography analyses. Serum levels of peptide-specific IgG were measured by means of an enzyme-linked immunosorbent assay (ELISA) employing a method previously reported (18). In brief, each peptide was

Table 1. HCV-derived peptides used in this study and serum reactivity^{a)}

Peptides			Serum reactivity	
Number	Region	Sequence	HCV patients ($n=12$)	HDs ($n=10$)
1	C 35–44	YLLPRRGPR	11	0
2	C 132–140	DLMGYIPLV	0	0
3	C 150–158	ALAHGVRAL	0	1
4	C 168–176	NLPGCSFSI	0	1
5	C 178–187	LLALLSCLTV	1	0
6	E1 220–229	ILHTPGCV	3	0
7	E2 363–372	SMVGNWAKV	0	0
8	E2 401–411	SLLAPGAKQNV	0	3
9	NS3 1073–1081	CINGVCWTV	0	0
10	NS3 1169–1178	LLCPAGINAV	1	2
11	NS3 1287–1296	KLVALGINAV	1	0
12	NS3 1406–1505	TGAPVTYSTY	1	0
13	NS4B 1789–1797	SLMAFTAAV	0	0
14	NS4B 1807–1816	LLFNILGGWV	0	0
15	NS4B 1851–1859	ILAGYGAGV	0	1
16	NS5A 2252–2260	ILDSFDPLV	0	0
17	NS5B 2692–2701	RLIVFPDLGV	1	0
18	NS5B 2727–2785	GLQDCTMLV	2	1

^{a)} We used 18 peptides containing HLA-A2 binding motifs that were previously shown to represent target epitopes of HLA-A2-restricted CTL responses directed to HCV antigens. We measured the levels of peptide-specific IgG in the sera of 12 patients with HCV-1b infection and 10 healthy donors (HDs). The cut-off value for this anti-HCV core 35–44 peptide at a serum dilution of 1:100 was determined as 0.18 (the mean <0.03> plus 3SD <0.05 times 3> of 10HDs). Abbreviations: C, core; E, envelope; NS, non-structure.

dissolved in dimethylsulfoxide (DMSO) and then stored at -20°C . Peptides (20 $\mu\text{g}/\text{well}$) diluted with 0.1 M carbonate buffer containing a chemical cross-linker, disuccinimidyl suberate (DSS) (PIERCE, Rockford, Ill., U.S.A.), were bound to the ELISA plate. The wells were rinsed three times with 0.05% Tween 20-PBS (PBST). The plates were blocked overnight at 4°C with Block Ace (Yukijirushi, Tokyo). Plasma or serum samples were diluted at 1:100, 1:200, and 1:400 with 0.05% Tween 20-Block Ace, and 100 $\mu\text{l}/\text{well}$ of samples was added to each well. After incubation at 37°C for 2 hr, the plates were washed with PBST three times and incubated at 37°C for 2 hr with 100 $\mu\text{l}/\text{well}$ of 1:1,000-diluted rabbit anti-human IgG (γ -chain-specific) (DAKO, Glostrup, Denmark). After washing three times, 100 $\mu\text{l}/\text{well}$ of 1:100-diluted anti-rabbit IgG-conjugated horseradish peroxidase-dextran polymer (EnVision, DAKO) was added to each well, and the plates were incubated at room temperature for 40 min. After washing, 100 $\mu\text{l}/\text{well}$ of tetramethylbenzidine substrate solution (KPL, Guildford, U.K.) was added, and the reaction was stopped by the addition of 1.0 M of phosphoric acid. Cut-off values were calculated as the mean + 3SD of optical density (OD) of sera from HDs taken as a negative control, and it was 0.18 for the screening assay and 0.2 for the fine epitope analysis with desalted peptides, or 0.093 with peptides of >90% purity.

Absorption and elution of peptide-reactive IgG. To test the specificity of anti-peptide IgG in the serum samples, 100 $\mu\text{l}/\text{well}$ of serum samples (1:100 dilution with 0.05% Tween 20-Block Ace) was absorbed by immobilized peptides (20 $\mu\text{g}/\text{well}$) in wells of the plate for 2 hr at 37°C . The same procedure was repeated three times, and the levels of peptide-specific IgG in the supernatant were measured by ELISA. For an elution test, Abs bound to the peptide-immobilized plates by the first absorption were eluted by 30 $\mu\text{l}/\text{well}$ of 0.5 M NaCl/50 mM citrate buffer (pH 3.0), neutralized by 1.0 M Tris-HCl (pH 7.5) with 0.05% Tween 20-Block Ace, and the levels of peptide-specific IgG in the eluted fraction were measured by ELISA.

Assay for peptide-specific CTLs. The method used for the detection of peptide-specific CTLs has been reported elsewhere (11, 13). In brief, PBMCs (1×10^5 cells/well) were cultured with 10 μM of each peptide (quadricate) in wells of a U-bottom-type 96-well micro-culture plate (Nunc, Roskilde, Denmark) in 200 μl of culture medium. The medium consisted of 45% RPMI-1640 (Invitrogen, Carlsbad, Calif., U.S.A.), 45% AIM-V (Invitrogen), 10% FCS, 100 U/ml of IL-2 (Shionogi, Osaka, Japan), and 0.1 mM MEM non-essential amino acid solution (Invitrogen). On days 3, 6, and 9, half of the medium was removed and replaced with new medi-

um containing the corresponding peptide (20 $\mu\text{g}/\text{ml}$). On day 12 of the culture, cells of each of the quadricate cultures were divided and placed into four wells and tested for their ability to produce IFN- γ in response to T2 cells pre-loaded with either a corresponding peptide or an HIV-peptide as a negative control in duplicate assays. The background IFN- γ production in response to the HIV peptide (<50 ng/ml) was subtracted from the values given in the data. The CD8 $^{+}$ cells were positively purified using the CD8 Isolation Kit (DYNAL, Oslo, Norway) in certain experiments. For the inhibition test, 20 $\mu\text{g}/\text{ml}$ of anti-HLA class I (W6/32, IgG2a), anti-CD8 (Nu-Ts/c, IgG2a), anti-CD4 (Nu-Th/i, IgG1b), anti-HLA-class II (H-DR-1, IgG2a), and anti-CD14 (H14, IgG2a, as an irrelevant control) monoclonal antibodies (mAbs) were used as reported previously (11).

Statistics. Statistical analyses were performed using Student's *t*-test, Mann-Whitney *U* test, and the χ^2 test. Values of $P < 0.05$ were considered statistically significant.

Results

Detection of Humoral Response to HCV-Peptides

We measured the levels of IgG reactive to each of 18 peptides in the sera of 12 patients with HCV-1b infection and 10 HDs. These subjects included both HLA-A2 $^{+}$ and -A2 $^{-}$. The cut-off value for this anti-HCV core 35–44 at a serum dilution of 1:100 was determined as 0.18 (the mean <0.03> plus 3SD <0.05 times 3> of 10HDs). Significant levels (>0.18 OD at a serum dilution of 1:100) of IgG reactive to HCV core 35–44 were detected in the sera from all patients except for one case, but were not detected at all from any of the HDs tested. The ELISA results are shown in Fig. 1. In contrast, significant levels of IgG reactive to the remaining 17 peptides were scarcely detected or undetectable in the patients (Fig. 1) or HDs (data not shown), respectively. Therefore, the HCV core 35–44 peptide and the other control peptides with >90% purity were used in the following experiments. The peptide specificity of the IgG reactive to HCV core 35–44 was confirmed by absorption and elution tests. As expected, anti-HCV core 35–44 Ab was absorbed by the corresponding peptide, but not by an irrelevant peptide (HCV NS5A 2252–60), and it was further eluted from the corresponding peptide, but not by an irrelevant peptide, bound fraction (Fig. 2).

A fine epitope of the HCV core 35–44 peptide recognized by the patients' sera was further analyzed using 15 decamer peptides possessing overlap sequences spanning HCV core 27–50. The reactivity of the patients' IgG to the overlapping peptides was examined and the

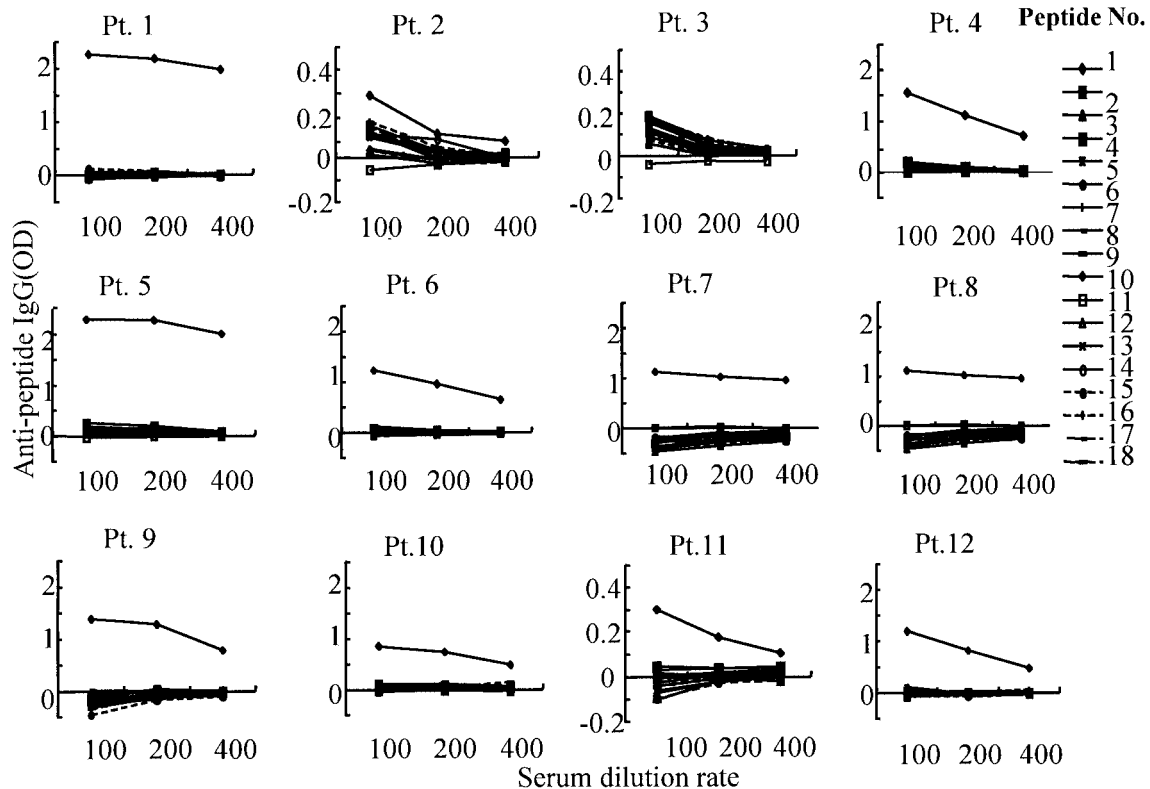


Fig. 1. Detection of anti-peptide IgG. Optical density (OD) values of each sample were assessed in serially diluted serum samples in order to estimate peptide-reactive IgG levels by ELISA. Representative results of 12 HCV⁺ patients are shown.

representative results and summary of the data of 5 patients' sera are shown in Fig. 3A and Table 2, respectively. Seven peptides spanning HCV core 31–46 expressed positive reactivity in a majority of the cases. The common overlapping sequence of these seven peptides is LPRR at position 37–40 of the HCV core protein. Thus, this sequence, LPRR, is considered to be the fine epitope of HCV core 35–44 peptide recognized by the patients' IgG. The reactivity of the IgG to 4-mer and 7-mer peptides containing LPRR with/without N- or C-terminal GGG spacer sequences was further analyzed (Fig. 3B). The patients' IgG reacted with the native sequence decamer peptides containing LPRR. In contrast, the IgG could not bind to the 4-mer or 7-mer peptides. These results, together with the highest reactivity of the 35–44 peptide, suggest that three-dimensional conformation affected by the nested sequences might be important for the recognition of LPRR sequence by the IgG.

Induction of Peptide-Specific Cellular Response

PBMCs from HLA-A2⁺ patients with HCV infection ($n=5$) and HLA-A2⁺ HDs without HCV infection ($n=9$) were cultured with each of the 18 peptides (>90% purity) or a control HIV-peptide for 12 days,

and were examined for their IFN- γ production in response to the corresponding peptide-pulsed T2 cells. The HCV core peptides at positions 35–44, 132–140 and 178–187, and NS3 peptide at position 1287–1296 induced significant levels ($P>0.05$ and net value >100 pg/ml) of IFN- γ from two, four, one, and one of five patients, respectively. In contrast, none of the peptides induced significant levels of IFN- γ in the HDs. Representative results of each of five cases whose PBMCs showed positive IFN- γ production in response to at least one peptide and a HD are given in Fig. 4A. Levels of IFN- γ produced by these PBMCs were significantly inhibited by anti-class I (W6/32) or anti-CD8 mAb, but not by any of the other mAbs tested (Fig. 4B). These results suggest that this cellular response was largely mediated by CD8⁺, but not CD4⁺ T cells in an HLA-class I-restricted manner.

The cytotoxicity of these peptide-stimulated PBMCs was confirmed by a 6 hr ⁵¹Cr-release assay, and the representative results of the cytotoxicity to T2 cells pulsed with each of the three HCV core peptides are shown in Fig. 4C. These PBMCs showed significant levels of cytotoxicity against the T2 cells that were pre-loaded with a corresponding peptide, but not with an HIV peptide, taken as the negative control. Further, the HLA

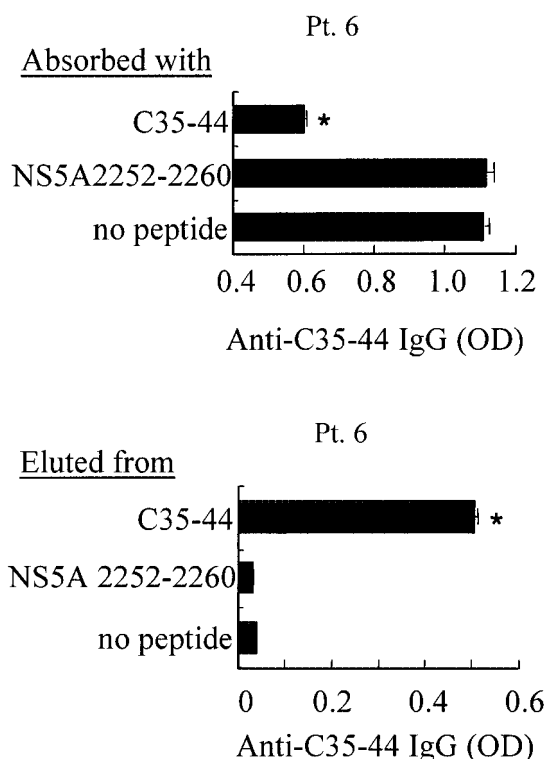


Fig. 2. Specificity of anti-peptide IgG. Each serum sample was absorbed, with either HCV core 35–44 peptide or an irrelevant (HCV NS5A 2252–2260) peptide used as the negative control, three times at 37 C followed by peptide-specific IgG ELISA. For the elution test, IgG molecules bound to the peptide-immobilized plates by the first absorption were eluted by acid and subjected for measurement of the levels of peptide-specific IgG by ELISA. The representative results from the sera of Pts. 6 and 9 are shown in this figure. Values represent the mean $\pm$ SD. *Values are differed from the no peptide or irrelevant controls, $P < 0.05$ by Student's *t*-test.

restriction and specificity of the CTL activity was confirmed by the inhibition assay. Specifically, the cytotoxicity against T2 cells pulsed with HCV core 35–44 peptide was inhibited by anti-HLA-class I (W6/32) or CD8 mAb, but not by any of the other mAbs tested, indicating that the peptide-specific cytotoxicity was largely mediated by CD8⁺ T cells in an HLA-class I-restricted manner (Fig. 4D). These results suggest that the HCV core peptides at positions 35–44 and 132–140 are suitable candidates for use as a prophylactic as well as therapeutic vaccine for HLA-A2⁺ patients with HCV-related diseases.

Discussion

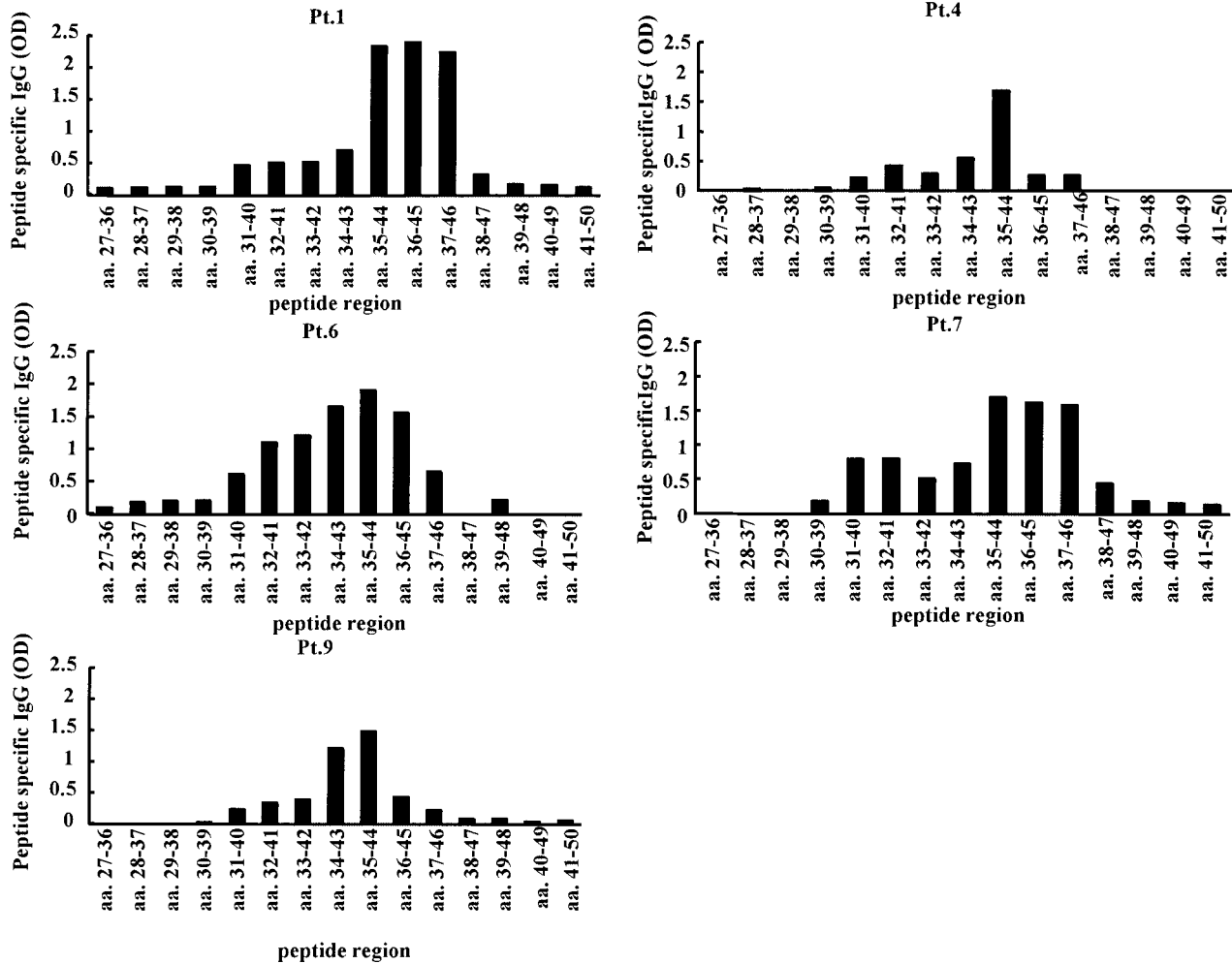
HCV infection is a serious worldwide health problem, approximately 170 million people in the world are persistently infected with HCV and have a high risk of LC and HCC at later stages. Most of them do not live in

developed countries, and thus have a rare chance of receiving either diagnosis or treatment. One solution will come, in part, from introducing an economical approach. The presently available modalities, such as IFN as a therapeutic agent and a third generation of enzyme/radio immunoassay (EIA-3/RIA-3) as a diagnostic tool, are too expensive for a majority of the infected individuals who are not living in well-developed countries. Therefore, development of both new diagnostic and therapeutic modalities with lower prices will increase the chance of these infected subjects to receive diagnosis and treatment. One way to achieve these goals would be to determine highly immunodominant HCV-peptides recognized by both cellular and humoral immunities, and provide them for the development of new therapeutic and diagnostic modalities (8, 25). Our results indicated that one peptide (HCV core at position 35–44) among the 18 kinds of peptides tested was recognized for more than 90% of the patients with HCV infection. This epitope peptide has already been reported as being capable of inducing HLA-A2-restricted CTLs (1, 6, 23–25). Therefore, our results along with those results, suggest that this core 35–44 peptide is a suitable candidate for prophylactic and therapeutic vaccines to treat HLA-A2⁺ patients with HCV-related diseases.

Our results show that significant levels of IgG specific to the HCV core 35–44 peptide were detectable in a majority of the HCV-infected individuals without HLA-A2 restriction. It is well known that serologically higher immunogenicity is located at the N-terminal of the HCV core protein (2, 5, 8, 22). Therefore, this region, at position 10–55, was used as one of the three key antigens for the third generation of EIA/RIA. Leucine, at position 36, and arginine or leucine, at positions 43 or 44, may be aggretopes for binding to HLA-A2 molecules (9, 20). This assumption is supported by the results from the fine epitope analysis of the HCV core 35–44 peptide using overlapping peptides. Namely, the common sequence of the overlapping peptides recognized by the patients' sera is LPRR, at position 37–40 of the HCV core protein. The results of this study, along with those of previous reports (1, 3, 6, 8, 20, 23–25), clearly indicate that the sequence at position 37–40 is one of the most immunodominant regions among the N-terminal of the HCV core protein recognized by both cellular and humoral immunities.

There is no apparent HLA-A2 restriction for the existence of anti-HCV core 35–44 peptide-reactive Ab, and the detailed mechanisms involved in this phenomenon are unclear at the present time. We also previously reported the existence of IgG reactive to certain CTL-epitope peptides derived from non-mutated self-anti-

(A)



(B)

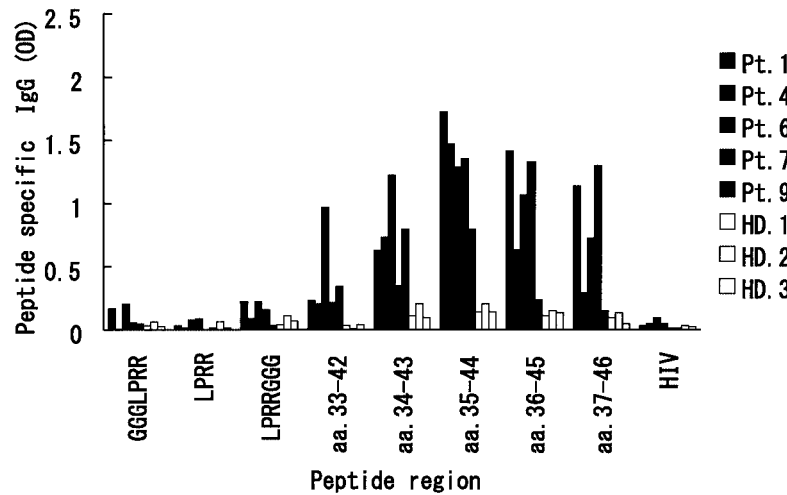


Fig. 3. Fine epitope analysis of HCV core 35–44 peptide recognized by patients' IgG. A: Representative ELISA results of the reactivity of five patients' sera (1:100 diluted) to 15 decamer peptides possessing overlapping sequences spanning HCV core 27–50. The cut-off value in these experiments was 0.15. B: Reactivity of 1:100 diluted sera from the five patients and three healthy donors (HDs) to the sequence LPRR containing peptides.

Table 2. Sequences of the overlapping peptides and a summary of the reactivity

Region	Peptide Sequence	Ab reactivity				
		Pt.1	Pt.4	Pt.6	Pt.7	Pt.9
aa.27–36	GGQIVGGVYL	—	—	—	—	—
aa.28–37	GQIVGGVYLL	—	—	—	—	—
aa.29–38	QIVGGVYLLP	—	—	—	—	—
aa.30–39	IVGGVYLLPR	—	—	—	—	—
aa.31–40	VGGVYLLPRR	+	+	+	+	+
aa.32–41	GGVYLLPRRG	+	+	+	+	+
aa.33–42	GVYLLPRRGP	+	+	+	+	+
aa.34–43	VYLLPRRGPR	+	+	+	+	+
aa.35–44	YLLPRRGPRL	+	+	+	+	+
aa.36–45	LLPRRGPRLG	+	+	+	+	+
aa.37–46	LPRRGPRLG V	+	+	+	+	+
aa.38–47	PRRGPRLGVR	+	—	—	—	—
aa.39–48	RRGPRLGVRA	—	—	—	—	—
aa.40–49	RGPRLGVRAT	—	—	—	—	—
aa.41–50	GPRLGVRATR	—	—	—	—	—

Sequences of the overlapping peptides and a summary of the reactivity of five patients' sera are summarized. Boxed sequence (LPRR) shows the fine epitope of HCV core 35–44 reactive IgG.

gens without apparent HLA-A2 restriction in both HDs and cancer patients (10, 14, 16–18, 21). One of the explanations might be that the HCV core 35–44 peptide is naturally processed by means of proteasomes, and transported into ER with a chaperone in HCV-infected cells. The peptide might be loaded to HLA-A2 or other undefined HLA-class I molecules, if any, followed by presentation on the cell surface as a HLA-peptide complex in certain subjects. In the remaining subjects, the unloaded peptides might be directly secreted from ER to outside the cells. Alternatively, naturally processed HCV core 35–44 might be directly secreted from the cytoplasm to outside the cells via an undefined pathway. Further studies are needed to resolve this issue.

The other important issue to be elucidated is the biological features of Abs to HCV core 35–44 peptide. Increases in the IgG levels of the CTL-epitope peptides of non-mutated self-peptides by peptide vaccination showed a close correlation with long-term survival in patients with advanced stages of lung cancer and gastric cancer (10, 16, 17, 21), whereas the class of IgG to these peptides was either lacking or unbalanced in atopic dermatitis patients (14, 18), suggesting its positive role in host defense. These peptide-specific Abs, however, failed to react to the whole molecules of the mother proteins. Furthermore, they did not react to tumor cells, and thus their biological features are not clarified at the present time. The reactivity of this peptide-specific Ab to HCV core protein as well as its activity to neutralize HCV remains to be answered in the near future.

This peptide-reactive Ab was undetectable in the sera

of the following subjects: patients with autoimmune diseases, subjects with HBV but not HCV infection, HDs vaccinated with HBV recombinant protein, and HDs without HBV vaccination, all taken as negative controls (data not shown). Based on these results, the sensitivity and disease specificity of the anti-HCV core 35–44 IgG was calculated as 93.3 and 100%, respectively. This point could be one of the advantages as compared to the reported peptide-based immunoassay using HCV core 21–40 peptide, which has cross-reactivity with HBV infection (8). These results may provide new insight for the development of both therapeutic and diagnostic modalities for HCV infection.

The sequence of the HCV core 35–44 peptide has partial cross-reactivity to that of HIV and HTLV-I. Specifically, the sequence of HCV core 39–43 is identical to that of the HIV envelope protein and to that of HTLV-I rex protein 5–9 by database analysis. The sequence of HCV core 38–42 is also identical to that of HIV at protein 54–58. Therefore, we tested if anti-HCV core 35–44 activity was detectable in the sera of subjects with HIV infection ($n=3$) and HTLV-I infection ($n=10$), and it was detected in all three and eight of ten subjects, respectively (Takao et al., unpublished results). This cross-reactivity, however, needs to be carefully studied since the amount of samples available for analysis was too small to both test the cross-infection of HCV in these patients and the peptide specificity by means of absorption and elution tests. This study is now in progress in our laboratory.

This study was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology,

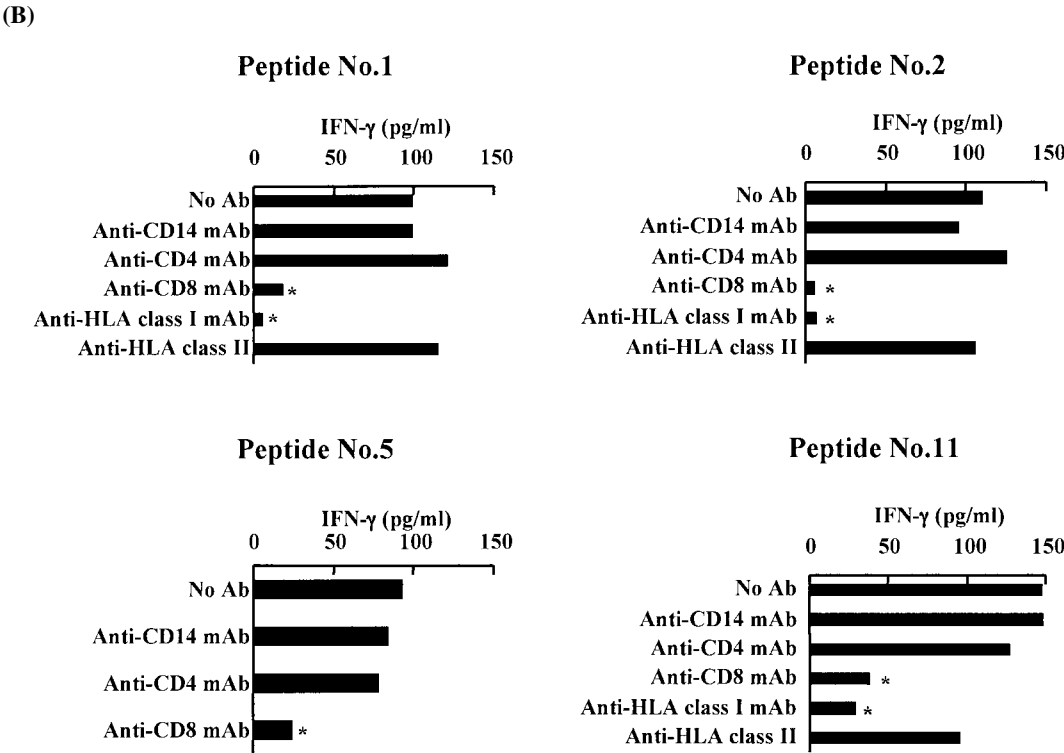
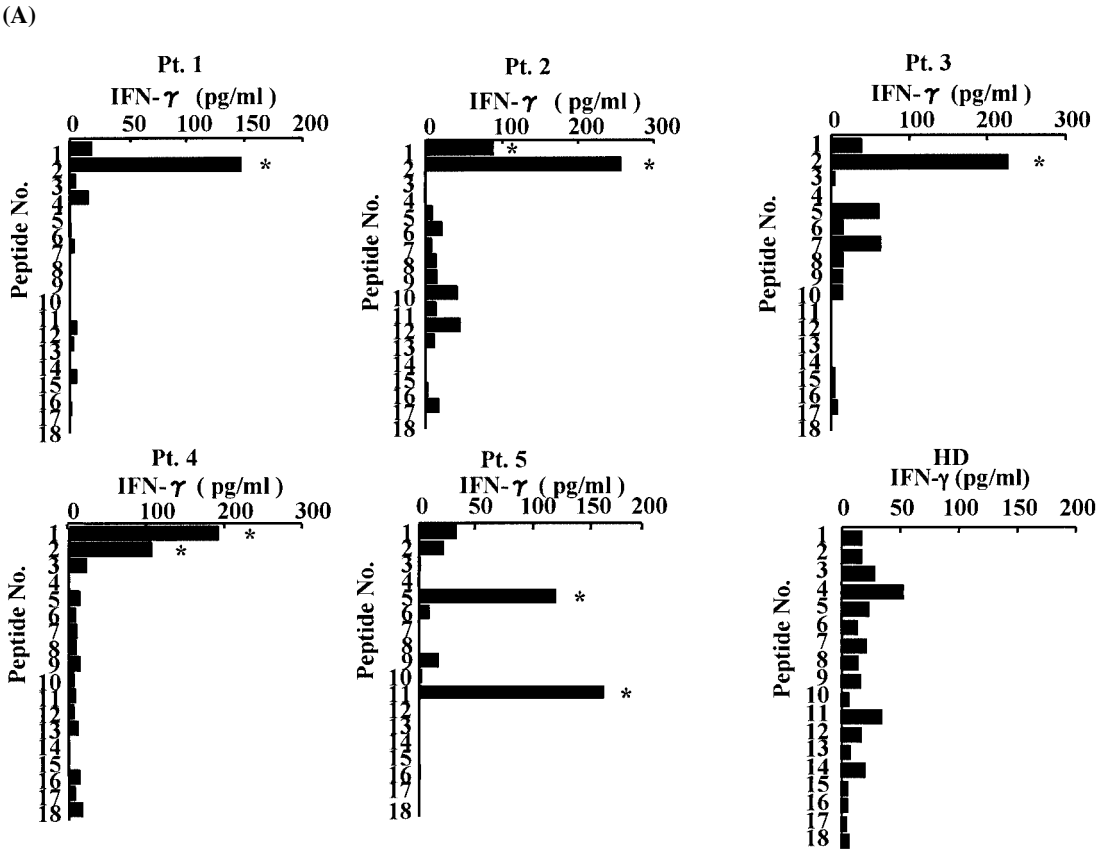
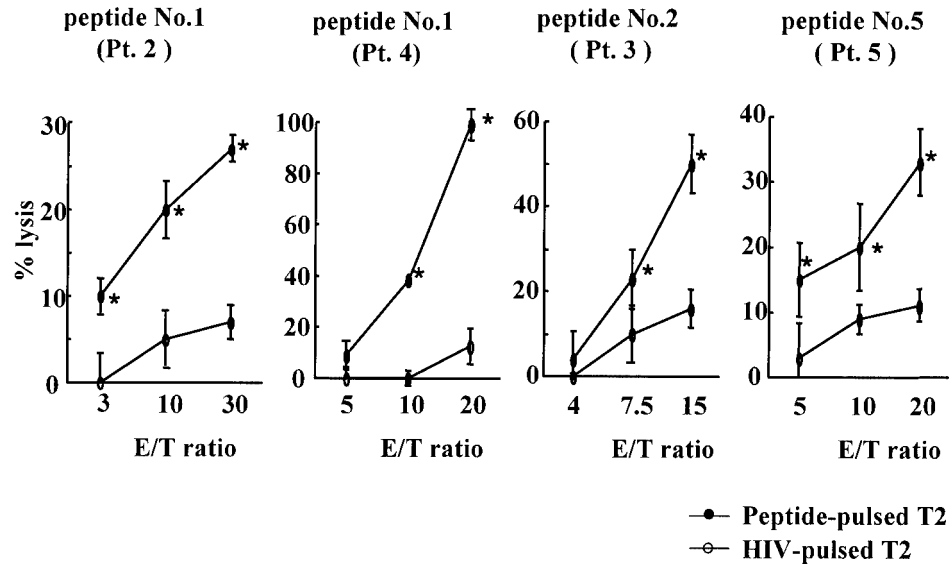


Fig. 4.

(C)



(D)

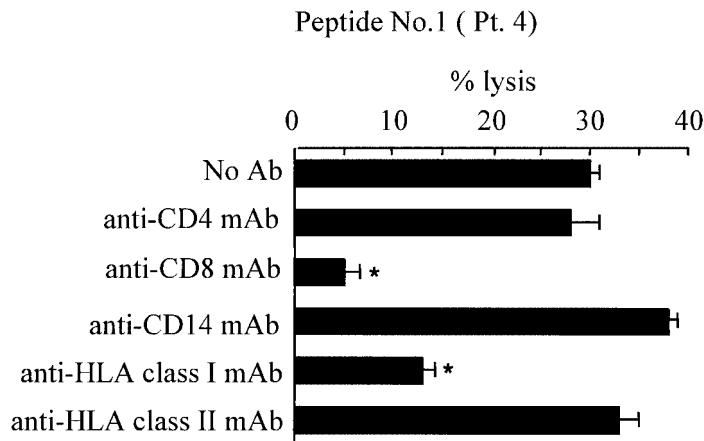


Fig. 4. Cellular responses to peptides. A: IFN- γ production. PBMCs from HLA-A2⁺ HCV⁺ patients ($n=5$) and HDs ($n=5$) were stimulated with each of the 18 different peptides in four different wells (15×10^4 /well). On culture day 14, the peptide-stimulated PBMCs ($80-120 \times 10^4$ /well) from each well were independently collected and divided into four equal portions. Two portions were separately tested for their ability to produce IFN- γ in response to T2 cells pulsed with a corresponding peptide, while the remaining two portions were tested using a negative control peptide (HIV). Background IFN- γ production in response to the HIV peptide (<50 pg/ml) was subtracted. The representative results of all five patients and of one HD whose PBMCs showed significant levels of IFN- γ production in response to at least one HCV-peptide are shown. * $P < 0.05$ by Student's t -test. B: Ab-inhibition of IFN- γ production. IFN- γ production of the peptide-stimulated PBMCs in response to the peptide-pulsed T2 cells in the presence of the indicated mAbs. Anti-CD14 mAb served as negative controls. * $P < 0.05$. C: Cytotoxicity. Peptide-stimulated PBMCs were tested for their cytotoxicity against T2 cells pulsed with each of the three HCV core peptides or an HIV-peptide as the negative control by a standard 6-hr ^{51}Cr -release assay at three different effector-to-target cell (E/T) ratios. The representative results of patients 2-5 are shown in this figure. * $P < 0.05$ by Student's t -test. D: Ab-inhibition of the cytotoxicity. Peptide-stimulated PBMCs were tested for their cytotoxicity against T2 cells pulsed with HCV core 35-44 peptide in the presence of anti-HLA-class I, anti-HLA-class II, anti-CD8, or anti-CD4 mAb. Anti-CD14 mAb served as the negative control. The 6-hr ^{51}Cr -release assay was performed at an E/T ratio of 10 to 1. * $P < 0.05$ by Student's t -test.

Japan (no.12213134 to K.I., and Cooperation of Innovative Technology and Advanced Research in Evolutional Area (CITY AREA) to A.Y.), and from the Ministry of Health, Labour and Welfare, Japan (no. H14-trans-002, 11-16, and H12-cancer-004 to K. I.).

References

- Alexander, J., Del Guercio, M.F., Fikes, J.D., Chesnut, R.W., Chisari, F.V., Chang, K.M., Appella, E., and Sette, A. 1998. Recognition of a novel naturally processed, A2 restricted, HCV-NS4 epitope triggers IFN-gamma release in absence of detectable cytopathicity. *Hum. Immunol.* **59**: 776–782.
- Akatsuka, T., Donets M., Scaglione, L., Ching, W.M., Shih, J. W.-K., Di Bisceglie, A.M., and Feintone, S.M. 1993. B cell epitopes on the hepatitis C virus nucleocapsid protein determined by human monospecific antibodies. *Hepatology* **18**: 503–510.
- Battegay, M., Fikes, J., Di Bisceglie, A.M., Wentworth, P.A., Sette, A., Celis, E., Ching, W.M., Grakoui, A., Rice, C.M., Kurokohchi, K., Berzofsky, J.A., Hoofnagle J.H., Feinstone, S.M., and Akatsuka, T. 1995. Patients with chronic hepatitis C have circulating cytotoxic T cells which recognize hepatitis C virus-encoded peptides binding to HLA-A2.1 molecules. *J. Virol.* **69**: 2462–2470.
- Cai, Q., Zhang, X., Tian, L., Yuan, M., Jin, G., and Lu, Z. 2002. Variant analysis and immunogenicity prediction of envelope gene of HCV strains from China. *J. Med. Virol.* **67**: 490–500.
- Cerino, A., Boender, P., Lamonica, N., Rosa, C., Habets, W., and Mondelli, M.U. 1993. A human monoclonal antibody specific for the N-terminus of the hepatitis-C virus nucleocapsid protein. *J. Immunol.* **151**: 7005–7015.
- Cerny, A., McHutchison, J.G., Pasquinelli, C., Brown, M.E., Brothers, M.A., Grabscheid, B., Flower, P., Houghton, M., and Chisari, F.V. 1995. Cytotoxic T lymphocyte response to hepatitis C virus-derived peptides containing the HLA A2.1 binding motif. *J. Clin. Invest.* **95**: 521–530.
- Chander, G., Sulkowski, M.S., Jenckes, M.W., Torbenson, M.S., Herlong, H.F., Bass, E., and Gebo, K.A. 2002. Treatment of chronic hepatitis C: a systematic review. *Hepatology* **36** (Suppl): S135.
- El Awady, M.K., El-Demellawy, M.A., Khalil, S.B., Galal, D., and Goueli, S.A. 2002. Synthetic peptide-based immunoassay as a supplemental test for HCV infection. *Clin. Chim. Acta* **325**: 39–46.
- Encke, J., Zu, Putlitz, J., Geissler, M., and Wands, J.R. 1998. Genetic immunization generates cellular and humoral immune responses against the nonstructural proteins of the hepatitis C virus in a murine model. *J. Immunol.* **161**: 4917–4923.
- Harada, M., Kobayashi, K., Matsueda, S., Nakagawa, M., Noguchi, M., and Itoh, K. 2003. Prostate-specific antigen-derived epitopes capable of inducing cellular and humoral response in HLA-A24+ prostate cancer patients. *Prostate* **57**: 152–159.
- Hida, N., Maeda, Y., Katagiri, K., Takasu, H., Harada, M., and Itoh, K. 2002. A simple culture protocol to detect peptide-specific cytotoxic T lymphocyte precursors in the circulation. *Cancer Immunol. Immunother.* **51**: 219–228.
- Hunziker, I.P., Zurbriggen, R., Glueck, R., Engler, O.B., Reichen, J., Dai, W.J., Pichler, W.J., and Cerny, A. 2001. Perspectives: towards a peptide-based vaccine against hepatitis C virus. *Mol. Immunol.* **38**: 475–484.
- Ito, M., Shichijo, S., Tsuda, N., Ochi M., Harashima, N., Saito, N., and Itoh, K. 2001. Molecular basis of T cell-mediated recognition of pancreatic cancer cells. *Cancer Res.* **61**: 2038–2046.
- Kawamoto, N., Yamada, A., Ohkouchi, S., Maeda, T., Tanaka, S., Hashimoto, T., Saijio, Y., Saijio, S., Nukiwa, T., Shichijio, S., Aizawa, H., and Itoh, K. 2003. IgG reactive to CTL-directed epitopes of self-antigens is either lacking or unbalanced in atopic dermatitis patients. *Tissue Antigens* **61**: 1–10.
- Kurokohchi, K., Arima, K., and Nishioka, M. 2001. A novel cytotoxic T-cell epitope presented by HLA-A24 molecule in hepatitis C virus infection. *J. Hepatol.* **34**: 930–935.
- Mine, T., Gouhara, R., Hida, N., Imai, N., Azuma, K., Rikimaru, T., Katagiri, K., Nishikori, M., Sukehiro, A., Nakagawa, M., Yamada, A., Aizawa, H., Shirouzu, K., Itoh, K., and Yamana, H. 2003. Immunological evaluation of CTL precursor-oriented vaccines for advanced lung cancer patients. *Cancer Sci.* **94**: 548–556.
- Noguchi, M., Kobayashi, K., Suetsugu, N., Tomiyasu, K., Suekane, S., Yamada, A., Itoh, K., and Noda, S. 2003. Induction of cellular and humoral immune responses to tumor cells and peptides in HLA-A24 positive hormone-refractory prostate cancer patients by peptide vaccination. *Prostate* **1**: 80–92.
- Ohkouchi, S., Yamada, A., Imai, N., Mine, T., Harada, K., Shichijio, S., Maeda, Y., Saijio, Y., Nukiwa, T., and Itoh, K. 2002. Non-mutated tumor-rejection antigen peptides elicit type-I allergy in the majority of healthy individuals. *Tissue Antigens* **59**: 259–272.
- Pumeechockchai, W., Bevit, D., Agarwal, K., Petropoulou, T., Langer, B.C., Belohradsky, B., Bassendine, M.F., and Toms, G.L. 2002. Hepatitis C virus particles of different density in the blood of chronically infected immunocompetent and immunodeficient patients: implications for virus clearance by antibody. *J. Med. Virol.* **68**: 335–342.
- Rammensee, H.G., Friede, T., and Stevanovic, S. 1995. MHC ligands and peptide motifs: first listing. *Immunogenetics* **41**: 178–228.
- Sato, Y., Shomura, H., Maeda, Y., Mine, T., Une, Y., Akasaka, Y., Kondo, M., Takahashi, S., Shinohara, T., Katagiri, K., Sato, M., Okada, S., Matsui, K., Yamada, A., Itoh, K., and Todo, S. 2003. Immunological evaluation of peptide vaccination for patients with gastric cancer based on pre-existing cellular response to peptide. *Cancer Sci.* **9**: 802–808.
- Tanaka, T., Lau, J.Y., Mizokami, M., Orito, E., Tanaka, E., Kiyosawa, K., Yasui, K., Ohta, Y., Hasegawa, A., Tanaka, S., et al. 1995. Simple fluorescent enzyme immunoassay for detection and quantification of hepatitis C viremia. *J. Hepatol.* **23**: 742–745.
- Urbani, S., Uggeri, J., Matsuura, Y., Miyamura, T., Penna,

- A., Boni, C., and Ferrari, C. 2001. Identification of immunodominant hepatitis C virus (HCV)-specific cytotoxic T-cell epitopes by stimulation with endogenously synthesized HCV antigens. *Hepatology* **33**: 1533–1543.
- 24) Vertuani, S., Bazzaro, M., Gualandi, G., Micheletti, F., Marastoni, M., Fortini, C., Canella, A., Marino, M., Tomatis, R., Traniello, S., and Gavioli, R. 2002. Effect of interferon-alpha therapy on epitope-specific cytotoxic T lymphocyte responses in hepatitis C virus-infected individuals. *Eur. J. Immunol.* **32**: 144–154.
- 25) Ward, S., Lauer, G., Isba, R., Walker, B., and Klennerman, P. 2002. Cellular immune responses against hepatitis C virus: the evidence base 2002. *Clin. Exp. Immunol.* **128**: 195–203.