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Hepatitis B core antigen stimulates interleukin-10 secretion by both T cells and monocytes from peripheral blood of patients with chronic hepatitis B virus infection

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SUMMARY

In chronic hepatitis B virus (HBV) infection, immune responses to hepatitis B core antigen (HBcAg) are weak. Interleukin (IL)-10 is a potent immunosuppressive cytokine which we reported recently to be secreted in response to HBcAg by peripheral blood mononuclear cells (PBMCs) from patients with chronic HBV infection or healthy controls. Using an enzyme-linked immunospot assay, we compared the ability of HBcAg to stimulate IL-10 production by PBMC with that of lipopolysaccharide (LPS), phytohaemagglutinin-P and hepatitis C virus-derived antigens in 16 patients with chronic HBV infection and six healthy controls. Frequencies of IL-10 spot-forming cells (SFC) in response to HBcAg were comparable to those obtained with LPS in patients with chronic HBV infection. Frequencies of IL-10 SFC in response to HBcAg or to LPS were significantly higher in patients with chronic HBV infection than in healthy controls. IL-10 SFC in response to HBcAg consisted of 26–35% T cells, 62–70% monocytes and less than 1% B cells in patients with chronic HBV infection. Only monocytes contributed to IL-10 production in controls. Frequencies of HBcAg stimulated IL-10 SFC representing T cells and monocytes were significantly higher in patients with elevated serum alanine aminotransferase (ALT) and detectable HBV DNA than in patients with normal ALT and undetectable HBV DNA. The potent ability of HBcAg to stimulate IL-10 production by PBMC may contribute importantly to immune tolerance toward HBV.

Keywords chronic hepatitis B virus infection enzyme-linked immunospot assay interleukin 10 hepatitis B core antigen

INTRODUCTION

In chronic hepatitis B virus (HBV) infection cellular immune responses are weak or absent, except during acute exacerbation [1–4]. The immunological and virological basis for viral persistence in chronic HBV infection has not been elucidated fully, although cytokine imbalance, T cell exhaustion, anergy and viral mutations have been suggested as possible mechanisms [1].

Hepatitis B core antigen (HBcAg) possesses unique immunological features conveying the properties of both T cell-independent and -dependent antigens: as little as 0·025 μg of HBcAg can elicit antibody production without need for an adjuvant; immunization with HBcAg primes preferentially type 1 helper T (Th1) cells, and HBcAg is an effective carrier for heterogeneous epitopes [5–7]. However, we found previously in patients with chronic HBV infection that peripheral blood mononuclear cells (PBMC) and lymphocytes infiltrating the liver secreting the potent immune-suppressor interleukin (IL)-10 [8–14] in response to HBcAg outnumber HBcAg-specific interferon (IFN)-γ-secreting cells [15]. In addition, IL-10-secreting cells were induced upon stimulation of PBMC from healthy controls with HBcAg. Thus HBcAg may promote HBV infection by stimulating production of immunosuppressive IL-10 in excess of immunostimulatory IFN-γ. Relationships between IL-10 production by PBMC stimulated with HBV antigens and disease activities of chronic HBV infection remain unclear [16,17], although IL-10 production by PBMC stimulated with HBcAg or HBeAg has been reported to decrease with successful treatment in patients with chronic HBV infection [18].

In the present study we found that HBcAg stimulated IL-10 production by CD4$^+$ and CD8$^+$ T cells, as well as by monocytes from patients with chronic HBV infection. Frequencies of HBcAg-stimulated IL-10 immunospot-forming cells (SFC)

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Table 1. Characteristics of chronic hepatitis B patients with elevated serum ALT and detectable HBV DNA (group A), patients with normal ALT and undetectable HBV DNA (group N) and healthy controls

	Group A	Group N	Control
Number of subjects	7	9	6
Age (years)	42·5 ± 3·5	34·7 ± 2·2	28·2 ± 0·4
Gender (M:F)	6 : 1	8 : 1	4 : 2
ALT (IU/l)	154·2 ± 44·6	23·8 ± 2·6	18·9 ± 0·3
HBV DNA (LGE/ml)	5·7 ± 0·6	<3·7	<3·7

Values are means ± s.d. ALT, alanine aminotransferase; HBV, hepatitis B virus.

among PBMC were significantly higher in patients with serum alanine aminotransferase (ALT) elevations and detectable HBV DNA than in other patients with normal ALT concentrations and undetectable HBV DNA. Additionally, we found that HBcAg also stimulated IL-10 production by monocytes from healthy controls.

PATIENTS AND METHODS

Subjects

Blood samples were obtained from 16 patients with chronic HBV infection and from six healthy controls who had normal ALT concentrations and no serological markers of hepatitis virus infection. All patients were HBsAg-positive, HBsAb-negative and HBcAb-positive with serum in a 1 : 200 dilution. Patients were separated into two groups: group A, patients with elevated serum ALT and detectable HBV-DNA measured by commercially available transcription-mediated amplification (TMA) assay with a detection limit of 3·7 LEG/ml; and group N, patients with normal serum ALT and undetectable HBV-DNA (Table 1). Patients had not received immunomodulating drugs during the preceding 3 years and had no other cause of chronic liver injury, such as HCV infection or excessive alcohol intake. Informed consent was obtained from all subjects, and the study was approved by the Ethical Review Committee of Jichi Medical School.

Antigens and mitogens

Recombinant HBcAg purchased from the Institute of Immunology (Tokyo, Japan) was more than 95% pure according to high-performance liquid chromatography. Lipopolysaccharide (LPS) and phytohaemagglutinin (PHA-P) were obtained from Sigma (Atlanta, GA, USA). HCV-derived core antigens (JCC-2 or C7) were generous gifts from the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) and the Advanced Life Science Institute (Saitama, Japan), respectively.

Isolation of PBMC, CD3$^+$ T cells, B cells and monocytes

PBMC isolated from heparinized venous blood by gradient centrifugation using Ficoll-Hypaque (Amersham-Pharmacia Biotech, Uppsala, Sweden) were suspended in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (RPMI-1640/10% FCS). The cells were kept cool to avoid adhesion and activation of monocytes.

To determine which cells among PBMC were responsible for IL-10 production, PBMC were incubated for 18 h in a 14 ml polypropylene round-bottomed tube (Falcon 2059, BD Biosciences, San Jose, CA, USA) with 10 μg/ml HBcAg, 1 μg/ml LPS or medium alone at 37°C in a humidified 5% CO_2 atmosphere. Then CD3$^+$ T cells, B cells and monocytes were isolated from stimulated PBMC by antibody-coated magnetic beads. CD3$^+$ T cells were isolated with MACS CD3$^+$ microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), monocytes with a monocyte negative-isolation kit (Dynal, Oslo, Norway) and B cells with CD19-positive isolation beads and Detacha-beads (Dynal), according to the manufacturers' instructions.

Enzyme-linked immunospot (ELISpot) assay

Frequencies of IL-10-secreting cells among PBMC, isolated T cells, B cells and monocytes were measured using an ELISpot assay as described previously [15]. In brief, 2×10^4 PBMC were added to each well coated with antihuman IL-10 antibody (Mabtech, Nacka, Sweden) for stimulation with 10 μg/ml of HBcAg, 1 μg/ml of LPS, 10 μg/ml PHA-P, 10 μg/ml of HCV derived antigens (JCC-2 and C7) or medium alone. For T cells, B cells and monocytes isolated from HBcAg-stimulated PBMC, 10^5 T or B cells or 10^4 monocytes were added to each well coated with antihuman IL-10 antibody. After incubation for 20 h, spots reflecting IL-10-secreting cells were visualized and counted. Responses were considered significant when a minimum of five SFC were present per well and the number was at least twice that in wells without the stimulus. All results are expressed as means of duplicate SFC in the presence of stimulus minus SFC without stimulus.

Phenotype analysis

In selected patients, ELISpot assays were performed using CD4$^+$ or CD8$^+$ cell-depleted non-adherent PBMC to analyse the phenotype of IL-10-secreting T cells. Non-adherent PBMC (lymphocytes) separated readily from monocytes which weakly express CD4 but remain adherent to the culture dish after 4 h of PBMC incubation with or without HBcAg. Non-adherent PBMC then were incubated with magnetic Dynabeads (Dynal A.S) coated with anti-CD4 or -CD8 monoclonal antibody, and cells bound to the two types of beads were depleted from unbound cells. The two types of unbound cells (CD4 depleted or CD8 depleted) were added to each well of 96-well nitrocellulose membrane-bottomed microtitre plates; well bottoms were coated with antihuman IL-10 antibody. Thus frequencies of specific IL-10-secreting cell types could be determined. To evaluate the contribution of B cells to IL-10 SFC, B cell depleted non-adherent PBMC also were analysed.

Statistical analysis

Data were analysed using a Kruskal–Wallis test or a Mann–Whitney *U*-test. A *P*-value less than 0·05 was considered indicative of statistical significance. Statistical analyses were carried out using a statistical software package STATVIEW version 5·0 for Macintosh (SAS Institute, Cary, NC, USA).

RESULTS

Cells secreting IL-10 in response to stimulation with HBcAg, LPS, PHA-P or HCV-derived antigens

Frequencies of IL-10 SFC in response to different antigens and mitogens are shown in Fig. 1. The median (and range) of

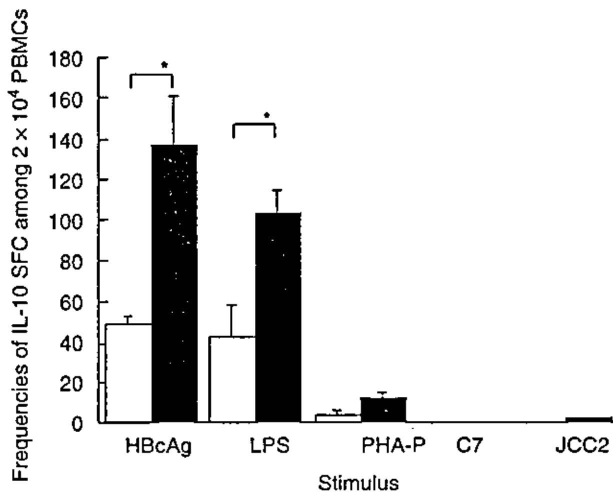

Fig. 1. Frequencies of IL-10-secreting cells upon stimulation with HBcAg, LPS, PHA-P and HCV-derived peptides (JCC-2 and C-7) per 2×10^4 PBMC. Open columns are frequencies of IL-10-secreting cells in healthy controls, and filled columns are those in patients with chronic HBV infection. *$P < 0.05$. IL, interleukin; HBcAg, hepatitis B core antigen; LPS, lipopolysaccharide; PHA-P, phytohaemagglutinin; HCV, hepatitis c virus; PBMC, peripheral blood mononuclear cell.

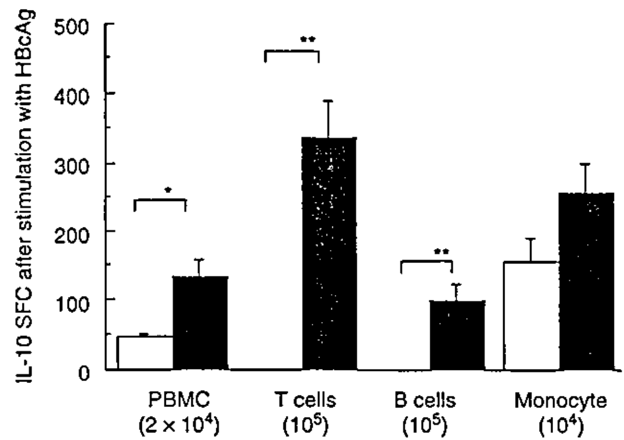

Fig. 2. Frequencies of IL-10-secreting cells in 2×10^4 PBMC, 10^5 CD3$^+$ T cells, 10^4 monocytes and 10^5 B cells separated from PBMC stimulated with HBcAg. Open columns are frequencies of IL-10-secreting cells in healthy controls and filled columns are frequencies in patients with chronic HBV infection.*$P < 0.05$, **$P < 0.01$. IL, interleukin; PBMC, peripheral blood mononuclear cell; HBV, hepatitis B virus.

frequencies of IL-10 SFC/2×10^4 PBMC upon stimulation with HBcAg in patients with chronic HBV infection and in healthy controls were 126 (56–502) and 45 (31–66), respectively; those with LPS stimulation were 94 (44–166) and 41 (18–70), respectively. IL-10 SFC with PHA-P stimulation were 9 (7–29) and 4 (2–7), respectively. Few IL-10 SFC were detected in response to HCV-derived antigens in either patients with chronic HBV infection or healthy controls. Both HBcAg and LPS induced significantly more IL-10 SFC than PHA-P and HCV-derived antigens in patients with chronic HBV infection ($P < 0.05$). Frequencies of IL-10 SFC induced by either HBcAg or LPS were significantly higher in patients with chronic HBV infection than in healthy controls ($P < 0.05$). Frequencies of HBcAg-induced IL-10 SFC were higher, although not significantly, than those of LPS-induced IL-10 SFC in patients with chronic HBV infection.

Frequencies of IL-10 SFC representing T cell, B cell and monocyte fractions of PBMC

In a preliminary study, T cells isolated from PBMC of patients with chronic HBV infection did not respond to HBcAg in the absence of antigen-presenting cells (APC). Thus we stimulated PBMC with HBcAg or LPS before separating them into T cell, B cell and monocyte fractions. The composition of T cells, B cells and monocytes in PBMC stimulated with HBcAg or LPS were 26–45%, 5–9% and 16–28%, respectively.

Among PBMC, monocytes, T cells and a few B cells secreted IL-10 in response to stimulation with HBcAg in patients with chronic HBV infection; monocytes were the main IL-10-producing cells in healthy controls (Fig. 2). Patients with increased serum ALT and detectable serum HBV DNA had higher frequencies of HBcAg-induced IL-10-secreting T cells and monocytes than those with normal ALT and undetectable HBV DNA ($P < 0.03$ and $P < 0.01$, respectively; Table 2). Frequencies of IL-10 SFC in monocytes stimulated with LPS

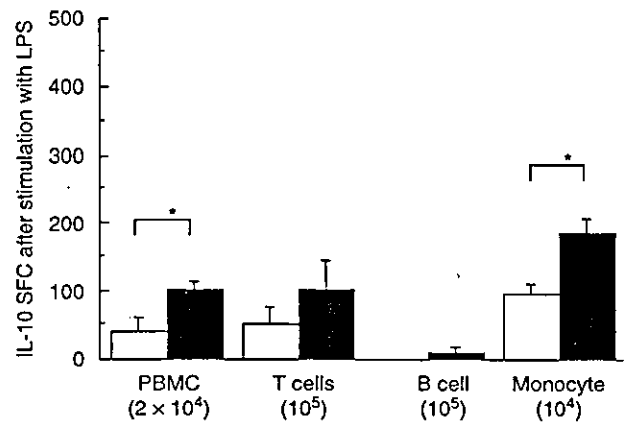

Fig. 3. Frequencies of IL-10-secreting cells in 2×10^4 PBMC, 10^5 CD3$^+$ T cells, 10^4 monocytes and 10^5 B cells separated from PBMC stimulated with LPS. Open columns are frequencies of IL-10-secreting cells in healthy controls, and filled columns are frequencies in patients with chronic HBV infection. *$P < 0.05$.

were higher in patients with chronic HBV infection than in healthy controls (Fig. 3).

T cell subsets secreting IL-10 in response to HBcAg stimulation

T cell subsets of non-adherent PBMCs that secreted IL-10 in response to HBcAg were analysed by the conventional magnetic bead depletion methods in two patients with increased serum ALT and detectable HBV DNA and in two patients with normal ALT and undetectable HBV DNA. In both patients with increased serum ALT and detectable HBV DNA, the frequency of IL-10 SFC in non-adherent PBMC exposed to HBcAg was reduced more by depletion of CD4$^+$ cells than by depletion of CD8$^+$ cells. In contrast, in both patients with normal serum ALT and undetectable HBV DNA, the frequency of IL-10 SFC among non-adherent PBMC in response to HBcAg was reduced almost equally by depletion of CD4$^+$ cells and depletion of CD8$^+$ cells (Fig. 4).

Table 2. Frequencies of IL-10 SFC among 2×10^4 PBMC, 10^5 T cells, 10^5 B cells and 10^4 monocytes in response to HBcAg in chronic hepatitis B patients with normal ALT levels and undetectable HBV DNA (group N), and patients with elevated ALT and detectable HBV DNA (group A)

	2×10^4 PBMC	10^5 T cells	10^5 B cells	10^4 monocytes
Group N	83 (63–146)*	293 (45–348)**	70 (25–265)	124 (38–323)***
Group A	146 (69–502)*	496 (395–596)**	80 (22–178)	362 (69–502)***

Values are medians (and ranges). SFC, spot-forming cell; PBMC, peripheral blood mononuclear cell; HBcAg, hepatitis B core antigen; ALT, alanine aminotransferase; HBV, hepatitis B virus. $^*P = 0{\cdot}03$, $^{**}P = 0{\cdot}02$, $^{***}P = 0{\cdot}007$.

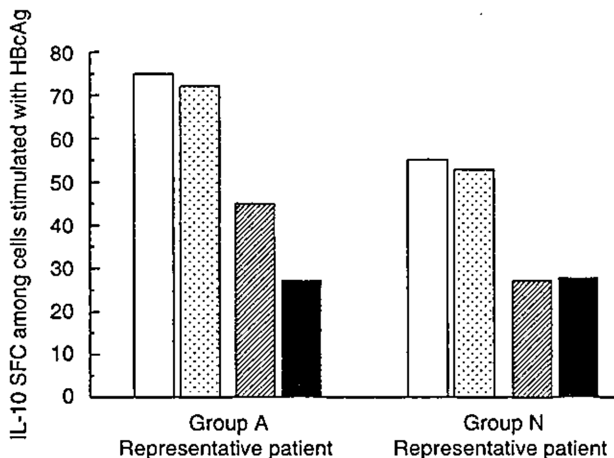

Fig. 4. Phenotypes of non-adherent IL-10-secreting cells in a patient with elevated serum ALT and detectable HBV DNA (group A representative patient) and a patient with normal ALT and undetectable HBV DNA (group N representative patient). Two patients were studied from each group and similar results were obtained within each pair of patients with similar laboratory findings. Non-adherent PBMC collected from HBcAg-stimulated PBMC were depleted of CD4+, CD8+ or B cells, and frequencies of IL-10-secreting cells among fractionated and unfractionated non-adherent PBMC were assayed. Frequencies of IL-10 SFC are expressed as numbers among 10^5 non-adherent PBMC before depletion. Open columns represent IL-10-secreting cells among unfractionated non-adherent PBMC; open columns with black stippling among B cell-depleted non-adherent PBMC; hatched columns among CD8+ T cell-depleted non-adherent PBMC; and filled columns among CD4+ T cell-depleted non-adherent PBMC. IL, interleukin; ALT, alanine aminotransferase; HBV, hepatitis B virus; PBMC, peripheral blood mononuclear cell; SFC, spot-forming cell.

DISCUSSION

In the present study, we showed that HBcAg stimulates IL-10 secretion by peripheral blood T cells, monocytes and B cells in patients with chronic HBV infection. Approximately 0·34% of T cells, 2·5% of monocytes and 0·12% of B cells secreted IL-10 in response to HBcAg at 10 μg/ml. IL-10 SFC among PBMC in response to HBcAg stimulation were estimated to include 62–70% of monocytes, 26–35% of T cells and less than 1% B cells. Not only CD4+ T cells but also CD8+ T cells secreted IL-10 in response to stimulation with HBcAg.

Frequencies of HBcAg-induced IL-10 SFC in peripheral blood T cells and monocytes both were significantly higher in patients with elevated ALT and detectable HBV DNA than in patients with normal ALT and undetectable HBV DNA. We have shown previously that frequencies of HBcAg-stimulated IFN-γ SFC in the peripheral blood are higher in patients with elevated serum ALT and detectable HBV DNA than in patients with normal ALT and undetectable HBV DNA [15]. IL-10 secretion by human monocytes has been reported to be augmented by the proinflammatory cytokine IFN-γ [14]; stimulation of IL-10 secretion by HBcAg and its augmentation by IFN-γmay prevent development of severe liver damage by suppressing immune responses to HBV infection. However, excessive IL-10 production also may contribute to viral persistence and weak cellular immune responses to HBV antigens in patients with chronic HBV infection.

Recently, a concept of anergic CD4+ and CD8+ T cell induction by immature dendritic cells primed by PBMC-derived IL-10 has attracted much attention [19,20]. Deranged APC function has been reported in HBV transgenic mice as well as in humans with chronic HBV infection [21,22]. IL-10 produced in response to HBcAg stimulation may prime dendritic cells and monocytes to induce anergic CD4+ and CD8+ T cells in chronic HBV infection. IL-10-secreting CD8+ T cells are considered to be anergic, non-cytolytic cells that prevent proliferation of cytolytic T cells expressing type 1 cytokines [23–26]. In the present study, we found that a portion of T cells secreting Il-10 in response to HBcAg stimulation were CD8+. Based on negative correlations of serum ALT with frequencies of IL-10-secreting CD8+ T cells and extent of hepatic fibrosis, Prezzi *et al.* [27] proposed that IL-10-secreting CD8+ T cells act to prevent tissue damage caused by cytolytic T cells expressing type 1 cytokines in chronic HCV infection. This hypothesis may also be applicable to HBV infection, although further studies are necessary.

Our study also showed that HBcAg stimulates IL-10 secretion by monocytes obtained from healthy people upon stimulation with HBcAg. However, acute HBV infection in healthy adults is generally self-limited, eliciting vigorous immune responses. Although IL-10 is thought generally to be immunosuppressive, this cytokine has been reported to induce potent cytotoxic T lymphocytes in the presence of IL-2 [28]. We have reported that frequencies of HBcAg-specific Th1 cells assessed by HBcAg-stimulated IFN-γSFC increase markedly before peak acute exacerbation in chronic hepatitis B, followed by a decrease after the peak [15]. Thus IL-10 may be bifunctional in HBV infection, participating in strong antiviral T cell responses in the early phase of acute HBV infection or acute exacerbation of a chronic infection and suppressing responses in the recovery phase and in chronic HBV infection.

In conclusion, HBcAg stimulates IL-10 production by T cells as well as monocytes in patients with chronic HBV infection, and by monocytes in healthy people. Both CD4+ and CD8+ T cells in the peripheral blood of patients with chronic HBV infection secrete IL-10 in response to stimulation with HBcAg. IL-10 may play a role in modulating antiviral responses in HBV infection, contributing to protection from severe liver damage but also to persistence of HBV infection.

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Identification of novel hepatitis C virus-specific cytotoxic T lymphocyte epitopes by ELISpot assay using peptides with human leukocyte antigen-A*2402-binding motifs

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The human leukocyte antigen (HLA)-A*2402 is common in Asians. The authors attempted to identify epitopes for HLA-A*2402-restricted, hepatitis C virus (HCV)-specific CD8$^+$ T cells by an enzyme-linked immunospot (ELISpot) assay using peripheral blood CD8$^+$ T cells from HLA-A*2402-positive hepatitis C patients and synthetic HCV peptides based on HLA-A*2402-binding motifs and the amino acid sequence of type 1b HCV. Ten novel epitopes were identified in five of seven HLA-A*2402-positive patients with acute or short-term chronic HCV infection (<3 years), but in none of four with longer-term chronic infection (>10 years). Only one of the ten epitopes proved to be definitely HLA-A*2402-restricted. Another epitope was identified in one of two HLA-A*2402-negative acute hepatitis C patients. In two of the six patients with positive CD8$^+$ T cell responses, the targeted epitopes were multiple. The same epitope was targeted in two patients. When patients with unresolved acute HCV infection were treated with alpha interferon, peripheral blood HCV-specific CD8$^+$ T cells decreased with resolution of the hepatitis. In conclusion, CD8$^+$ T cell responses to HCV infection are heterogeneous. One definite HLA-A*2402-restricted and ten probably non-HLA-A*2402-restricted epitopes were identified. Patients with short-term HCV infection are suitable for searching for novel HCV epitopes, but peripheral blood HCV-specific CD8$^+$ T cells decrease markedly after loss of antigenic stimulation.

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INTRODUCTION

CD8$^+$ cytotoxic T lymphocytes (CTLs) are thought to play an important role in elimination of hepatitis C virus (HCV), while also contributing to pathogenesis in this infection (Chisari, 1997; Cerny & Chisari, 1999; Rehermann & Chisari, 2000). Virus-specific CTLs recognize viral antigens on the infected cells in a human leukocyte antigen (HLA) class I molecule-restricted manner, then lysing the cells (Gotch *et al.*, 1987; Cannon *et al.*, 1988). Although reports concerning HCV-specific CTL epitopes are accumulating (Ward *et al.*, 2002), which epitopes are most active in viral clearance or immunopathogenesis *in vivo* remains to be determined. In HLA-B44-positive patients with chronic HCV infection, we previously demonstrated an inverse relationship between virus loads and peripheral blood CTL activities specific for HCV nucleoprotein amino acid residues 88–96 (Hiroishi *et al.*, 1997). Accordingly, the identification of new epitopes recognized by HCV-specific CTLs that play a role in elimination of HCV may suggest new ways to suppress growth of HCV and prevent persistent infection. A universally immunogenic vaccine against HCV infection would require multiple epitopes, preferably from conserved regions of HCV, with recognition by CTLs that is restricted by HLA molecules occurring commonly in the population to be immunized.

HLA-A24 is one of the most common HLA-A antigens in Asians (Chandanayingyong, 1986), occurring in more than 60% of Japanese (Date *et al.*, 1996). To study the immunopathogenesis of HCV infection in Japanese and other Asians and to develop HCV-specific CTL vaccines for these populations, identification of HCV-specific CTL epitopes with recognition restricted by HLA-A24 is therefore important. An HLA-A24 allele in Japanese people is almost exclusively HLA-A*2402 (Date *et al.*, 1996). Yet few HLA-A*2402-restricted, HCV-specific CTL epitopes have been reported, in contrast to more numerous reports concerning HLA-A2.1-restricted, HCV-specific CTL epitopes (Ward *et al.*, 2002), representing the HLA most common in Caucasians.

A recently reported enzyme-linked immunospot (ELISpot) assay provides a rapid, inexpensive and efficient way to define a given HLA class I molecule-restricted, novel virus-specific $CD8^+$ T cell epitope and to characterize the breadth of CTL responses (Altfeld *et al.*, 2000). To identify HLA-A*2402-restricted, HCV-specific $CD8^+$ T cell epitopes, we synthesized 87 peptides derived from the total protein content of HCV and carrying HLA-A*2402-binding motifs (Ibe *et al.*, 1996) and assessed the ability of the peptides to stimulate $CD8^+$ T cells by counting interferon (IFN)-γ-releasing cells in HLA-A*2402-positive patients with acute or chronic hepatitis C using the ELISpot assay. Using the epitopes identified, we then studied the effects of treatment with IFN-α on frequencies of HCV-specific $CD8^+$ T cells in two HLA-A*2402-positive patients with unresolved acute hepatitis C.

METHODS

Subjects. Three HLA-A*2402-positive patients with acute hepatitis C and eight with chronic hepatitis C, as well as two HLA-A*2402-negative patients with acute hepatitis C, were studied (Table 1). As controls, one patient with acute hepatitis B, one with fatty liver and three healthy subjects with HLA-A24 were also studied. All hepatitis C patients had detectable HCV RNA in serum and had elevated serum concentrations of alanine aminotransferase (ALT). Diagnosis of chronic hepatitis was based on continuous elevation of serum ALT for more than 6 months. Diagnosis of acute hepatitis was based on acute clinical onset of hepatitis and confirmation of previously negative anti-HCV antibody. The study was approved by the Ethical Review Committees of Jichi Medical School and the Institute of Clinical Medicine of the University of Tsukuba. Informed consent was obtained from all subjects.

Synthetic peptide library. We synthesized 87 peptides of 8–11 amino acids in length that carried HLA-A*2402-binding motifs (tyrosine or phenylalanine at position 2 and leucine, isoleucine, phenylalanine or tryptophan at the C terminus; Ibe *et al.*, 1996). The peptides were based on the amino acid sequence of the genotype 1b HCV-J strain (accession no. D90208). Peptides were synthesized by and purchased from Mimotopes and were more than 80% pure according to high-performance liquid chromatography. The peptides were grouped into 17 mixtures of five or six peptides each for experimental convenience (Mixtures A–Q; Table 2). Two previously reported HLA-A*2402-restricted CTL epitopes, HCV NS3 amino acid residues 1031–1039 (Kurokohchi *et al.*, 2001) and 1100–1108 (Ito *et al.*, 2001) were contained in mixture E.

Isolation of $CD8^+$ T cells and monocytes. Peripheral blood mononuclear cells (PBMC) were separated from heparinized peripheral blood by gradient centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech). $CD8^+$ T cells were isolated from PBMC by positive selection using antibody-conjugated magnetic

Table 1. Characteristics of the subjects studied

Subject	Phase	Aetiology†	Duration	HLA (serotype or genotype)
1	Acute	HCV-1	3 months	A*2402, A*1101, B*5201, B*3902, Cw*0702, Cw*1202
2	Acute	HCV-1	5 months	A*2402, A*2601, B*5401, B*4006, Cw*0801, Cw*0803
3	Acute	HCV-2	5 months	A*2402, B51, B52
4	Chronic	HCV-1	1 year	A*2402, A*2601, B*3501, B*4002, Cw*0303, Cw*0304
5	Chronic	HCV-2	1 year	A*2402, A*2601, B7, B59, Cw1, Cw7
6	Chronic	HCV-2	2 years	A*2402, A*0206, B*5201, B*5901, Cw*0102, Cw*1202
7	Chronic	HCV-2	2·5 years	A*2402, A*3101, B*4801, B*5101, Cw*0304, Cw*0801
8	Chronic	HCV-1	> 10 years	A*2402, A2, B7, B39, Cw7
9	Chronic	HCV-1	> 15 years	A*2402, B54, B61, Cw1, Cw3
10	Chronic	HCV-1	> 15 years	A*2402, A31, B60, Cw3
11	Chronic	HCV-1	> 20 years	A*2402, A2, B7, B61, Cw3, Cw7
12	Acute	HCV-1	2 months	A*2601, A*3101, B*3501, B*5101, Cw*0303, Cw*1402
13	Acute	HCV-1	4 months	A*0201, A*0206, B52, B46, Cw1
14	Acute	HBV	5 months	A24, A31, B56, B61, Cw3, Cw4
15	Chronic	Fatty liver	> 5 years	A24, A26, B54, B61, Cw1, Cw3
16	Healthy			A24, A11, B48, B55, Cw1, Cw3
17	Healthy			A24, A26, B60, B61, Cw7, Cw8
18	Healthy			A24, A33, B61, Cw3

†HCV-1 or -2 indicates the HCV genotype of infected individuals.

Table 2. Peptide library for the ELISpot assay

Mixture	HCV peptides
A	E1 213–220, NS3 1541–1548, NS5A 2153–2160, NS5A 2384–2391, NS5B 2594–2601
B	Core 85–93, Core 129–137, Core 173–181, E1 234–242, E1 360–368
C	E2 464–472, E2 488–496, E2 717–725, E2 770–778, E2 790–798
D	NS2 834–842, NS2 837–845, NS2 885–893, NS2 910–918, NS2 975–983
E	NS2 1017–1025, NS3 1031–1039, NS3 1100–1108, NS3 1267–1275, NS3 1292–1300
F	NS3 1374–1382, NS4B 1716–1724, NS4B 1727–1735, NS4B 1767–1775, NS4B 1773–1781
G	NS5A 2132–2140, NS5A 2280–2288, NS5B 2432–2440, NS5B 2635–2643, NS5B 2870–2878
H	Core 135–144, Core 176–185, Core/E1 191–200, E1 284–293, E2 630–639
I	E2 717–726, E2 767–776, NS2 822–831, NS2 910–919, NS2 932–941
J	NS2 947–956, NS3 1031–1040, NS3 1081–1090, NS3 1243–1252, NS3 1443–1452
K	NS3 1463–1472, NS3 1556–1565, NS4B 1759–1768, NS4B 1792–1801, NS4B 1854–1863, NS5A 1990–1999
L	NS5A 2121–2130, NS5A 2132–2141, NS5A 2146–2155, NS5A 2292–2301, NS5B 2521–2530, NS5B 2944–2953
M	Core 34–44, E1 275–285, E2 593–603, E2 611–621, E2 616–626
N	E2 787–797, E2 790–800, NS2 833–843, NS2 847–857, NS3 1130–1140
O	NS3 1158–1168, NS3 1375–1385, NS3 1416–1426, NS3 1520–1530, NS3 1625–1635
P	NS4A 1672–1682, NS5A 2013–2023, NS5A 2089–2099, NS5B 2456–2466, NS5B 2613–2623
Q	NS5B 2694–2704, NS5B 2801–2811, NS5B 2833–2843, NS5B 2866–2876, NS5B 2973–2983

beads according to the manufacturer's instructions (Dynal). Beads were detached from the isolated cells using the DetachaBead system (Dynal). The yield of $CD8^+$ T cells was 5–20 % of PBMC. Monocytes were isolated from the $CD8^+$ T cell-depleted PBMC by negative selection using a monocyte negative isolation kit (Dynal). Use of isolated $CD8^+$ T cells as effector cells and monocytes as antigen-presenting cells in an ELISpot assay reduced the number of non-specific spots by removal of IFN-γ-secreting $CD4^+$ T cells and natural killer cells and also increased the sensitivity. Purities of isolated $CD8^+$ T cells and monocytes on flow cytometry were >95 % and >80 %, respectively.

ELISpot assay. Analysis of anti-peptide immune responses of peripheral blood $CD8^+$ T cells was performed using an IFN-γ-based ELISpot assay kit (Mabtech). Briefly, 10^5 $CD8^+$ T cells, 10^4 monocytes as antigen-presenting cells and a peptide mixture or individual peptides at 10 μg ml^{-1} each were placed in duplicate in 96-well plates with a PVDF membrane at the bottom (MAIP S45; Millipore). Well bottoms were coated with anti-IFN-γ monoclonal antibody (mAb). Cells were cultured for 40 h at 37 °C in a humidified 5 % CO_2 atmosphere. No peptide was added to the negative control wells. After culture, IFN-γ spot-forming cells (SFCs) were visualized as described previously (Lalvani *et al.*, 1997). Responses were considered significant when a minimum of five SFCs were present per well, representing at least twice the number of SFCs in negative control wells. In preliminary studies, monocytes did not present IFN-γ SFCs in response to stimulation with HCV peptides.

To enrich peptide-specific $CD8^+$ T cells that might recognize a known HLA-A*2402-restricted epitope, 2×10^6 PBMC pulsed with 10 μg peptide 1031–1039 ml^{-1} (Kurokohchi *et al.*, 2001) were cultured in a 24-well flat-bottom plate for 9 days in RPMI 1640 medium supplemented with 10 % human serum AB blood type and 50 U recombinant human interleukin (rhIL)-2 ml^{-1} added on day 2. The cells were harvested after 9 days of culture and $CD8^+$ T cells were isolated for an ELISpot assay.

Generation of HCV-specific CTLs. HCV-specific CTLs were generated as described previously (Hiroishi *et al.*, 2002). Briefly, PBMC were suspended at a cell density of 10^6 cells ml^{-1} in RPMI 1640 medium supplemented with 10 % human AB serum and a single peptide was added on day 0. Cells were incubated at 37 °C in a humidified 5 % CO_2 atmosphere. On day 2, rhIL-2 was added at a final concentration of 20 U ml^{-1}. On day 7, the culture was re-stimulated with the single peptide and irradiated autologous PBMC. Cytotoxic activity of peptide-induced effector cells was assessed on days 14–16.

CTL assay. The cytotoxic activity of peptide-induced effector cells was assessed using a standard 4 h sodium chromate (^{51}Cr) release assay. Briefly, Epstein–Barr virus-transformed B-lymphoblastoid cell lines (B-LCL) were labelled with 100 μCi (3·7 MBq) ^{51}Cr. The ^{51}Cr-labelled B-LCL were suspended in RPMI 1640 medium supplemented with 10 % fetal calf serum and incubated overnight at 37 °C with a synthetic peptide or infected with recombinant vaccinia virus (rVV) that endogenously expressed HCV antigens (K. Funatsuki & H. Ishiko, unpublished results). An m.o.i. of 5 was used for an 18 h incubation with rVV. Then, after incubating the effector cells with the target cells for 4 h at 37 °C in a humidified 5 % CO_2 atmosphere, supernatants were collected and radioactivity was measured with a gamma counter.

RESULTS

Screening of $CD8^+$ T cell epitopes by an ELISpot assay using the peptide mixtures

When the isolated $CD8^+$ T cells from 13 patients with HCV infection (subjects 1–13) were stimulated with peptide mixtures A–Q composed of HCV peptides with HLA-A*2402-binding motifs, eight of the 17 peptide mixtures elicited significant IFN-γ SFC responses (Table 3). Mixtures C and J each elicited responses from two patients' cells, but the other peptide mixtures each elicited responses in only one patient's cells. IFN-γ SFC responses to the peptide mixtures were observed in cells from two of three HLA-A*2402-positive patients with acute hepatitis C, three of four with chronic hepatitis C lasting for less than 3 years and none of four with chronic hepatitis C for more than 10 years. In two of the five patients who demonstrated a

Table 3. Number of IFN-γ SFCs per 10^5 CD8$^+$ T cells in response to stimulation with the individual peptide mixtures A–Q

Numbers in bold indicate positive IFN-γ SFC responses.

Subject	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	0	2	0	2	2	1	3	0	0	0	2	0	1	3	**94**	0	2
2	0	1	**56**	1	0	0	**13**	**9**	2	2	**51**	2	1	1	1	1	1
3	0	0	1	2	0	1	1	2	2	1	2	0	0	2	1	2	0
4	0	0	1	0	0	0	0	0	**8**	**49**	0	0	0	0	0	0	1
5	0	0	0	0	0	0	1	0	1	0	1	0	2	0	1	0	1
6	0	1	0	0	2	0	1	0	0	**44**	1	0	0	1	2	0	0
7	1	1	**7**	1	1	0	1	0	1	1	1	0	1	0	0	1	0
8	0	0	1	0	1	0	1	2	0	1	1	0	1	0	0	0	1
9	1	0	1	1	0	1	1	0	0	1	0	0	0	1	1	0	0
10	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1
11	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0
12	1	1	2	2	0	1	0	1	1	2	0	0	0	2	0	**79**	1
13	1	1	1	1	1	0	0	1	0	1	0	0	1	1	0	0	1
14	1	0	1	0	0	0	1	0	0	0	1	1	0	0	0	0	0
15	0	0	1	0	0	0	1	1	1	0	0	0	0	0	1	0	0
16	0	0	2	1	0	0	0	1	0	0	1	0	1	0	1	0	0
17	0	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0
18	0	1	0	1	1	0	1	0	0	1	0	0	0	1	0	1	1

positive IFN-γ SFC response to peptide mixtures, a response was observed against more than one peptide mixture. Although peptide mixture E contained two previously reported HLA-A*2402-restricted CTL epitopes, it did not elicit an IFN-γ SFC response from any patient's cells. Unexpectedly, one of two HLA-A*2402-negative patients with acute hepatitis C also demonstrated an IFN-γ SFC response to one of the peptide mixtures. None of the control subjects (subjects 14–18) demonstrated a positive IFN-γ SFC response to any peptide mixture.

Peptide specificity of HCV-specific CD8$^+$ T cells

To identify the peptides that elicited IFN-γ SFC responses, we assessed responses using isolated CD8$^+$ T cells and the individual peptides making up the peptide mixture that had elicited the initial response. The following 11 peptides that elicited a significant IFN-γ SFC response were identified: peptide 1375–1385 from mixture O in patient 1; peptide 790–798 from mixture C, peptide 2280–2288 from mixture G, peptide 284–293 from mixture H and peptides 1759–1768 and 1990–1999 from mixture K in patient 2; peptide 910–919 from mixture I and peptides 947–956 and 1243–1252 from mixture J in patient 4; peptide 1443–1452 from mixture J in patient 6; peptide 790–798 from mixture C in patient 7; peptide 2456–2466 from mixture P in patient 12 (data not shown). Peptide 790–798 elicited a response in both patients 2 and 7 who shared HLA-A*2402 and HLA-Cw*0801 molecules.

The magnitude of IFN-γ SFC responses to single-peptide stimulation ranged from 4 to 139 SFC per 10^5 CD8$^+$ T cells and summed frequencies of the SFCs in patients 2 and 4 were 146 and 193 SFC per 10^5 CD8$^+$ T cells, respectively. Of the 11 peptides, two were 9-mers, seven were 10-mers and two were 11-mers. These epitopes were distributed throughout the entire HCV protein; one epitope was localized in each of the E1 and E2 regions, two in the NS2 region, three in the NS3 region, two in the NS4 region and three in the NS5 region. None of the 11 epitopes had been reported previously.

The individual peptides previously reported as HLA-A*2402-restricted, HCV-specific CTL epitopes did not elicit an *ex vivo* IFN-γ SFC response in cells from any patient studied. The assay was repeated after CD8$^+$ T cells were expanded by stimulating CD8$^+$ T cells from patients 1 and 4 with the known HLA-A*2402-restricted, HCV-specific CTL epitope peptide 1031–1039 (Kurokohchi *et al.*, 2001) for 9 days in the presence of rhIL-2 to enrich CD8$^+$ T cells with specificity for the peptide. Although stimulation of the CD8$^+$ T cells with the epitope peptides identified in the present study enriched peptide-specific CD8$^+$ T cells, peptide 1031–1039-specific CD8$^+$ T cells could not be enriched to attain a detectable level (Fig. 1).

HLA restriction of peptide recognition

Using peptide–HLA-A*2402 dimer staining (Greten *et al.*, 1998), we examined whether recognition by CD8$^+$ T cells of the epitope peptides identified was truly restricted by the HLA-A*2402 molecule. Although all 11 peptides effectively bound to HLA-A*2402 dimer proteins, only HCV NS5A peptide 2280–2288–HLA-A*2402 dimer complexes could stain CD8$^+$ T cells of patient 2, indicating that recognition by CD8$^+$ T cells of the peptides other than the

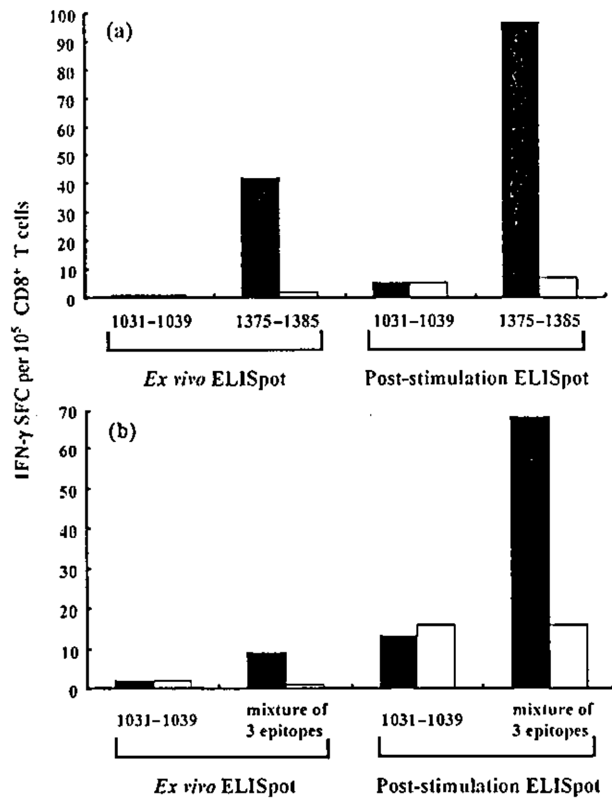

Fig. 1. Frequencies of IFN-γ SFCs in CD8$^+$ T cells assessed by either an *ex vivo* or a post-stimulation ELISpot assay in patients 1 (a) and 4 (b). In a post-stimulation assay, PBMC obtained from patient 1 at 5 months from onset of acute hepatitis and patient 4 at 5 months after the completion of 24 weeks of IFN-α therapy were stimulated with a known HLA-A*2402-restricted epitope, peptide 1031–1039 and cultured in the presence of 50 U rhIL-2 ml^{-1} for 9 days preceding an ELISpot assay. The newly identified epitope peptides (peptide 1375–1385 for patient 1 and a mixture of peptides 910–919, 947–956 and 1243–1252 for patient 4) were used as positive control peptides. Solid bars, Peptide-stimulated SFCs; open bars, non-peptide-stimulated SFCs.

peptide 2280–2288 was probably restricted by HLA class I molecules other than HLA-A*2402 (H. Morita & M. Imawari, unpublished results).

To define the HLA molecules that restricted recognition of peptides by CD8$^+$ T cells other than peptide 2280–2288, we attempted to induce CTL lines by stimulating PBMC from patients with the individual peptides. We could generate CTLs specific for peptides 910–919, 947–956 and 1243–1252 from PBMC of patient 4 and CTLs specific for peptide 1443–1452 from PBMC of patient 6. The peptide-induced CTLs lysed not only peptide-pulsed autologous B-LCL but also B-LCL that had been infected with rVV, resulting in HCV protein expression including the peptide sequence in infected cells (data not shown). HLA restriction of peptide recognition by CTLs was studied using a panel of autologous and allogeneic B-LCL with known HLA haplotypes as target cells. CTLs induced by peptide 910–919 or 1243–1252 selectively lysed B-LCL expressing HLA-Cw3 (HLA-Cw*0303 or HLA-Cw*0304 or both) that had been pulsed with the individual peptides (Fig. 2a and b), indicating that recognition of both HCV NS2 peptide 910–919 and NS3 peptide 1243–1252 was restricted by HLA-Cw*0303 and HLA-Cw*0304 molecules. However, since the NS3 peptide 1243–1252-specific CTL lysis restricted by HLA-Cw*0303 and HLA-Cw*0304 was less than the total lysis for the peptide, the recognition of the peptide also might be restricted by HLA-B*4002 (Fig. 2b). CTL induced by peptide 947–956 selectively lysed peptide-pulsed B-LCL expressing HLA-B61 (HLA-B*4002 or HLA-B*4006; Fig. 2c) indicating that recognition of HCV NS2 peptide 947–956 was restricted by HLA-B*4002 and HLA-B*4006 molecules. CTL induced by peptide 1443–1452 selectively lysed peptide-pulsed B-LCL expressing HLA-A*0206 (Fig. 2d), indicating that the recognition of the peptide 1443–1452 was restricted by an HLA-A*0206 molecule, although the possibility that the CTLs also could recognize the targets in an HLA-A*0207 molecule-restricted manner cannot be ruled out from the data in Fig. 2(d). CTLs induced by peptide 1443–1452 did not lyse peptide-pulsed B-LCL expressing HLA-A*0201 (data not shown).

Although we could not establish peptide-specific CTL lines, HCV E2 protein peptide 790–798 induced an IFN-γ SFC response in cells from patients 2 and 7, who shared HLA class I alleles HLA-A*2402 and HLA-Cw*0801. Since peptide–HLA-A*2402 dimer complexes did not stain PBMC from patient 2 or 7 (H. Morita & M. Imawari, unpublished results), recognition of peptide 790–798 by CD8$^+$ T cells is likely to be restricted by an HLA-Cw*0801 molecule. However, the possibility that HLA-A*2402 restricted the recognition of peptide 790–798 cannot be ruled out completely, since the dimer might not work efficiently.

Recognition of truncated and overlapping HCV peptides by CD8$^+$ T cells

To define further the epitopes within the peptides that elicited an IFN-γ SFC response, truncated and overlapping peptides were synthesized and assayed for their ability to elicit a response from CD8$^+$ T cells obtained from patients 4, 6 and 7 (Table 4).

Peptide 1443–1452 truncated by 1 amino acid at its C terminus (peptide 1443–1451 or GFTGDFDSV by one-letter code) identified in patient 6 evoked 1·5 times as many IFN-γ SFC as the original peptide, although the truncated peptide lost the HLA-A*2402-binding motif. Further truncation at the C terminus to produce peptide 1443–1450 or at the N terminus to produce peptide 1444–1452 led to loss of antigenicity. Thus, peptide 1443–1451 was defined as the minimal and optimal epitope for HCV-specific CD8$^+$ T cells. The amino acid sequence of HCV NS3 protein

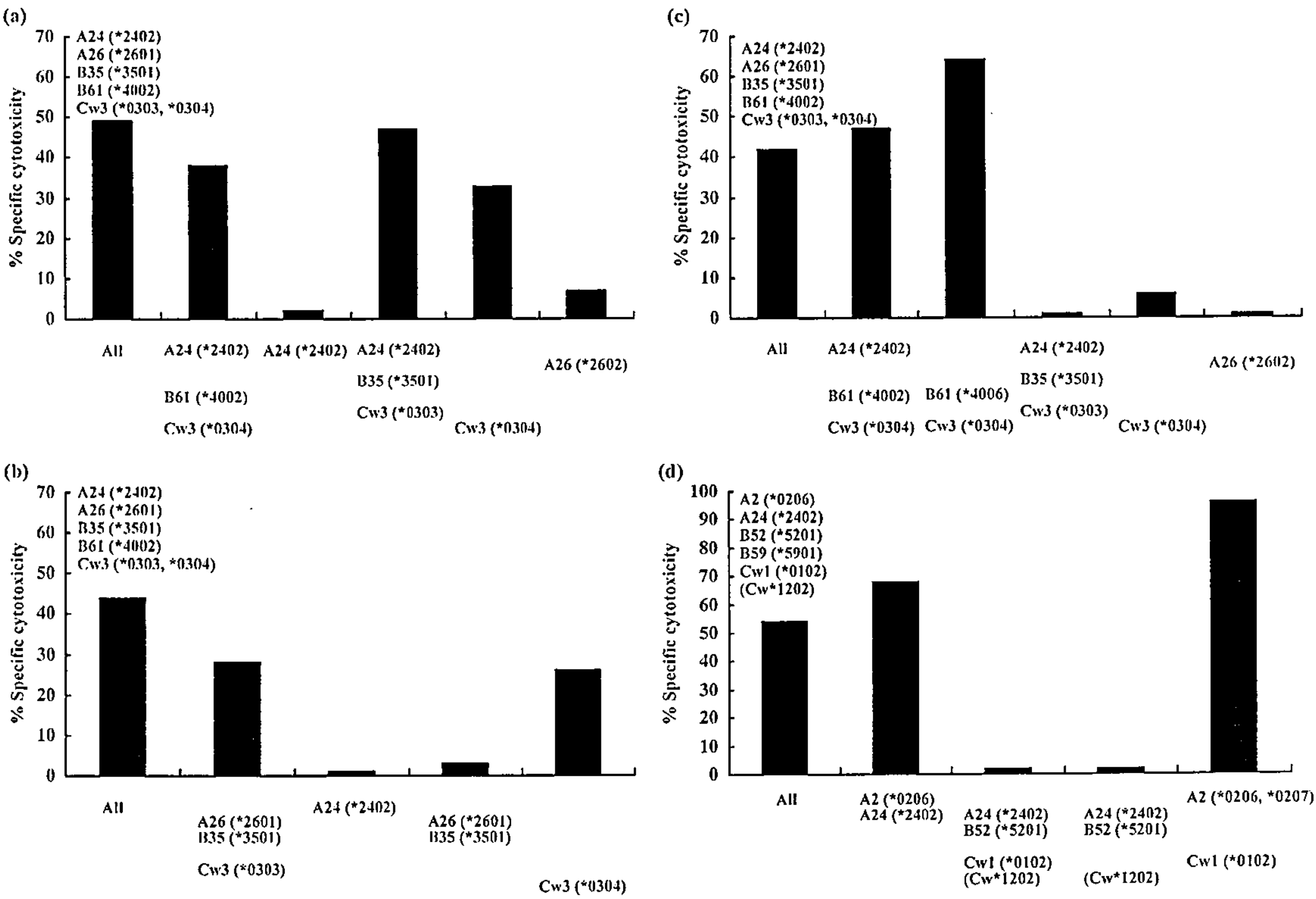
(a)
% Specific cytotoxicity
70
60
50
40
30
20
10
0
A24 (*2402)
A26 (*2601)
B35 (*3501)
B61 (*4002)
Cw3 (*0303, *0304)
All
A24 (*2402)
B61 (*4002)
Cw3 (*0304)
A24 (*2402)
A24 (*2402)
B35 (*3501)
Cw3 (*0303)
Cw3 (*0304)
A26 (*2602)
(c)
% Specific cytotoxicity
70
60
50
40
30
20
10
0
A24 (*2402)
A26 (*2601)
B35 (*3501)
B61 (*4002)
Cw3 (*0303, *0304)
All
A24 (*2402)
B61 (*4002)
Cw3 (*0304)
B61 (*4006)
Cw3 (*0304)
A24 (*2402)
B35 (*3501)
Cw3 (*0303)
Cw3 (*0304)
A26 (*2602)
(b)
% Specific cytotoxicity
70
60
50
40
30
20
10
0
A24 (*2402)
A26 (*2601)
B35 (*3501)
B61 (*4002)
Cw3 (*0303, *0304)
All
A26 (*2601)
B35 (*3501)
Cw3 (*0303)
A24 (*2402)
A26 (*2601)
B35 (*3501)
Cw3 (*0304)
(d)
% Specific cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
A2 (*0206)
A24 (*2402)
B52 (*5201)
B59 (*5901)
Cw1 (*0102)
(Cw*1202)
All
A2 (*0206)
A24 (*2402)
A24 (*2402)
B52 (*5201)
Cw1 (*0102)
(Cw*1202)
A24 (*2402)
B52 (*5201)
(Cw*1202)
A2 (*0206, *0207)
Cw1 (*0102)

Fig. 2. HLA restriction of peptides 910–919 (a), 1243–1252 (b), 947–956 (c) and 1443–1452 (d) recognition by CTLs induced by the individual peptides. PBMC stimulated with peptides 910–919, 1243–1252 and 947–956 (from patient 4) and PBMC stimulated with peptide 1443–1452 (from patient 6) were assayed for cytotoxicity directed at autologous and allogeneic B-LCL of known HLA class I haplotypes that had been pulsed with the individual peptides. The effector-to-target ratio was 20. Specific cytotoxicity expressed as a percentage was calculated by subtracting cytotoxicity of effector cells to non-peptide-pulsed B-LCL from cytotoxicity to peptide-pulsed B-LCL. HLA molecules of CTLs and those shared by target cells are indicated at the top and bottom, respectively.

residues 1443–1451 is well conserved among members of the same type and among different types of HCV except for the amino acid at position 1444, which can be either phenylalanine or tyrosine. HCV peptide 1443–1452 with tyrosine at position 1444 stimulated IFN-γ production by CD8$^+$ T cells from patient 6 as effectively as HCV peptide 1443–1452 with phenylalanine at position 1444 (data not shown).

Table 4. Recognition of truncated and overlapping peptides by the CD8$^+$ T cells isolated from PBMC of patients 4, 6 and 7

	HCV peptide	Amino acid sequence	SFCs*
Patient 4	910–919	PYFVRAQGLI	35
	910–918	PYFVRAQGL	2
Patient 6	1443–1452	GFTGDFDSVI	40
	1443–1451	GFTGDFDSV	67
	1444–1452	FTGDFDSVI	2
	1443–1450	GFTGDFDS	4
Patient 7	790–798	LYGVWPLLL	15
	790–797	LYGVWPLL	2

*No. of IFN-γ-secreting cells per 10^5 CD8$^+$ T cells in response to the individual synthetic peptides.

Since both peptides 790–798 and 910–919 had an HLA-A*2402-binding amino acid residue at the position next to the C terminus, peptides with truncation of the HLA-A*2402-binding amino acid at the C terminus were synthesized and assayed for antigenicity. Neither of the truncated peptides retained antigenicity, indicating that C-terminal amino acids of peptides 790–798 and 910–919 were essential for antigenicity. The effect of truncation of the N-terminal amino acids was not studied. No studies of effects of truncation were performed for the other eight peptides.

HCV-specific CD8$^+$ T cell epitopes that were identified and their HLA restriction are shown in Table 5.

Sequential analysis of HCV-specific CD8$^+$ T cell responses in two patients with unresolved acute hepatitis treated with IFN-α

To study the effects of IFN therapy on HCV-specific CD8$^+$ T cell responses, we monitored changes in frequency of IFN-γ-releasing CD8$^+$ T cells in the peripheral bloods in patients 4 and 7 by ELISpot assay. The individual HCV epitope peptides were used to carry out assays during and after treatment with IFN-α or consensus IFN (Tong *et al.*, 1997) (Fig. 3). In both patients, frequencies of HCV peptide-specific, IFN-γ-releasing CD8$^+$ T cells in the peripheral blood decreased upon IFN therapy in association

Table 5. Novel HCV-specific CD8$^+$ T cell epitopes identified and their HLA restriction

HCV protein	Amino acid residues	Sequence	HLA restriction	Patient
E1	284–293	VFLVSQLFTF	ND	2
E2	790–798†	LYGVWPLLL	Cw*0801?	2, 7
NS2	910–919†	PYFVRAQGLI	Cw*0303, 0304	4
NS2	947–956	TYVYDHLTPL	B*4002, 4006	4
NS3	1243–1252	AYAAQGYKVL	Cw*0303, 0304	4
NS3	1375–1385	FYGKAIPIEAI	ND	1
NS3	1443–1451‡	GFTGDFDSV	A*0206	6
NS4B	1759–1768	AFWAKHMWNF	ND	2
NS5A	1990–1999	DFKTWLQSKL	ND	2
NS5A	2280–2288	KFPPALPIW	A*2402	2
NS5B	2456–2466	VYSTTSRSASL	ND	12

ND, Not determined.

†Amino acid(s) at the N terminus may be able to be truncated although the amino acid at the C terminus cannot be truncated.

‡Defined as minimal and optimal epitope.

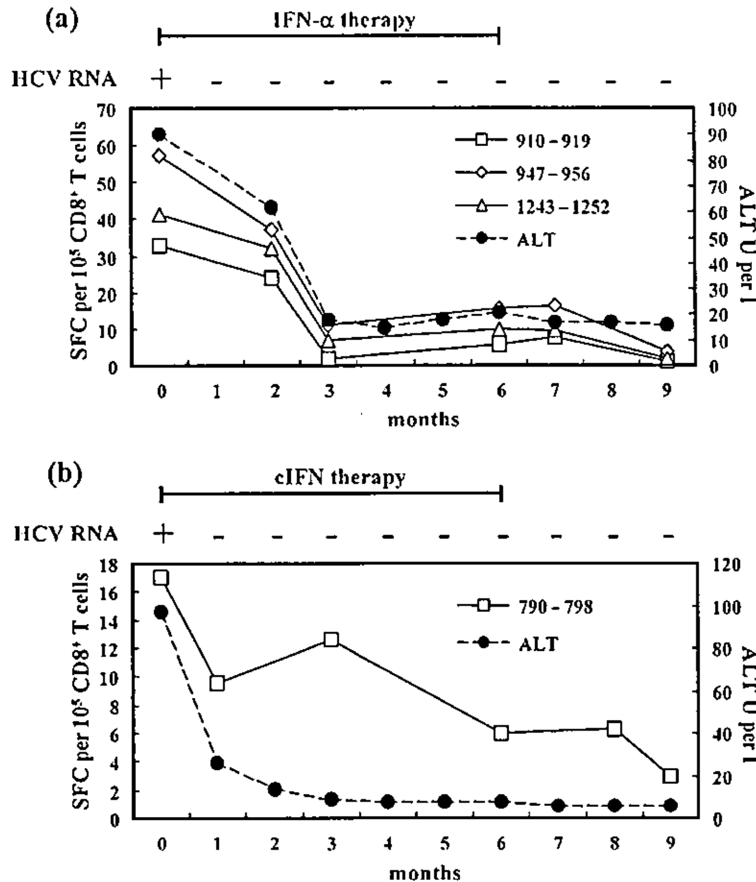

Fig. 3. Sequential analysis of HCV-specific $CD8^+$ T cell responses in two patients with unresolved acute hepatitis who were treated with IFN-α. (a) Patient 4 was treated with 10^7 units IFN-α daily for the first 2 weeks, followed by injection of the same dose of IFN-α three times weekly for the following 22 weeks. (b) Patient 7 was treated with $1{\cdot}8\times10^7$ units consensus IFN (cIFN) daily for the first 2 weeks, followed by injection of the same dose of cIFN three times weekly for the following 22 weeks. Both patients showed sustained virological responses.

with disappearance of serum HCV RNA and with ALT normalization. They were nearly undetectable at and after completion of therapy.

DISCUSSION

A proportion of patients with chronic HCV infection remain resistant to antiviral therapies including recently developed treatment modalities such as IFN-α2b plus ribavirin (Poynard *et al.*, 1998) and pegylated IFN (Zeuzem *et al.*, 2000). Such treatment failure may be partly a reflection of insufficient antiviral immune responses. Augmentation of HCV-specific $CD8^+$ CTL responses by therapeutic vaccines could enhance HCV elimination by IFN therapy, leading to a better treatment outcome. Development of a universally immunogenic vaccine would require identification of as many CTL epitopes as possible, especially those recognized by CTLs in association with common HLA class I molecules in the population.

In the present study, we sought to identify HCV-specific, $CD8^+$ T cell epitopes, with recognition restricted by HLA-A*2402, the most frequent HLA class I allele in Japanese and other Asians (Chandanayingyong, 1986). We screened the epitopes by an ELISpot assay based on IFN-γ release by $CD8^+$ T cells obtained from HLA-A*2402-positive patients with acute or chronic hepatitis C in response to peptide stimulation. Eighty-seven peptides were synthesized based on HLA-A*2402-binding motifs and the amino acid sequence of type 1b HCV. We could identify 10 HCV-specific CTL epitopes that induced IFN-γ release by $CD8^+$ T cells from a total of five of seven HLA-A*2402-positive patients with acute or relatively early chronic hepatitis C but not in any of four patients with persisting chronic hepatitis. The findings indicate that the response of HCV-specific CTLs to the panel of peptides is very low in patients with prolonged HCV infection. Consistent with this interpretation, an HLA-B*3501-restricted CTL epitope peptide that induced strong HCV-specific CTL responses in peripheral blood cells in the acute phase of HCV infection reportedly failed to induce CTL responses in seven of seven patients with chronic hepatitis C (Ibe *et al.*, 1998). Frequencies of HLA-B*3501-restricted, HCV-specific CTL also have been reported to be very low in the peripheral blood of patients with chronic hepatitis C, although CTLs were detectable among the PBMC by flow cytometric analysis using HLA-B*3501 tetramers (Sobao *et al.*, 2001). In still other reports, frequencies (Lechner *et al.*, 2000; Rehermann *et al.*, 1996; He *et al.*, 1999) and IFN-γ-production potential (Gruener *et al.*, 2001; Wedemeyer *et al.*,

2002) of antiviral CTLs were low in patients with chronic HCV infection.

Unexpectedly, only one of the ten CTL epitopes identified in HLA-A*2402-positive patients was found to be definitely HLA-A*2402-restricted. In addition, the frequency of $CD8^+$ T cells that responded to stimulation with the epitope was far less than for other CTL epitopes in this patient. The HLA class I molecules that restricted recognition of the other five epitopes by $CD8^+$ T cells were thought to be HLA-Cw*0303 and HLA-Cw*0304 for two epitopes, HLA-B*4002 and HLA-B*4006 for one, HLA-A*0206 for one and probably HLA-Cw*0801 for another. The HLA class I molecules that restricted recognition of the remaining four epitopes by $CD8^+$ T cells have not yet been defined. One more $CD8^+$ T cell epitope was identified using the peptides with HLA-A*2402-binding motifs in one of two HLA-A*2402-negative patients with acute hepatitis C, although the HLA class I molecule restricting recognition of the epitope has not been determined. In two of the six patients with positive $CD8^+$ T cell responses the target epitopes were multiple and only one of the 11 peptides was targeted in more than one patient. These findings indicate that a universally immunogenic HLA-A*2402-restricted, HCV-specific $CD8^+$ T cell epitope may not exist; epitopes with recognition by $CD8^+$ T cells restricted by HLA molecules other than HLA-A*2402 presumably were contained in the synthetic peptides with HLA-A*2402-binding motifs. CTL responses to HCV infection are heterogeneous, as concluded by Lauer *et al.* (2002).

We analysed sensitivity and specificity of previously reported HLA-A*2402-restricted, HCV-specific CTL epitopes (Kurokohchi *et al.*, 2001; Ito *et al.*, 2001) in HLA-A*2402-positive patients with acute and relatively recently acquired chronic hepatitis C. These two epitope peptides did not induce IFN-γ SFC responses, suggesting that their immunogenicity might be low compared with other epitopes. However, HCV NS3 peptide 1031–1039 identified by Kurokohchi *et al.* (2001) has been reported to induce HCV-specific CTLs in three of four HLA-A*2402-positive patients with chronic hepatitis C. It has been reported that *in vitro* expansion of $CD8^+$ T cells by stimulation with known HLA-A2-restricted CTL epitopes and culture in the presence of rhIL-2 revealed the existence of $CD8^+$ T cells specific for the peptide, although IFN-γ SFC responses *ex vivo* could not be induced (Lauer *et al.*, 2002). However, we could not confirm immunogenicity of the 1031–1039 epitope, even after stimulation and expansion with culture in the presence of rhIL-2. The limited number of patients in our study may have happened to lack CTLs responsive to stimulation with peptide 1031–1039; alternatively, the IFN-γ-based ELISpot assay might detect a $CD8^+$ T cell population that is functionally different from the CTLs identified by Kurokohchi *et al.* (2001). Consistent with this speculation, a 'stunned' $CD8^+$ T cell population has been reported to emerge in the acute phase of HCV infection, retaining potent HCV-specific cytotoxicity but only limited capacity for IFN-γ production (Lechner *et al.*, 2000; Thimme *et al.*, 2001). CTLs responsive to the 1031–1039 peptide may belong to such a population.

Reliability of T cell epitope prediction based on HLA-binding motifs or algorithms (Rammensee *et al.*, 1999) has been reported to be limited (Lauer *et al.*, 2002; Anthony *et al.*, 2002; Day *et al.*, 2001). Therefore, establishing that no immunodominant HLA-A*2402-restricted, HCV-specific $CD8^+$ T cell epitope exists would require screening HLA-A*2402-restricted, HCV-specific $CD8^+$ T cell epitopes by an IFN-γ ELISpot assay using overlapping peptides that span the entire HCV protein; such a study, in progress in our laboratory, may identify new HLA-A*2402-restricted, HCV-specific $CD8^+$ T cell epitopes without known HLA-A*2402-binding motifs. This study is intended to define the hierarchy of immunodominance of CTL epitopes in patients with HCV infection. Lauer *et al.* (2002) demonstrated multiple unpredicted specificities of HCV-specific $CD8^+$ T cell epitopes by an ELISpot assay using overlapping peptides that spanned the entire HCV protein, but none of the new epitopes that they found corresponded to those identified in the present study.

Using the $CD8^+$ T cell epitopes currently identified, we sequentially monitored frequencies of $CD8^+$ T cells secreting IFN-γ in response to stimulation with the epitope peptides during and after treatment of two patients with unresolved acute hepatitis C with IFN-α. Although effects of IFN-α therapy on HCV-specific $CD8^+$ T cell responses have been reported from several laboratories (Löhr *et al.*, 1999; Vertuani *et al.*, 2002; Barnes *et al.*, 2002), results are conflicting. Löhr *et al.* (1999) have reported that augmentation of HLA class I-restricted tumour necrosis factor (TNF)-α responses by IFN-α therapy contributes to a better treatment outcome in patients with chronic hepatitis C. The decline of serum HCV RNA during IFN-α therapy has been described as having two phases: a rapid early phase, thought to reflect direct inhibition of HCV replication by IFN-α; and a slower second phase, thought to be mediated by cellular immune responses, especially those of CTLs (Neumann *et al.*, 1998). Augmentation of TNF-α-releasing HCV-specific $CD8^+$ T cell responses by IFN-α may beneficially affect the second phase of HCV RNA decline. However, in our present study, numbers of HCV epitope peptide-sensitized $CD8^+$ T cells in peripheral blood declined in parallel with decreases and disappearance of serum HCV RNA and with ALT normalization. The reason why our results differed from those of Löhr *et al.* (1999) is not known, but it could involve differences in degree of chronicity of disease, decrease rates of serum HCV RNA, doses of IFN-α or ethnicity of patients.

In conclusion, we have newly identified one definite HLA-A*2402-restricted, HCV-specific $CD8^+$ T cell epitope and 10 probably non-HLA-A*2402-restricted epitopes by an IFN-γ-based ELISpot assay using synthetic HCV peptides with HLA-A*2402-binding motifs. We could find HCV-specific CTL epitopes only in patients with acute or relatively

early chronic hepatitis C. $CD8^+$ T cell responses to HCV infection were heterogeneous. The findings indicate a need to identify as many HCV-specific $CD8^+$ T cell epitopes as possible in large numbers of patients with acute or recently acquired chronic hepatitis C to understand better how immune responses eliminate HCV and contribute to pathogenesis. The ultimate aim is development of new strategies for enhancing immune responses for more effective control of HCV infection.

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