Abstract: Background and Aims: Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTL) may contribute to viral clearance and liver cell injury in patients with chronic hepatitis C. In the present study, we attempted to determine the serial HCV-specific CTL activity during interferon-beta (IFN-β) therapy in patients with chronic hepatitis C and whether there is any relationship between the CTL response and clinical response to IFN-β therapy.

Methods: Eight HLA-A2-positive patients with chronic hepatitis C were treated initially with 6 million U/ml of IFN-β every day for 8 weeks and then 3 times weekly for the subsequent 16 weeks. Peripheral blood mononuclear cells (PBMC) were collected before the start, 4 weeks after the start, and after the end of IFN treatment and were stimulated with 2 peptides corresponding to core sequences, which were previously reported to have an HLA-A2 restricted-CTL epitopes. Cytolytic activity was determined by a standard 51Cr-release assay using allogenic HLA-matched EBV-transformed B lymphoblastoid cell lines (B-LCL).

Results: HCV-specific CTL responses were detected in 2 of the 8 patients before treatment with IFN-β. One of 2 patients was not observed HCV-specific CTL responses after 4 weeks of IFN-β treatment, however these two patients showed CTL responses at the end of IFN-β treatment, and finally HCV-RNA was negative. In addition, HCV-specific CTL responses were observed in 4 patients after 4 weeks of IFN-β treatment. Three of these 4 patients showed CTL responses only at 4 weeks after IFN-β treatment. However, there were no differences between clinical parameters or between IFN efficacy in HCV-specific CTL response-positive (n=4) and -negative (n=4) patients at 4 weeks after the start of IFN-β treatment.

Conclusions: These findings suggest that there are few relations between peripheral
HCV-specific CTL response and clinical response to IFN therapy in patients with chronic hepatitis C, although IFN enhances the host immune response against HCV synergistically with antiviral activities.

**Key words**: chronic hepatitis C, cytotoxic T lymphocyte, interferon, host immune response, target cell

**INTRODUCTION**

Interferon (IFN) is an essential component of therapy for patients with chronic hepatitis C virus (HCV) infection. In general, the efficacy of IFN is mainly determined by the HCV genotype and quantity of serum HCV-RNA before treatment; however, the host immune response may play an important role in the elimination of HCV during IFN treatment. The host cellular immune defense, including the CD4+ and CD8+ T-cell responses, is activated in patients with HCV infection. HCV-specific cytotoxic T lymphocyte (CTL) response is thought to cause hepatic injury and determine the course of an HCV infection. Peripheral blood mononuclear cells (PBMC) obtained from patients with chronic hepatitis C showed CTL responses to core, E1, E2, NS3, NS4, and NS5 epitopes presented as synthetic peptides on human leukocyte antigen (HLA) class I-matched target cells. The presence of intrahepatic HCV-specific CTLs in patients with chronic hepatitis C has also been reported. The CTL response appears to be relatively weak and has an inverse relationship with the HCV virus titer, suggesting that the induction of CTL response might be a useful therapeutic antiviral strategy.

Although the specificity of HCV CTL epitopes has been studied in detail, there has been little study of CTL activity during IFN treatment in patients with chronic hepatitis C. IFN has been shown to up-regulate the expression of HLA class I antigen on liver cells, suggesting that it enhances HCV viral antigen presentation. Since several HLA-A2-restricted HCV CTL epitopes have already been reported, the present study was designed to determine HLA-A2-restricted cytotoxic T cell activity during interferon beta (IFN-β) therapy in patients with chronic hepatitis C and whether there is any relationship between CTL response and clinical response to IFN-β therapy.

**MATERIALS AND METHODS**

**Patients**

Eight patients with chronic hepatitis C and HLA-A2 molecules, two HCV-negative healthy subjects with HLA-A2 molecules, and two chronic hepatitis C patients without HLA-A2 molecules participated in this study after giving written informed consent. The characteristics of the 8 patients with chronic hepatitis C are
Table 1 Clinical and virological data of 8 patients with chronic HCV infection

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age, Sex</th>
<th>HCV genotype</th>
<th>HCV-RNA (KIU/ml)</th>
<th>ALT (IU/l)</th>
<th>Histology</th>
<th>Response to IFN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46, F</td>
<td>1b</td>
<td>9.8</td>
<td>19 133 15</td>
<td>A1/F1</td>
<td>CR</td>
</tr>
<tr>
<td>2</td>
<td>53, M</td>
<td>2a</td>
<td>31</td>
<td>25 112 10</td>
<td>A1/F1</td>
<td>CR</td>
</tr>
<tr>
<td>3</td>
<td>63, M</td>
<td>2a</td>
<td>52</td>
<td>182 109 17</td>
<td>A2/F2</td>
<td>CR</td>
</tr>
<tr>
<td>4</td>
<td>47, M</td>
<td>1b</td>
<td>1.9</td>
<td>149 48 48</td>
<td>A2/F1</td>
<td>CR</td>
</tr>
<tr>
<td>5</td>
<td>49, M</td>
<td>2a</td>
<td>6.1</td>
<td>254 230 49</td>
<td>A2/F2</td>
<td>CR</td>
</tr>
<tr>
<td>6</td>
<td>48, F</td>
<td>1b</td>
<td>(10)*</td>
<td>207 110 58</td>
<td>A1/F1</td>
<td>NR</td>
</tr>
<tr>
<td>7</td>
<td>60, F</td>
<td>1b</td>
<td>260</td>
<td>61 81 53</td>
<td>A1/F2</td>
<td>NR</td>
</tr>
<tr>
<td>8</td>
<td>55, M</td>
<td>1b</td>
<td>680</td>
<td>76 51 72</td>
<td>A2/F1</td>
<td>NR</td>
</tr>
</tbody>
</table>

*Meq/ml; CR, complete response; NR, non-response

shown in Table 1. All patients were seropositive for the antibody to HCV (EIA III; Ortho Diagnostics, Tokyo, Japan), had histologically proven chronic hepatitis, and were seronegative for hepatitis B surface antigen. Histopathological assessment of the grade of hepatic fibrosis and necro-inflammatory reaction were based on the new Inuyama classification for chronic hepatitis. None of the patients had previously been treated with IFN. Serum HCV-RNA was quantified using an Amplicore HCV Monitor ver. 2 assay kit (Roche Molecular Systems, Inc., Branchburg, NJ) or branched-chain DNA assay kit (Quantiplex HCV RNA, Chiron Corp. Emeryville, CA) and was detected by reverse-transcriptase polymerase chain reaction (RT-PCR). Genotype analysis of HCV was performed according to the method described by Okamoto et al. Patients were treated initially with 6 million U/ml of IFN-β (Toray Co., Ltd., Tokyo, Japan) every day for 8 weeks and then 3 times weekly for the subsequent 16 weeks. Complete response (responders) was defined as undetectable serum HCV-RNA by RT-PCR at 6 months after the IFN treatment (Table 1).

Stimulation of PBMC with synthetic peptides

Heparinized blood samples were collected before the start, 4 weeks after the start, and after the end of IFN treatment, and PBMC were obtained by Ficoll-Hypaque density gradient centrifugation and were cryopreserved in a CELL BANKER (DIA-IATRON Co., Ltd., Tokyo, Japan). Two peptides (pep-1, YLLPRRGPRL [35-44]; pep-2, DLMGYIPLV [132-140]) corresponding to core sequences that were previously reported to have an HLA-A2 restricted CTL were used for in vitro stimulation of PBMC according to the modified method described by Kita et al. Cryopreserved PBMC (rapidly thawed at 37°C and washed three times in cold Hank’s balanced salt solution (HBSS; GIBCO BRL, Grand Island, NY) were suspended at a cell density of 4 x 10⁸ cells/ml/well in 10% human AB serum-AIMV (LIFE TECHNOLOGIES, Rockville, MD), and a synthetic peptide (10
μg/ml), PPD (5 μg/ml) and IL-2 (20 U/ml) were added on day 0. The cells were incubated at 37°C in a humidified 5% CO₂ atmosphere. On day 5, the culture was stimulated with PPD (5 μg/ml) and IL-2 (20 U/ml). On day 8, the cultures were restimulated with mitomycin C-treated (50 μg/ml) autologous PBMC (1×10⁶ cells/ml), a synthetic peptide (10 μg/ml), and IL-2 (20 U/ml).

**Cytotoxicity assays**

Allogenic HLA-matched EBV-transformed B lymphoblastoid cell lines (B-LCL) were established as target cells from normal donors according to the method described by Cerny et al. The cells were incubated overnight with synthetic peptides at 10 μg/ml. Target cells were labeled with 100 μCi of ⁵¹Cr (Amersham Co., Arlington Heights, IL) for 1 hour and washed three times with HBSS. Cytologic activity was determined in a standard 4-h ⁵¹Cr-release assay using U-bottom 96-well plates containing 10,000 cells/well. All assays were performed in triplicate. Percent cytotoxicity was determined from the formula 100×[(experimental release–spontaneous release)/(maximum release–spontaneous release)] at an effector-to-target cell ratio of 20:1. Maximum release was determined by lysis of target cells with 10% Triton X-100 (Sigma Chemical Co., St. Louis, MO). HCV-specific CTL responses were considered as positive by the statistical analysis to compare the difference between the percent cytotoxicity of pep-1 or pep-2-pulsed target cells and that of nonpulsed target cells.

**Statistical analysis**

Values for results are presented as means±SD. Welch’s t-test was used to compare the difference between the percent cytotoxicity of peptide-pulsed target cells and that of nonpulsed target cells and a CTL response was considered positive when the p value was less than 0.05. Fisher’s exact probability test was used to compare the difference between biochemical and virological parameters and the difference between efficacy of IFN in CTL-positive patients and that in CTL-negative patients with chronic hepatitis C at 4 weeks after IFN treatment.

**RESULTS**

In this study, 5 of the 8 patients with chronic HCV infection were considered to have shown a complete response to IFN-β treatment (Table 1). HLA-A2-restricted CTL response was observed in PBMC from 5 of 8 patients at 1 to 3 points in this study (Table 2). There were no significant differences between % cytotoxicities of pep-1- or pep-2-pulsed PBMC and unpulsed PBMC from 2 healthy controls with HLA-A2 molecules and 2 chronic hepatitis C patients without HLA-A2 molecules. As shown in Table 2, HCV-specific CTL responses to pep-2 were detected in 2 of the 8 patients with chronic HCV infection before treatment with IFN-β. These two patients (patients 2 and 3) showed HCV-specific CTL responses
for pep-1 or pep-2 even at the end of IFN-β treatment, and finally HCV-RNA was negative after six month (Table 1). In addition, HCV-specific CTL responses to pep-1 or pep-2 were observed in 4 patients with chronic HCV infection after 4 weeks of IFN-β treatment. Three of these 4 patients showed CTL responses only at this point during IFN-β treatment. Only 1 of these 3 patients was defined as IFN responders.

Representative HCV-specific CTL responses in two patients with chronic hepatitis C are shown in Fig. 1. Patient 3 was a 63-year-old man who was considered to have shown a complete response to IFN-β treatment. His HCV genotype was 2a, and his HCV-RNA level before treatment was low. HCV-specific CTL responses to pep-2 were observed before and at 4 weeks after the start of IFN treatment, and those to pep-1 were observed at the end of IFN treatment (Fig. 1a). Patient 7 was a 60-year-old woman who was considered to have shown no response to IFN-β treatment. Her HCV genotype was 1b, and her HCV-RNA level before treatment was high. HCV-specific CTL response to pep-1 or pep-2 was not observed before IFN treatment, but she showed a newly induced CTL response to pep-2 at 4 weeks after IFN treatment (Fig. 1b).

A comparison of HCV-specific CTL response-positive (n=4) and -negative (n=4) patients at 4 weeks after the start of IFN-β treatment showed that there were no differences in the ALT levels before and at 4 weeks after the start of IFN treatment, HCV genotype distribution, HCV-RNA levels, and IFN efficacy (Table 3).

**DISCUSSION**

In this study, HLA-A2-restricted HCV-specific CTL responses were observed from PBMC of patients with chronic hepatitis C, and HCV-specific CTL responses were observed at 4 weeks after IFN treatment. However, clinical correlates of HCV-specific CTL response positivity were not observed in this study. Before IFN therapy, two patients (patient 2 and 3) with chronic hepatitis C
showed HCV-specific CTL responses. These two patients showed complete responses to IFN-β treatment. Hoffman et al. showed that the presence of HCV-specific CD4+ response in peripheral circulation may be associated with a favorable response to IFN therapy18, and Nelson et al. showed that the detectable intrahepatic HCV-specific CTL activity before IFN treatment developed a complete response to IFN18. These studies suggest that the host immune response may be important in determining the outcome of IFN therapy for chronic hepatitis C. Our two patients with HCV-specific CTL response before IFN treatment also developed a CR to IFN. However, it is difficult to judge whether the presence of HCV-specific CTL response before IFN treatment was involved in the eradication of HCV in our two patients.
Table 3 Comparison of clinical and virological data in patients negative for HCV-specific CTL activity and those positive for HCV-specific CTL activity at 4 weeks after IFN treatment.

<table>
<thead>
<tr>
<th></th>
<th>Negative (n=4)</th>
<th>Positive (n=4)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>man: woman</td>
<td>3:1</td>
<td>2:2</td>
<td>NS</td>
</tr>
<tr>
<td>ALT (IU/l) 0 W</td>
<td>158.8±99.0</td>
<td>84.5±69.3</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>125.0±76.0</td>
<td>93.5±35.4</td>
<td>NS</td>
</tr>
<tr>
<td>Genotype 1b:2a</td>
<td>2:2</td>
<td>3:1</td>
<td>NS</td>
</tr>
<tr>
<td>HCV-RNA (KIU/ml)</td>
<td>13.0±15.7</td>
<td>250.5±306.5</td>
<td>NS</td>
</tr>
<tr>
<td>IFN response CR:NR</td>
<td>3:1</td>
<td>2:2</td>
<td>NS</td>
</tr>
</tbody>
</table>

CR, complete response; NR, non-response; NS, not significant.

because these patients had a low titer of serum HCV-RNA (<100 KIU/ml) and genotype 2a, which are favourable predictive factors of IFN response. Hiroishi et al. showed that a high HCV-specific CTL response to nucleoprotein residues 88-96 was associated with a low titer of serum HCV-RNA in HLA B44-positive patients infected with HCV\(^\text{10}\). In this study, 3 of 5 CR patients with a low titer of serum HCV-RNA (<100 KIU/ml) as well as 3 NR patients with a high titer of serum HCV-RNA (>100 KIU/ml) had no detectable HLA-A2-restricted HCV-specific CTL response before IFN treatment. Therefore, CTLs recognizing pep-1 and pep-2, which were HLA-A2-restricted HCV CTL epitopes used in this study, might not play a major role in suppression of the outgrowth of HCV.

IFN has been shown to up-regulate the expression of HLA class I antigen on liver cells\(^\text{10}\), and it is therefore possible that IFN may directly augment CTL activity or enhance viral antigen processing and presentation. Indeed, IFN treatment led to an elevated production of various cytokines such as IL-4, IL-6, IL-10, tumour necrosis factor-alpha (TNF-alpha) and transforming growth factor-beta (TGF-beta)\(^\text{19,20}\). These cytokines are involved in the induction of CTL activity. In this study, 3 patients observed HLA-A2-restricted HCV-specific CTL responses at 4 weeks after IFN-\(\beta\) treatment. Although only one of the 3 patients showed a complete response to IFN, the other one patient was negative for serum HCV-RNA at 4 weeks after the start of IFN treatment. In this study, no difference was found between clinical parameters in HCV-specific CTL response-positive (\(n=4\)) and negative (\(n=4\)) patients at 4 weeks after the start of IFN-\(\beta\) treatment. This is the first study to show serial HCV-specific CTL activity during IFN treatment. But our data base is small, further study is needed to clarify the relationship between HCV-specific CTL activity and IFN efficacy.

The results of this study are not necessarily evaluating all of the HCV-specific CTL activity. There are various HCV-specific binding motifs even if it limits only HLA-A2 molecules\(^\text{11}\). It is possible that there were only a few patients who were positive for HCV-specific CTL activity in this study because only two peptides
corresponding to HCV core sequences were used. In addition, there is a possibility that cryopreserved PBMC influenced the results of CTL activity. Racial differences should also be considered in the selection of peptides. It would also be of value to examine serial change in HCV-specific CTL activity of intrahepatic lymphocytes during IFN treatment. IFN possesses both antiviral and immunomodulatory activities. Elucidation of the mechanisms underlying host immune response against HCV during IFN treatment would contribute to the establishment of better therapeutic approaches.

REFERENCES

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