A minimal and optimal cytotoxic T cell epitope within hepatitis C virus nucleoprotein

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Amino acid residues 81–100 of the hepatitis C virus (HCV) nucleoprotein contain a cytotoxic T cell epitope that is recognized by cytotoxic T lymphocytes (CTLs) in association with human leukocyte antigen B44. With panels of truncated and overlapping peptides, the minimal and optimal epitope recognized by CTLs was shown to be a 9-mer peptide (residues 88–96). The peptide can stimulate effectively CTLs that are able to recognize endogenously synthesized and processed HCV nucleoprotein.

Cytotoxic T lymphocytes (CTLs) are thought to be a major host defence against virus infection. Human CTLs recognize endogenously synthesized and processed virus proteins in association with human leukocyte antigen (HLA) class I molecules, and eliminate virus-infected cells (Gotch et al., 1987; Cannon et al., 1988). The size of the peptides in association with HLA class I molecules is usually 8–10 amino acids long.

Hepatitis C virus (HCV) infection frequently persists and results in chronic hepatitis, cirrhosis and, eventually, hepatocellular carcinoma. Post-transfusion HCV infection showed a substantial decrease after the introduction of an HCV antibody screening assay, but sporadic cases of acute HCV infection still occur. Interferon is effective in less than 50% of patients with chronic HCV infection (Hino et al., 1994). By genetic mutation, the HCV has been shown to escape recognition by neutralizing antibodies (Shimizu et al., 1994). The lack of an effective neutralizing humoral immune response to HCV may be correlated in part with virus persistence (Farci et al., 1992). However, insufficient CTL responses may also be responsible for virus persistence in HCV infection. It has been reported that HCV-specific CTLs exist in both peripheral blood lymphocytes (PBLs) (Kita et al., 1993) and liver-infiltrating lymphocytes (Koziel et al., 1992) of patients with chronic HCV infection. We previously demonstrated the presence of immunodominant CD8+ CTLs that recognize HCV nucleoprotein by repeated stimulation with a synthetic peptide of PBLs of patients with chronic HCV infection (Kita et al., 1993). The epitope was mapped to HCV nucleoprotein residues 81–100, the amino acid sequence of which is highly conserved between members of the same type and between the different types of HCV (Weiner et al., 1991). This recognition is restricted by HLA B44. CTLs that recognized the epitope could not be generated in any of eight HLA B44-negative patients with HCV infection (Kita et al., 1994), suggesting that the epitope preferentially stimulates HLA B44-restricted CTLs. We recently observed that HCV-specific CTL activity correlates negatively with serum titres of HCV RNA in patients with chronic HCV infection and HLA B44; a finding that suggests that CTLs suppress the outgrowth of HCV (K. Hiroishi, H. Kita, M. Kojima, H. Okamoto, T. Moriyama, T. Kaneko, S. Ohnishi, T. Aikawa, N. Tanaka, Y. Yazaki, K. Mitamura & M. Imawari, unpublished results). Therefore, vaccines based on CTL epitopes may be useful for the prevention and treatment of HCV infection.

This report describes the identification of a minimal and optimal sequence recognized by HLA B44-restricted CTLs specific for the HCV nucleoprotein using panels of truncated and overlapping peptides.

Two patients with the HLA B44 phenotype were studied. Patient 1 had received multiple blood transfusions, had chronic HCV infection, and had eliminated HCV with interferon therapy. The patient's HLA class I haplotype was A24, B35 and B44. Patient 2 had suffered...
Table 1. Recognition of truncated and overlapping HCV nucleoprotein synthetic peptides by NP9-stimulated PBLs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Specific cytotoxicity (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>81-100</td>
<td>YPWPLYGNEGLGWAGWLLSP</td>
<td>Patient 1: 83, Patient 2: 54</td>
</tr>
<tr>
<td>81-99</td>
<td>YPWPLYGNEGLGWAGWLLS</td>
<td>Patient 1: 79, Patient 2: 46</td>
</tr>
<tr>
<td>81-98</td>
<td>YPWPLYGNEGLGWAGWLL</td>
<td>Patient 1: 80, Patient 2: 51</td>
</tr>
<tr>
<td>82-100</td>
<td>PWPLYGNEGLGWAGWLLSP</td>
<td>Patient 1: 78, Patient 2: 50</td>
</tr>
<tr>
<td>83-97</td>
<td>WPPLYGNEGLGWAGWL</td>
<td>Patient 1: 21, Patient 2: 17</td>
</tr>
<tr>
<td>83-95</td>
<td>WPPLYGNEGLGWAG</td>
<td>Patient 1: 9, Patient 2: 0</td>
</tr>
<tr>
<td>83-93</td>
<td>WPPLYGNEGL</td>
<td>Patient 1: 6, Patient 2: 1</td>
</tr>
<tr>
<td>83-91</td>
<td>WPLYGNEGL</td>
<td>Patient 1: 2, Patient 2: 0</td>
</tr>
<tr>
<td>85-97</td>
<td>LYGNEGLGWAGWL</td>
<td>Patient 1: 18, Patient 2: 9</td>
</tr>
<tr>
<td>87-97</td>
<td>GNEGLGWAGWL</td>
<td>Patient 1: 18, Patient 2: 12</td>
</tr>
<tr>
<td>85-93</td>
<td>LYGNEGLGW</td>
<td>Patient 1: ND, Patient 2: 3</td>
</tr>
<tr>
<td>86-94</td>
<td>YGNEGLGWA</td>
<td>Patient 1: 8, Patient 2: 2</td>
</tr>
<tr>
<td>87-95</td>
<td>GNEGLGWAG</td>
<td>Patient 1: 4, Patient 2: 2</td>
</tr>
<tr>
<td>88-96</td>
<td>NEGLGWAG</td>
<td>Patient 1: 77, Patient 2: 60</td>
</tr>
<tr>
<td>89-97</td>
<td>EGLGWAGWL</td>
<td>Patient 1: ND, Patient 2: 1</td>
</tr>
<tr>
<td>88-95</td>
<td>NEGLGWAG</td>
<td>Patient 1: 2, Patient 2: ND</td>
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<tr>
<td>89-96</td>
<td>EGLGWAG</td>
<td>Patient 1: 8, Patient 2: ND</td>
</tr>
<tr>
<td>87-96</td>
<td>GNEGLGWAGW</td>
<td>Patient 1: 77, Patient 2: ND</td>
</tr>
<tr>
<td>88-97</td>
<td>NEGLGWAGWL</td>
<td>Patient 1: 19, Patient 2: ND</td>
</tr>
</tbody>
</table>

* Cytotoxicity assay was performed at an effector to target cell ratio of 20 (patient 1) or 40 (patient 2). Autologous (patient 1) and allogeneic HLA B44-positive target cells (patient 2) were sensitized with 10 μg of each peptide per ml. The percentage of specific cytotoxicity was calculated by subtracting the percentage of cytotoxicity of effector cells to non-peptide-pulsed BCLs from that of peptide-pulsed BCLs. ND, Not determined.

from post-transfusion acute HCV infection. The patient’s HLA class I haplotype was A11, A33, B44, B55 and Cw1. Both patients had normal liver functions and had no detectable HCV when studied in 1994–1995. Before interferon therapy patient 1 had been infected with genotype 2b/IV HCV (Chan et al., 1992; Okamoto et al., 1992) and the amino acid sequence of residues 81–100 was that of wild-type 2b/IV HCV, with a cysteine at position 91. Patient 2 had been infected with wild-type 2a/III HCV with a leucine at position 91 of the core protein. The HCV genotype and the sequence of a portion of the nucleoprotein gene of HCV were determined as reported previously (Okamoto et al., 1992).

The HLA B44-restricted CTLs were generated from PBLs by repeated stimulation with the 20-mer synthetic peptide of residues 81–100 of genotypes 1b/II and 2a/III HCV (NP9 peptide; YPWPLYGNEGLGWAGWLLSP), as reported previously (Kita et al., 1993). Briefly, PBLs were suspended at a cell density of 1 × 10⁶ cells/ml in RPMI-1640 medium supplemented with 10% human AB serum and 10 μg of the NP9 peptide per ml. Cells were incubated in a 24-well plate at 37 °C in a humidified 5% CO₂ atmosphere. On day 2, Lymphocult T (Biotest Diagnostics) was added as a source of interleukin-2 to a final concentration of 10% (10 U/ml). On day 7, the culture was re-stimulated with the NP9 peptide and irradiated autologous PBLs (30 Gy). On days 14–16, the cytotoxic activity of peptide-induced effector cells was assessed against autologous or allogeneic HLA B44-positive Epstein-Barr virus-transformed B cell lines (BCLs) that had been sensitized with peptides or infected with recombinant vaccinia virus expressing HCV nucleoprotein (VAC-HCVc) as shown with a 5 h europium (Eu)-release assay (Kita et al., 1993). The truncated and overlapping peptides that spanned the HCV nucleoprotein sequence between residues 81 and 100 were synthesized with an ABI 430A peptide synthesizer (Applied Biosystems) and purified by high-performance liquid chromatography (HPLC). Alternatively, the sequences were synthesized and purified in Neosystem Laboratoire (Strasbourg, France) or Chiron Mimotope (Clayton, Australia). All peptides were more than 90% pure on HPLC.

The HLA B44-restricted CTLs that recognized the nucleoprotein residues 81–100 of genotypes 1b/II and 2a/III HCV could be generated in both patients, although patient 1 had only been infected with genotype 2b/IV HCV. Since patient 1 had received multiple blood transfusions and as CTLs stimulated with the 20-mer peptide of residues 81–100 of genotype 2b/IV HCV do not recognize the 20-mer peptide of residues 81–100 of genotype 2b/IV HCV (Kita et al., 1995 b), patient 1 might also have been infected with genotype 1b/II or 2a/III HCV. Alternatively, since HLA B44-
restricted CTLs specific for genotypes 1b/II and 2a/III HCV can recognize the 9-mer peptide of residues 88–96 of genotype 2b/IV HCV, though less efficiently than the peptides of genotypes 1b/II and 2a/III HCV (K. Hiroishi, K. Ando, K. Kita, H. Okamoto, T. Kaneko, T. Moriyama & M. Imawari, unpublished results), genotype 2b/IV HCV might have primed CTLs specific for genotypes 1b/II and 2a/III HCV in vivo in patient 1.

The fine specificity of the HLA B44-restricted CTLs was assessed with panels of truncated and overlapping peptides. Cytotoxic activity was observed exclusively against peptides containing a 9-mer peptide of HCV nucleoprotein residues 88–96, NEGLGWAGW, but the addition of leucine to the carboxyl terminus of the peptide decreased recognition by CTLs (Table 1). No cytotoxic activity was observed against the other 9-mer and 8-mer peptides. The 9-mer peptide 88–96 was recognized by CTLs 5- to 10-fold better than the 20-mer peptide 81–100 and the 18-mer peptide 81–98. The 11-mer peptide 87–97 was recognized by CTLs with approximately 1% of the efficiency for peptide 88–96 and with less than 10% of the efficiency for peptides 81–98 and 81–100 (Fig. 1). These data suggest that residues 88–96 represent the minimal and also the optimal epitope within HCV nucleoprotein residues 81–100.

Recently it has been reported that natural peptide ligands for HLA B44 are 9 or 10 amino acids in length, with glutamic acid at position 2 (residue 89) and hydrophobic tryptophan at position 9 (residue 96). In addition, the amino acid at residue 97 of the HCV nucleoprotein is leucine, which is also the amino acid reported at the carboxyl terminus of the HLA B44 binding motif. Thus, peptide 87–97 may also bind HLA B44 through glutamic acid at position 2 (residue 89) and leucine at position 10 (residue 97), and the binding may affect binding of peptide 87–97 to HLA B44 through amino acids at positions 2 and 9. This might lead to decreased recognition of peptide 87–97 by CTLs.

It has been reported that stimulation with peptides may generate CTLs that recognize the peptide used for stimulation but not the native antigen (Schild et al., 1991). We tested the efficiency of the 9-mer synthetic peptide 88–96 in generating CTLs in patient 1 and tested whether it could stimulate CTLs that recognized endogenously synthesized HCV nucleoprotein. The 9-mer peptide 88–96 was able to stimulate CTLs more effectively than the 20-mer peptide 81–100 (Fig. 2a), and the CTLs recognized endogenously synthesized HCV antigen efficiently (Fig. 2b). The 9-mer peptide was also able to stimulate CTLs in patient 2, not only when serum HCV was not detectable but also when HCV later reappeared in the serum. In addition, the 9-mer peptide was able to stimulate CTLs in two other HLA B44-positive patients with persistent HCV infection (data not shown).

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**Fig. 1.** Recognition of HCV nucleoprotein peptides 88–96 (●), 87–97 (▲), 81–98 (■) and 81–100 (▲) by CTLs generated by stimulation with NP9 (peptide 81–100). The PBLs of patient 1 stimulated with NP9 were tested against autologous BCLs sensitized with the indicated concentrations of peptides at an effector to target cell ratio of 20. Specific cytotoxicity was calculated as described in the footnote to Table 1.

**Fig. 2.** Relative efficiencies of HCV nucleoprotein peptides 88–96 (●) and 81–100 (■) in CTL generation (a) and recognition of peptide 88–96 and endogenously synthesized HCV nucleoprotein by PBLs stimulated with peptide 88–96 (b). The PBLs of patient 1 were stimulated with the indicated concentrations of peptides 88–96 and 81–100 and assayed for cytotoxicity against autologous BCLs sensitized with 10 μM of peptide 88–96 at an effector to target cell ratio of 20. Specific cytotoxicity was calculated as described in the footnote to Table 1. The PBLs of patient 1 stimulated with 10 μM of peptide 88–96 were also tested against targets sensitized with medium alone or 10 μM of peptide 88–96 or targets infected with wild-type vaccinia (VAC-W) or VAC-HCVc at an effector to target cell ratio of 20.
Since CTLs restricted by distinct HLAs recognize different epitopes, a universally immunogenic HCV CTL vaccine would require multiple epitopes, preferably from the conserved regions of HCV. The HCV infects and replicates in hepatocytes (Fong et al., 1991) and PBLs (Müller et al., 1993). Therefore, CTLs primed by such a vaccine must also have access to HCV-infected hepatocytes. It has been reported that intravenously injected CTLs specific for hepatitis B surface antigen (HBsAg) gain access to hepatocytes that express HBsAg through the fenestrae of the hepatic sinusoid endothelium but are not exposed to other HBsAg-positive tissues in which the vasculature has a continuous endothelium and basement membrane (Ando et al., 1994). This finding suggests that CTLs primed by peptide 88-96 would also be exposed to HCV-infected hepatocytes, although the peptide is an epitope for HLA B44-restricted CTLs generated from PBLs. The HLA B44 antigen is shared by the vasculature has a continuous endothelium and basement membrane (Ando et al., 1992). Evaluation of the reported amino acid sequence of HCV clones shows that, with the exception of residue 91, the amino acid sequence of residues 88-96 is conserved. The amino acid at position 91 is cysteine in genotype 1a/1 HCV, leucine or methionine in 1b/II, leucine in 2a/III, and cysteine in 2b/IV. Peptide 88-96 with either leucine or methionine at position 91 can stimulate cross-reactive CTLs, and peptide 88-96 with cysteine at position 91 can stimulate CTLs specific for itself (K. Hiroishi, K. Ando, K. Kita, H. Okamoto, T. Kaneko, T. Moriyama & M. Imawari, unpublished results). Thus, peptide 88-96 might be used as one of the multiple epitopes for a HCV CTL vaccine.

We recently reported that a helper T cell antigen enhances the generation of HCV-specific CTLs in vitro (Kita et al., 1995a). Such a helper T cell antigen may help to mount a sufficient CTL response to an HCV CTL vaccine.

In conclusion, the results demonstrate that the 9-mer HCV nucleoprotein peptide 88-96 is the minimal and optimal epitope to be recognized by CTLs and to stimulate HLA B44-restricted CTLs that recognize not only the peptide used for stimulation but also endogenously synthesized and processed related HCV nucleoproteins. Peptide 88-96 may be the basis for a vaccine to induce a sufficient anti-HCV CTL response in vivo to prevent HCV persistence in individuals with HLA B44 and to eliminate HCV from infected patients with HLA B44.

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References


virus or synthetic lipopeptide vaccine or primed in vitro with peptide. 
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