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Immunodominant CD4+ T-Cell Epitope within Nonstructural Protein 3 in Acute Hepatitis C Virus Infection

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In acute hepatitis C virus infection, 50 to 70% of patients develop chronic disease. Considering the low rate of spontaneous viral clearance during chronic hepatitis C infection, the first few months of interaction between the patient’s immune system and the viral population seem to be crucial in determining the outcome of infection. We previously reported the association between a strong and sustained CD4+ T-cell response to nonstructural protein 3 (NS3) of the hepatitis C virus and a self-limited course of acute hepatitis C infection. In this study, we identify an immunodominant CD4+ T-cell epitope (amino acids 1248 to 1261) that was recognized by the majority (14 of 23) of NS3-specific CD4+ T-cell clones from five patients with acute hepatitis C infection. This epitope can be presented to CD4+ T cells by HLA-DR4, -DR11, -DR12, -DR13, and -DR16. HLA-binding studies revealed a high binding affinity for 10 of 13 common HLA-DR alleles. Two additional CD4+ T-cell epitopes, amino acids 1388 to 1407 and amino acids 1450 to 1469, showed a very narrow pattern of binding to individual HLA-DR alleles. Our data suggest that the NS3-specific CD4+ T-cell response in acute hepatitis C infection is dominated by a single, promiscuous peptide epitope which could become a promising candidate for the development of a CD4+ T-cell vaccine.

Materials and Methods

Patients. NS3-specific CD4+ T-cell clones were isolated from five patients with acute hepatitis C infection. The diagnosis was based on the following criteria:

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incorporated into DNA was estimated with a beta counter (LKB/Pharmacia, Uppsala, Sweden). Triplicate cultures were assayed routinely, and the results are expressed as mean counts per minute. The stimulation index was calculated as the ratio of counts per minute obtained in the presence of antigen to that obtained without antigen. A stimulation index of $>3$ was considered significant.

Controls. To ensure that proliferation of PBMCs in response to HCV antigens is specific and confined to patients with HCV infection, PBMCs from 13 healthy volunteers and from patients with the following liver diseases unrelated to HCV were tested with HCV antigens: acute hepatitis B infection (two patients), autoimmune hepatitis (two patients), and cryptogenic liver disease (two patients). Proliferation assays were performed with protein concentrations from 0.1 to 10 g/ml. Stimulation indices in all control experiments were $<3$. In addition, for all HCV patients, PBMCs were routinely tested with buffers that were processed in parallel to the recombinant proteins. Significant proliferation was accepted only if no proliferation in response to control buffers was observed.

Generation of T-cell clones and specificity testing. Two million PBMCs were stimulated with $1 \mu$g of HCV protein per ml in 96-well U-bottom plates as described above. On day 6, recombinant interleukin 2 (IL-2) was added to a final concentration of 15 U/ml (kindly provided by Boehringer, Mannheim, Germany) in 10% fetal bovine serum. Generation of T-cell clones was accepted only if no proliferation in response to control buffers was observed. On day 10, cells were cloned at 0.5 cell/well in the presence of 15 U/ml (kindly provided by Boehringer, Mannheim, Germany) recombinant IL-2 to a final concentration of $10^4$ lymphoblastoid cells in 96-well V-bottom plates, washed, incubated for 1 h in medium supplemented with 2 mM L-glutamine (GIBCO), 50 IU/100 ml of penicillin per ml (Irvine Scientific). Large quantities of cells were grown in medium supplemented with 2 mM L-glutamine (GIBCO), 50 IU/100 ml of penicillin per ml (Irvine Scientific), 104 g of phytohemagglutinin per ml (HA16; Murex Diagnostics, Dartford, United Kingdom) per ml. After 3 to 5 weeks, growing clones were tested for specificity to HCV antigens. For this, $1 \times 10^5$ to $5 \times 10^5$ cells were added to $3 \times 10^5$ autologous, irradiated PBMCs with and without $1 \mu$g of HCV protein per ml and cultured for 5 days. The proliferation assay was performed as described for PBMCs.

For expansion, T-cell clones were stimulated every 3 to 5 weeks with irradiated autologous or allogeneic PBMCs. 15 U of IL-2 per ml, and 2 g of phytohemagglutinin/ml. Earlier restimulation usually led to an unacceptable rate of cell death. Therefore, care was taken to restimulate T-cell clones only after the activation marker CD25 to 90% of baseline. TACON immunofluorescence staining was performed on T-cell clones with the following combinations of conjugated antibodies: CD3 (MT301-FITC, kindly provided by E. P. Rieber, Institute for Immunology, Munich, Germany), CD4 (Leu-3a-PE; Becton Dickinson, Hamburg, Germany), CD8 (3B5-TRI-Color; Medac, Hamburg, Germany), CD25 (IL-2RI-FITC; Coulter, Hialeah, Fla.), LLA-DR (L243-PE; Becton Dickinson), and CD4 (S3.3-TRI-Color; Medac). Fluorescence-activated cell sorter (FACS) analysis was performed with a FACSscan (Becton Dickinson) as described previously (9).

Lymphokine assays. NS3-specific CD4+ T-cell clones were stimulated ($10^5$ cells/100 ml) with a combination of anti-CD2 (hybridomas 6G4 and 4B2) and anti-CD28 (hybridoma 15E8) monoclonal antibodies (1:4,000) and 1 ng of phorbolester acetate (Sigma, St. Louis, Mo.) per ml. Supernatants were collected after 24 h and stored at $-80^\circ$C. Supernatants were tested in a 96-well U-bottom plate with a beta counter (LKB/Pharmacia, Uppsala, Sweden). Triplicate cultures were assayed routinely, and the results are expressed as mean counts per minute. The stimulation index was calculated as the ratio of counts per minute obtained in the presence of antigen to that obtained without antigen. A stimulation index of $>3$ was considered significant.

Determination of HLA restriction. For determination of HLA restriction, proliferation assays were performed in the presence or absence of anti-HLA class II antibodies anti-DR (catalog no. 7730), anti-DP (catalog no. 7450), and anti-DQ (catalog no. 7540) (Becton Dickinson). Addition of 10 l of antibody per well led to optimal inhibition of T-cell stimulation. After identification of the presenting class II molecule, fine analysis was performed using the following partially matched, homozygous, lymphoblastoid cell lines as antigen-presenting cells (APC) (12): Schu (DRA1*0102, DRB1*1501, DRB5*0101, DQA1*0102, DQB1*0602, DPA1*0100, DPB1*0101, DPB1*0102, DQA1*0101, DQB1*0602, DPA1*0100, DPB1*0102, DQA1*0101, DQB1*0602, DPA1*0100, DPB1*0102). Significant proliferation was measured by using strongly validated in-house sandwich enzyme-linked immunosorbent assay techniques that have been described in detail elsewhere (22, 23).

### Table 1. Clinical data for patients with acute hepatitis C infection

<table>
<thead>
<tr>
<th>Infection type and patient no.</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Genotype</th>
<th>Mode of transmission</th>
<th>HLA pattern</th>
<th>Follow-up (mo)</th>
<th>Peak ALT (U/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute self-limited</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>F</td>
<td>36</td>
<td>1a</td>
<td>Sporadic</td>
<td>A2,10B27,51 DR12,2</td>
<td>41</td>
<td>973</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>64</td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Sporadic</td>
<td>A2 B51,w60 Cw3 DR6,11</td>
<td>16</td>
<td>1,248</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>38</td>
<td>NA</td>
<td>Intravenous drug abuse</td>
<td>A1,2 B8,51 Cw7 DR2,11,22</td>
<td>22</td>
<td>1,466</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>18</td>
<td>1b</td>
<td>Transfusion</td>
<td>A11 B7,51 Cw7 DR15</td>
<td>12</td>
<td>876</td>
</tr>
<tr>
<td><strong>Evolving chronic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>23</td>
<td>1b</td>
<td>Sexual</td>
<td>A2,28B27,51 Cw2 DR2,4</td>
<td>16</td>
<td>879</td>
</tr>
</tbody>
</table>

<sup>a</sup> F, female; M, male.

<sup>b</sup> ALT, alanine aminotransferase. Normal values are <24 U/liter for males and <19 U/liter for females.

<sup>c</sup> NA, not available.
spinner cultures. Cells were lysed at a concentration of 10^8/ml in PBS containing 1% Nonidet P-40 (NP-40) (Fluka Biochemika, Buchs, Switzerland), 1 mM phenylmethylsulfonyl fluoride (CalBioChem, La Jolla, Calif.), 5 mM sodium orthovanadate, and 25 mM iodoacetamide (Sigma Chemical). The lysates were cleared of debris and nuclei by centrifugation at 10,000 \( g \) for 20 min.

(ii) Affinity purification of HLA-DR molecules.

HLA class II molecules were purified by affinity chromatography as previously described (8, 20) using monoclonal antibody LB3.1 coupled to Sepharose 4B beads. Lysates were filtered through 0.8- and 0.4-µm-pore-size filters and then passed over the anti-DR column, which was then washed with 15 column volumes of 10 mM Tris in 1.0% NP-40–PBS and with 2 column volumes of PBS containing 0.4% n-octylglucoside. Finally, the DR was eluted with 50 mM diethylamine in 0.15 M NaCl containing 0.4% n-octylglucoside, pH 11.5. A 1/25 volume of 2.0 M Tris, pH 6.8, was added to the eluate to reduce the pH to 8.0, and the eluate was then concentrated by centrifugation in Centriprep 30 concentrators at 2,000 rpm (Amicon, Beverly, Mass.).

(iii) HLA class II peptide-binding assays.

Purified human class II molecules (5 to 500 nM) were incubated with various unlabeled peptide inhibitors and 1 to 10 nM125I-radiolabeled probe peptides for 48 h in PBS containing 5% dimethyl sulfoxide in the presence of a protease inhibitor cocktail. Radiolabeled probes used were HA Y307-319 (DR1), tetanus toxoid TT 830-843 (DR2w2a, DR5w11, DR7), MBP85-100Y (DR2w2b), a nonnatural peptide with the sequence YARFQSQTTLKQKT (DR4w4, DR4w14) (21), and for DR5w12, a peptide eluted from cell line C1R, EALIHQLKINPYVLS (6); there is no gene bank match. Also used as radiolabeled probes were the aforementioned nonnatural peptide for DR4 splits (DR4w15), TT 830-843 (DR8w2, DR8w3, DR9), and TT 830-843 with S836 substituted with A for DR6w19 (unpublished data). Radiolabeled peptides were iodinated by the chloramine-T method (4). Peptide inhibitors were typically tested at concentrations ranging from 120 µg/ml to 1.2 ng/ml.

### TABLE 2. Lymphokine profile of NS3-specific CD4+ T-cell clones

<table>
<thead>
<tr>
<th>Patient no. and clone</th>
<th>Cytokine level (ng/ml)</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.12</td>
<td>&lt;0.05</td>
<td>4.37</td>
<td>1.02</td>
</tr>
<tr>
<td>1.12a</td>
<td>1.12</td>
<td>&lt;0.05</td>
<td>&lt;3</td>
<td>0.89</td>
</tr>
<tr>
<td>2</td>
<td>2.9</td>
<td>1.13</td>
<td>11.1</td>
<td>7.09</td>
</tr>
<tr>
<td>2.11</td>
<td>2.18</td>
<td>10.2</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>2.12</td>
<td>&lt;0.2</td>
<td>&lt;3</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>2.18</td>
<td>&lt;0.1</td>
<td>17.7</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>2.20</td>
<td>&lt;0.2</td>
<td>&lt;3</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>2.30</td>
<td>5.0</td>
<td>12.9</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>2.78</td>
<td>&lt;0.1</td>
<td>&lt;0.2</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>2.79</td>
<td>&lt;0.1</td>
<td>&lt;0.2</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.11</td>
<td>0.4</td>
<td>6.4</td>
<td>0.56</td>
</tr>
<tr>
<td>3.14</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&gt;9.0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.29</td>
<td>&lt;0.1</td>
<td>0.75</td>
<td>0.24</td>
</tr>
<tr>
<td>5.34</td>
<td>&lt;0.1</td>
<td>0.21</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>

* For clones 2.18 and 2.30, fine-specificity was not determined, and these clones do not appear in Table 3.

### TABLE 3. Summary of NS3-specific CD4+ T-cell clones and HLA restriction

<table>
<thead>
<tr>
<th>Patient no. and time since onset</th>
<th>aa 1248–1261</th>
<th>HLA restriction (% anti-DR inhibition)</th>
<th>aa 1388–1407</th>
<th>HLA restriction (% anti-DR inhibition)</th>
<th>aa 1450–1469</th>
<th>HLA restriction (% anti-DR inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 5 mo</td>
<td>1.10</td>
<td>ND*</td>
<td>1.12</td>
<td>DRB1*1201 (69)</td>
<td>1.12a</td>
<td>DRB1*1201 (74)</td>
</tr>
<tr>
<td>2</td>
<td>2.9</td>
<td>DRB1*1101 (56)</td>
<td>2.11</td>
<td>DRB1*1101 (90)</td>
<td>2.20</td>
<td>DRB1<em>1101 (37) DRB1</em>1302 (71)</td>
</tr>
<tr>
<td>1 mo</td>
<td>2.78</td>
<td>DRB1*1101 (70)</td>
<td>2.79</td>
<td>DRB1<em>1101 (50) DRB1</em>1302 (100)</td>
<td>2.68</td>
<td>DRB1*1101 (78)</td>
</tr>
<tr>
<td>16 mo</td>
<td>3.11</td>
<td>DRB1<em>1101 (68) DRB1</em>1302 (100)</td>
<td>3.14</td>
<td>DRB1*1501 (77)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.110</td>
<td>ND*</td>
<td>3.110</td>
<td>ND*</td>
<td>3.118</td>
<td>ND*</td>
</tr>
<tr>
<td>6 mo</td>
<td>4.11</td>
<td>DRB1<em>1501 (98) DRB1</em>1501 (100)</td>
<td>4.31</td>
<td>DRB1*1501 (100)</td>
<td>4.39</td>
<td>DRB1*1501 (100)</td>
</tr>
<tr>
<td>5, 1 mo</td>
<td>5.29</td>
<td>DRB1<em>0401 (54) DRB1</em>1601 (86)</td>
<td>5.34</td>
<td>DRB1<em>0401 (65) DRB1</em>1601 (100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* ND, not done.
The data were then plotted, and the dose yielding 50% inhibition was measured. Peptides were tested in two to four completely independent experiments. The final concentrations of protease inhibitors were as follows: 1 mM phenylmethylsulfonyl fluoride, 1.3 nM 1.10-phenanthroline, 73 μM pepstatin A, 8 mM EDTA, and 200 μM Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) (all protease inhibitors from CalBioChem). The final detergent concentration in the incubation mixture was 0.05% NP-40. All assays were performed at pH 7.0. Class II peptide complexes were separated from free peptide by gel filtration on TSK2000 columns, and the fraction of bound peptide was calculated as previously described (20). In preliminary experiments, the titer of the DR preparation was determined in the presence of fixed amounts of radiolabeled peptides to ascertain the concentration of class II molecules necessary to bind 10 to 20% of the total radioactivity. All subsequent inhibition and direct binding assays were then performed with these class II molecule concentrations.

RESULTS

Isolation and characterization of NS3-specific clones. NS3-specific CD4⁺ T-cell clones were isolated from five patients with acute hepatitis C infection. The clinical data are summarized in Table 1. Four patients (no. 1 to 4) achieved spontaneous virus clearance and were HCV RNA negative as determined by PCR, with normal aminotransferase levels throughout the follow-up of 12 to 41 months. One patient (no. 5) developed chronic hepatitis C infection and remained HCV RNA positive with abnormal liver biochemistry until 5 months after disease onset, when a 6-month course of recombinant IFN-α2b was begun. This patient showed a sustained virological and biochemical response beyond 6 months after the end of treatment.

A strong NS3-specific CD4⁺ T-cell response in the peripheral blood (mean stimulation index, 22.9; range, 5.5 to 64) was present during the first 4 weeks of acute hepatitis infection in all five patients and was maintained throughout the follow-up in the four patients with self-limited disease (mean stimulation index, 34.6; range, 14.2 to 70.3). In contrast, in the patient who did not achieve viral clearance, the NS3 response disappeared 4 weeks after disease onset and remained undetectable thereafter (mean stimulation index, 1.7; range, 1.2 to 2.8). Seven peripheral T-cell cloning experiments were performed, yielding 45 NS3-specific CD4⁺ T-cell clones (median, six T-cell clones per cloning procedure; range, 2 to 9). For two patients, NS3-specific CD4⁺ T-cell clones were obtained at different times, during the acute phase of disease and during follow-up (patients 2 and 3). In all cases, the cloning was performed starting with NS3-specific T-cell lines stimulated in vitro with

![FIG. 2.](image)

![FIG. 3.](image)
our longest NS3 protein, spanning aa 1007 to 1534. Determination of the lymphokine profile revealed significant IFN-γ production by all T-cell clones; some T-cell clones also produced variable amounts of IL-4 and/or IL-5 (Table 2). Twenty-three clones responding specifically to the aa 1007 to 1534 NS3 protein could be expanded sufficiently for further testing with shorter protein fragments and synthetic 20-mer peptides to identify their fine-specificity. The characteristics of the T-cell clones are summarized in Table 3. All six cloning procedures for patients 1, 2, 3, and 5 yielded at least one clone specific for the peptides from aa 1242 to 1261 and aa 1248 to 1267, localizing the relevant epitope to aa 1248 to 1261 (median, three T-cell clones per patient; range, two to six; Fig. 2). Fourteen of the 23 CD4⁺ T-cell clones, for which the fine-specificity could be determined, were specific for aa 1248 to 1261, and for two patients (1 and 5), all NS3-specific CD4⁺ T-cell clones responded to that epitope (Table 3). A new set of amino- and carboxy-terminally truncated peptides was synthesized, and for three clones from different patients (T-cell clones 1.12, 2.11, and 3.11), the minimal epitope was defined as aa 1251 to 1259 (Fig. 3). Whereas T-cell clones 1.12 and 3.11 were virtually identical with regard to the response to the truncated peptides, T-cell clone 2.11 seemed to depend less on aa 1251 for stimulation. All NS3-specific CD4⁺ T-cell clones from patient 4, who is homozygous at the HLA-DR locus (DR15), responded to peptide 1388-1407 (Fig. 4); in addition, both cloning procedures for patient 3 yielded one CD4⁺ T-cell clone specific for that epitope that was also restricted by HLA-DR15. For patient 2, three NS3-specific CD4⁺ T-cell clones responded to a third peptide, aa 1450 to 1469 (Fig. 5). By the use of additional recombinant protein fragments from a different source (Chiron), the epitope mapping could be confirmed: T-cell clones specific for aa 1248 to 1261 and aa 1388 to 1407 could be stimulated by proteins aa 1192 to 1457 and aa 1192 to 1931, whereas T-cell clones specific for aa 1450 to 1469 could be stimulated only by protein aa 1192 to 1931 but not protein aa 1192 to 1457, which does not contain the complete sequence (data not shown). The relevant epitopes can therefore be generated by intracellular processing of proteins of different lengths, with different fusion proteins (GST or SOD) or unfused proteins and independently of whether the proteins have been expressed in E. coli or yeast.

**Determination of HLA restriction.** In inhibition experiments using anti-HLA class II antibodies, all clones were susceptible to inhibition by anti-HLA-DR antibodies (Fig. 6A, D, G, and I; Table 3). Subsequently, the exact restriction of our HCV-specific T-cell clones was mapped by using homozygous, lymphoblastoid cell lines as APC. For clones specific for aa 1248 to 1261, the HLA-DR alleles DRB1*1101, DRB1*1201, and DRB1*0401 were identified as restriction elements (Fig. 6A to I). Presentation by DR52 and DR53 also expressed by homozygous EBV lines could be excluded on the basis of lack of presentation by EBV lines expressing similar DR52 and/or DR53 alleles but different DRB1 allelic products. When a wider panel of lymphoblastoid cell lines was used, some clones recognized the peptide also when presented by other HLA-DR molecules, irrespective of DR alleles expressed by the patient from whom the T-cell clone was isolated: a fraction of
DRB1*1101-restricted clones was also stimulated by the peptide presented by the DRB1*1302 allele (Fig. 6B and C), and one clone restricted by DRB1*0401 was also stimulated by DRB1*1601 (Fig. 6I). This promiscuous recognition could be inhibited by anti-HLA-DR antibodies (Table 3) and was of similar avidity, as judged by the antigen sensitivity (Fig. 7).

Clones specific for aa 1450 to 1469 were restricted by the allele DRB1*1302 (without cross-reactivity to DRB1*1101; data not shown); all T-cell clones specific for aa 1388 to 1407 (from both patients 3 and 5) were restricted by DRB1*1501/DRB5*0101 (data not shown). In this case, because of the tight linkage disequilibrium between DRB1*1501 and DRB5*0101, which are coexpressed in all EBV lines available to us, it is
possible that either DRB1*1501 or DRB5*0101 could act as a restriction element, presenting the aa 1388 to 1407 peptide.

**HLA class II affinity determination.** Next, the capacities of the three epitopes described above to bind purified HLA-DR molecules in vitro were analyzed. Thirteen of the most common DR molecules, representative of more than 90% of DR types from the most common ethnic groups, were selected for this analysis. The results shown in Table 4. It was found that the degenerate and promiscuous NS3 aa 1248 to 1261 epitope bound with high affinity (50% inhibitory concentration [IC50], ≤500 nM) to 10 of the 13 molecules tested and appreciably (albeit weakly: IC50, 500 to 5,000 nM) to the remaining three molecules. In particular, all DR molecules shown above to be able to present this epitope to CD4+ T cells bound the NS3 aa 1248 to 1261 epitope, three of them (DRB1*1101, DRB1*1302, and DRB1*0401) with high affinity and one (DRB1*1201) with relatively weak but still significant affinity.

Synthetic peptides corresponding to the other two NS3 epitopes (aa 1388 to 1407 and 1450 to 1469) bound very selectively and with poor affinity. NS3 aa 1388 to 1407 bound its potential restricting element DRB5*0101 weakly (IC50, 1,887 nM), cross-reacted marginally (IC50, 17,391 nM) on DRB1*1101, and bound none of the remaining DR types tested. Similarly, NS3 aa 1450 to 1469 bound its likely restricting element DRB1*1302 only marginally (IC50, 35,000 nM), cross-reacted weakly on DRB1*0701, and bound no other DR type tested.

**DISCUSSION**

The acute phase of hepatitis C infection, in which clearance of the virus and resolution of the disease or virus persistence and chronic disease are determined, represents the perfect situation to identify mechanisms which are considered to play a pivotal role in the interaction between virus and host. Previously, it was demonstrated that a strong and persistent HCV-specific CD4+ T-cell response is associated with a self-limited course of acute hepatitis C infection (5). These data have most recently been confirmed by another group, who also demonstrated a significantly stronger HCV-specific CD4+ T-cell response in patients with acute self-limited hepatitis C infection than in patients with evolving chronic hepatitis C infection (16). In the first study, NS3 seemed to be the immunodominant viral antigen for CD4+ T lymphocytes, whereas the study of Missale et al. (16) found a strong CD4+ T-cell response to most viral antigens, including NS3, to be associated with viral clearance. A weaker association between an HCV-specific CD4+ T-cell response and viral clearance has also been described for patients with chronic hepatitis C infection who achieve a sustained response to IFN-α therapy (3, 7, 11). In those patients, however, the strongest CD4+ T-cell response detected was usually to core antigen and NS4. Although CD8+ cytotoxic T lymphocytes are generally thought to be the most important effector cells for the elimination of virally infected cells, in HCV infection, CD4+ T cells seem to play a central role in the antiviral immune response, possibly by inducing or maintaining cytotoxic activity or by directly secreting antiviral cytokines.

CD4+ T-cell responses to peptide epitopes within HCV NS4 and core antigen have previously been determined in proliferation assays using freshly isolated PBMCs (11, 16). This technique, however, may overestimate the number of CD4+ T-cell epitopes; in particular, weakly positive responses are difficult to interpret (4a). Moreover, no detailed analysis of HLA restriction is possible. To avoid these problems, we used NS3-specific CD4+ T-cell clones which had been isolated from polyclonal T-cell lines after stimulation with recombinant antigen to ensure that T cells are stimulated only by intracellularly processed peptides. Using that approach, we identified one immunodominant 14-aa epitope (aa 1248 to 1261) that was recognized by the majority of T-cell clones from four of five patients. It could be presented to T cells by at least five different HLA-DR alleles, and binding studies showed that 10 of 13 common HLA-DR alleles are able to bind the epitope with high affinity. Furthermore, fine-mapping with three different T-cell clones defined aa 1251 to 1259 as the putative minimal epitope. Another epitope (aa 1388 to 1407) was recognized by T-cell clones from two patients, and all these clones were HLA-DRB1*1501/DRB5*0101 restricted, suggesting that aa 1388 to 1407 may be an important CD4+ T-cell epitope for patients carrying HLA-DRB1*1501. In contrast to the immunodominant epitope aa 1248 to 1261, epitope aa 1388 to 1407 and the HLA-DRB1*1302-restricted epitope aa 1450 to 1469 bound only weakly to their likely restriction elements and did not exhibit broadly cross-reactive degenerate binding capacity for other DR alleles. These observations confirm earlier studies which had suggested that degenerate binding and promiscuous recognition are associated with high-affinity binding, while selective binding is associated with weak interactions (19). They also underline the influence of HLA-DR binding affinity in determining immunodominance.

It is not known which APC present NS3 epitopes to CD4+ T cells in vivo, or in what form NS3 sequences are taken up by APC. Since NS3 may not be contained in the viral particle, it is conceivable that NS3 or larger fragments of the viral polyprotein are liberated from lysed infected cells and taken up by surrounding macrophages. We could demonstrate that the relevant epitope was presented to CD4+ T cells after intracellular processing of various NS3 protein fragments (including a large NS3-NS4 protein) that contained the relevant sequence, irrespective of whether the proteins were expressed in E. coli or yeast and whether or not they were fused to SOD or GST. It can thus be anticipated that the three epitopes can also be presented by APC in vivo even though the exact form of the source antigen is unknown.

We thus observed that a strong NS3-specific CD4+ T-cell response, which is associated with viral clearance in acute hepatitis C infection, is dominated by the response to a single 14-aa epitope, aa 1248 to 1261, and can be mounted by patients with a diverse HLA background. Since viral heterogeneity and the high mutational rate of HCV are generally thought to be important factors in establishing chronic infection, we searched databases for NS3 sequences. Unexpectedly, aa 1248 to 1261 were completely conserved in all 33 genotype 1a, 1b, 1c, 2a, and 2b sequences (Table 5). Only genotype 3a shows a
change at position aa 1250 from lysine to asparagine, which lies outside the putative minimal epitope aa 1251 to 1259. Two other sequences which were not genotyped displayed one amino acid exchange each, only one of which lies within the minimal epitope. This may imply that viral escape is unlikely to be an important factor in the regulation of the CD4 T-cell response to aa 1248 to 1261.

However, we cannot exclude that by using only genotype 1a proteins to determine T-cell specificity we might have missed some viral epitopes with high variability. While it is evident that the presence of a CD4 T-cell response, which in the early phase of the disease focuses on conserved epitopes, is associated with viral clearance, the absence of the described epitope-specific CD4 T-cell response in patients developing a chronic course of disease does not necessarily imply that these individuals cannot mount an immune response against HCV proteins at that early stage; instead, their T-cell response may focus on variable epitopes of the virus, thereby offering the virus a chance to evade the immune attack. The reason these patients can’t respond to the conserved epitopes despite the presence of the appropriate HLA-DR alleles is unknown at present.

Interestingly, in patient 5, an initial response to epitope aa 1248 to 1261 was lost during the first 4 weeks of acute hepatitis C infection, and the patient subsequently developed chronic hepatitis C infection. This observation suggests that during the course of acute hepatitis C infection, the virus-specific immune response can be downregulated to promote viral persistence. It is not known whether HCV infection leads to exhaustion of HCV-specific T cells, as suggested for certain animal models of lymphocytic choriomeningitis virus infection (17), or whether, e.g., inefficient antigen presentation in the liver induces anergy or apoptosis. Another attractive hypothesis would be that the presence of viral proteins in the bile could induce oral tolerance to HCV (10, 24), which is supported by the clinical observation that patients with severe cholestasis clear the infection more frequently. Those mechanisms may be amenable to therapeutic intervention by a peptide vaccine with or without the addition of certain cytokines. Along these lines, studies with animal models to clarify any causal relationship of the CD4 T-cell response to NS3 and other HCV antigens with viral clearance and identification of regulatory mechanisms may lead to the development of a new therapeutic strategy for both primary immunization against HCV and the treatment of chronic hepatitis C infection.

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