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A Human CTL Recognizes a Caspase-8-Derived Peptide on Autologous HLA-B*3503 Molecules and Two Unrelated Peptides on Allogeneic HLA-B*3501 Molecules

Susanna Mandruzzato, Vincent Stroobant, Nathalie Demotte, and Pierre van der Bruggen

A CTL clone that recognizes autologous tumor cells was previously isolated from the blood of a head-and-neck cancer patient. The Ag identified as peptide FPSDSWCYF presented by autologous HLA-B*3503 molecules. This peptide was encoded by a mutated CASP-8 gene, which is implicated in the triggering of apoptosis. Here, we show that this CTL clone, which expresses a single TCR, also recognizes two unrelated peptides on allogeneic HLA-B*3501 molecules. One peptide, HIPDVITY, is encoded by squalene synthase, and the other one, QFADVIVLF, is encoded by 2-hydroxyphytanoyl-CoA lyase. Both genes are expressed ubiquitously. These antigenic peptides are processed and presented by HLA-B*3501 cells. The two HLA-B35 alleles are closely related. Our results might reinforce the notion that the recognition of allogeneic HLA molecules depends on the presence in their groove of a limited number of peptides processed from ubiquitous proteins. The Journal of Immunology, 2000, 164: 4130–4134.
Production of progressive deletions in cDNA 668

To generate progressive deletions from the 3′ ends of cDNA 668, and thereby obtain a large number of truncated cDNA clones, we used the Erase-a-base System (Promega, Madison, WI) as described (15).

Antigenic peptides and CTL assay

Peptides were synthesized on solid phase using F-moc for transient NH₂-terminal protection as described (16) and were characterized using mass spectrometry. All peptides were >80% pure, as indicated by analytical HPLC. Lyophilized peptides were dissolved at 20 mg/ml in 10 mM acetic acid, and stored at −20°C. Peptides were tested in chromium release assays where 1,000 51 Cr-labeled target cells were incubated for 30 min at room temperature in 96-well microplates with various concentrations of peptide before adding an equal volume containing 10,000 CTL 121. The indicated concentrations of peptide represent the final concentrations during the incubation of the target cells with the CTL. The assay was terminated after 4 h of incubation at 37°C.

RNA extraction, cDNA synthesis, and PCR amplification of TCRVa and TCRβ

Total RNA was prepared from CTL clone 121 using TRIZOL (Life Technologies), and first strand cDNA was synthesized with oligo(dT) and reverse transcriptase (Life Technologies). PCR was conducted by amplification with primers complementary to TCR Va and C region sequences (17) in a 25-μl reaction mixture containing 2.5 μl of cDNA, all four dNTPs (each at 0.2 mM), 0.8 μM of each primer, and 0.5 U of Dynazyme (Finnzymes Oy, Finland) on a DNA thermal cycler (Biometra, Thermotrioblock, Westburg, The Netherlands). Amplification was performed for 25 cycles to amplify TCRα and 28 cycles to amplify TCRβ, each consisting of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The PCR product of TCRβ was cloned into the pTZ18R vector and sequenced by the dideoxy-chain termination method (Thermosequenase™ cycle sequencing kit; Amersham). Nucleotide sequences were compared with available published TCR sequences (18–21). The TCR nomenclature proposed by the International Union of Immunological Societies was adopted (22).

Peptide binding assay

We designed a peptide binding assay similar to the assay on intact human B cells described by van der Burg and colleagues (23). Peptides were stripped by mild acid treatment (pH 2.9), after which cells were incubated overnight at 4°C with a fluorescein (FL)1-labeled reference peptide at a fixed concentration (250 nM), together with decreasing concentrations (50 μM to 0.18 μM) of the three peptides of interest. After washing, the effectiveness by which these peptides competed for binding to HLA molecules was assayed by measuring the amount of HLA-bound FL-labeled peptide with FACScan analysis. The reference peptide was LPSC(FL) (24), ADVEF, a Cys-derivative of the tyrosinase antigenic peptide presented by allo-HLA-B*3501, and LSPFPFDL, a Cys-derivative of the tyrosinase antigenic peptide presented by allo-HLA-B*3501.

Results and Discussion

By stimulating blood cells of patient BB49 with irradiated autologous tumor cells, we isolated CD8⁺ CTL clone 121. To identify the Ag recognized by this CTL, a cDNA library was prepared with RNA from the autologous tumor cells. DNA was extracted from pools of 100 recombinant clones and transfected into COS-7 cells together with a cDNA coding for allogeneic HLA-B*3501 molecules. Patient BB49 was typed HLA-B*3503 and not B*3501, but for convenience we used an HLA-B*3501 cDNA, which was already cloned in an appropriate expression vector. The only difference between allo-B*3501 and self-B*3503 molecules is a S to F

Table I. Defined peptide-MHC ligands for three crossreactive CTL clones

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>MHC Class I Presenting Molecule</th>
<th>Protein Containing the Peptide</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine CTL clone 2C</td>
<td>S I Y R Y Y G L</td>
<td>Self-H-2 K₁</td>
<td>None (random peptide library)</td>
</tr>
<tr>
<td></td>
<td>E Q Y K F Y S V</td>
<td>Allo-H-2 K₁⁴³</td>
<td>MLRQ (nuclear encoded mitochondrial protein)</td>
</tr>
<tr>
<td></td>
<td>L S P F F P D L</td>
<td>Allo-H-2 L₁⁴³</td>
<td>a-ketoglutarate dehydrogenase</td>
</tr>
<tr>
<td>Human CTL clone JL 12</td>
<td>F L R G R A Y G L</td>
<td>Self-HLA-B8</td>
<td>EBNA-3 protein from EBV</td>
</tr>
<tr>
<td></td>
<td>K P S P P Y F G L</td>
<td>Allo-HLA-B35</td>
<td>CTP synthase</td>
</tr>
<tr>
<td></td>
<td>K P I V V L H G Y</td>
<td>Allo-HLA-B35</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td></td>
<td>M P E A T Y Y G L</td>
<td>Allo-HLA-B35</td>
<td>None (analog substituted)</td>
</tr>
<tr>
<td></td>
<td>I P I A P V Y G M</td>
<td>Allo-HLA-B35</td>
<td>None (analog substituted)</td>
</tr>
<tr>
<td>Human CTL clone 121</td>
<td>F P S D S W C Y F</td>
<td>Self-HLA-B*3503</td>
<td>Mutated caspase-8</td>
</tr>
<tr>
<td></td>
<td>H I P D V I T Y</td>
<td>Allo-HLA-B*3503</td>
<td>Squalene synthase</td>
</tr>
<tr>
<td></td>
<td>Q F A D V I V L F</td>
<td>Allo-HLA-B*3503</td>
<td>2-Hydroxyphytanoyl-CoA lyase</td>
</tr>
</tbody>
</table>

3 Abbreviation used in this paper: FL, fluorescein.
change at position 116 in the F pocket of the HLA molecule, which is the pocket that interacts with the C terminus of the peptide (25, 26). The transfected cells were screened for the expression of the Ag by adding CTL 121 and then measuring TNF production after 1 day of coculture. Of the 800 cDNA pools that were tested, four proved positive. Bacteria from the four positive pools were subcloned, and in each of them we obtained several clones that transferred the expression of the Ag in cells expressing allo-B*3501 molecules. Results obtained with a representative clone from each pool are shown in Fig. 1. When these four positive cDNA clones were transfected into COS-7 cells together with a cDNA coding for self-B*3503 molecules, only cDNA 668 was able to transfer the expression of the Ag in cells expressing allo-B*3501 molecules. Results obtained with a representative clone from each pool are shown in Fig. 1. When these four positive cDNA clones were transfected into COS-7 cells together with a cDNA coding for self-B*3503 molecules, only cDNA 668 was able to transfer the expression of the Ag (Fig. 1). The coding sequence of cDNA clone 668 was found to be identical, with the exception of a mutation, to that of \textit{CASP-8} coding for caspase-8, which is implicated in the triggering of apoptosis (8). The antigenic peptide is FPSDSWCYF, which is not encoded by the wild-type \textit{CASP-8}. It produced half-maximal lysis of autologous EBV-B target cells at \(\frac{1}{2}\) nM and on \(.221\) cells expressing self-B*3503 molecules at \(\frac{1}{2}\) nM (Fig. 2).

cDNAs 167 and 576 correspond to 2-hydroxyphytanoyl-CoA lyase (accession no. HSA131753), which is also expressed in normal tissues and catalyzes the carbon-carbon bond cleavage during oxidation of some fatty acids (28). The peptide recognized by CTL 121 was identified with the same strategy as described for squalene synthase. The shortest antigenic peptide was a nonamer, QFADVIVLF (amino acids 177–185). Half-maximal lysis was obtained at a peptide concentration of \(\sim 10\) nM (Fig. 2).

Table II. Binding capacity of the three peptides on each HLA-B35 subtype

<table>
<thead>
<tr>
<th>Sequence Competitor</th>
<th>Peptide Origin</th>
<th>B*3501 (allo)</th>
<th>B*3503 (self)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPSSADVEF</td>
<td>Tyrosinase (positive control)</td>
<td>0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>SLFRAVITK</td>
<td>MAGE-A1 HLA-A3 (negative control)</td>
<td>&gt;50.0</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td>FPSDSWCYF</td>
<td>Caspase-8</td>
<td>&lt;0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>HIPDVITY</td>
<td>Squalene synthase</td>
<td>3.3</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td>QFADVIVLF</td>
<td>2-Hydroxyphytanoyl-CoA lyase</td>
<td>1.2</td>
<td>4.8</td>
</tr>
</tbody>
</table>

* Peptides were tested in a competition assay at 4°C with an overnight incubation time. Reference peptide was LPSC_{FL}AD -VEF, a Cys-derivative of the tyrosinase antigenic peptide presented by HLA-B3503, LPSSADVEF (24). It was used at a concentration of 250 nM. Binding capacity of the peptide is shown as the concentration of peptide needed to inhibit binding of the FL-labelled reference peptide to 50% (IC_{50}). Peptides with an IC_{50} < 5 \(\mu\)M are considered as high-affinity peptides, and peptides with an IC_{50} of 5–15 \(\mu\)M should also be considered as potential CTL epitopes (23).
needed to induce half-maximal lysis was cDNA were not (Fig. 1). But the amount of peptide HIPDVITY transfected with the squalene synthase cDNA and a HLA-B*3503

...ized by the CTL on self-B*3503 molecules, whereas COS-7 cells 

...ingly, the squalene synthase peptide, HIPDVITY, was also recog- 

...ived from mutated (Fig. 2). On the autologous EBV-B cell line, only the peptide 

...molecules, or .221 cells expressing self-B*3503 molecules 

...121 on autologous EBV-B cells, .221 cells expressing allo-B*3501 molecules, the three peptides were 

...recognized, as expected, with half-maximal lysis obtained at a peptide 

...centration of ~1 nM (Fig. 2).

...Each of the three peptides were titrated for recognition by CTL 

...121 on autologous EBV-B cells, .221 cells expressing allo-B*3501 molecules, or .221 cells expressing self-B*3503 molecules (Fig. 2). On the autologous EBV-B cell line, only the peptide derived from mutated CASP-8 resulted in recognition. Surprising- 

...ingly, the squalene synthase peptide, HIPDVITY, was also recog- 

...recognized by the CTL on self-B*3503 molecules, whereas COS-7 cells transfected with the squalene synthase cDNA and a HLA-B*3503 cDNA were not (Fig. 1). But the amount of peptide HIPDVITY needed to induce half-maximal lysis was ~10 nM, whereas only ~1 nM of the CASP-8-derived peptide was necessary. On .221 cells expressing allo-B*3501 molecules, the three peptides were recognized, as expected, with half-maximal lysis obtained at a peptide 

...centration of <10 nM.

...To determine whether the affinity of each of the three peptides 

...for two HLA-B35 subtypes influenced the recognition by the 

...CTL, we designed a peptide binding assay similar to the assay on intact human B cells described by van der Burg and colleagues (23). Briefly, the natural peptides were stripped from the HLA class I molecules by mild acid treatment, after which the .221.B*3501 or .221.B*3503 cells were incubated overnight at 4°C with a FL-labeled reference peptide at a fixed concentration, together with decreasing concentrations of the three peptides of interest. After washing, the effectiveness by which these peptides competed for binding to HLA molecules was assayed by measuring the amount of HLA-bound FL-labeled peptide with FACScan analysis. The concentrations needed to inhibit the binding of the FL-labeled peptide to 50% (IC 50) are indicated in Table II. Pepti- 

...did bind to self-B*3503 molecules, thus revealing that it was not recognized by CTL clone 121. For the two other peptides, the amount needed to induce lysis was corre- 

...lated to their affinity to self-B*3501. Concerning allo-B*3501 molecules, the three peptides can be considered to have a high affinity; the mutated peptide was the best competitor. Their recognition was not strictly correlated with their affinity.

...The two peptides recognized on allo-B*3501 molecules only partially match the HLA-B35 binding motif, i.e., P in position 2 and Y, F, M, L, or I at the C terminus (29). They share amino acids DVI in the center of the peptide (Table I). Interestingly, only a D at position 4 is shared by the three peptides recognized by CTL 121. This amino acid seems to be essential for recognition, because replacing in each of the three peptides the D at position 4 by an A prevents recognition by the CTL (data not shown).

...We verified that CTL 121 expresses only one TCR. RNA was 

...prepared from CTL 121 and RT-PCR amplification was conducted with primers complementary to the V and C regions of the TCR. Sequencing revealed that CTL 121 expresses Vβ14 and two Vα transcripts, Vα27 and Vα9 (Fig. 3). The Vα9 transcript is not functional because the sequences of the J and C regions are not in frame. Thus, CTL 121 expresses only one TCR. Taking into account the conserved conformation of the CDR1α loops that have been studied so far by crystallography (30), the CDR1α amino acid sequence of the TCR of CTL 121 was compared with the sequence of TCR A6 interacting with a tax peptide presented by HLA-A2. The crystal structure of this last TCR had revealed that it was the Q at position 30 of the CDR1α that interacts with a neutral G at position 4 of the peptide (31). Interestingly, in the TCR of CTL 121, there is a positively charged R at position 30, which may interact with the negatively charged D, which seemed to be essen- 

...tial for recognition and was present at position 4 of each of the three peptides recognized by CTL 121.

...The evidence that the peptides are processed was first obtained 

...by transfection into COS-7 cells. We also tested two tumor cell lines obtained from B*3501 patients for their ability to stimulate 

...CTL 121, because a unusually high expression can be achieved in transiently transfected COS-7 cells. The tumor cell lines scored

FIGURE 4. Stimulation of CTL 121 by HLA-B35 tumor cell lines. The tumor cell lines were renal cell carcinoma lines LE9211 and LB1047, HLA-B35 negative bladder carcinoma line LB1831, autologous head-and-neck squamous carcinoma line BB49, and melanoma line LG2. Bladder carcinoma line LB831 was transiently transfected with an HLA-B*3501 cDNA, obtained by RT-PCR from the RNA of LB1047 and cloned into expression vector pcDNAI/Amp. A total of 1,500 CTL were added into microwells containing 10,000 tumor cell lines. The production of TNF was measured after 24 h of coculture by testing the toxicity of the supernatants for the TNF-sensitive WEHI-164.13 cells.
positive, but the production of TNF by the CTL was not very high (Fig. 4). A bladder carcinoma line, transiently transfected with an allo-B*3501 cDNA, also stimulated the CTL to produce TNF (Fig. 4). Most probably, the number of peptide-HLA complexes was too low to result in maximal stimulation. This is in accordance with the 10–15% lysis of the .221 cells transfected with allo-B*3501 that was observed in absence of synthetic peptide (Fig. 2). Whether or not the recognition of allogeneic HLA molecules by CTL is dependent on the presence of a peptide remains a subject of debate (3, 4, 32, 33). Here we show that CTL 121 clearly required the presence of a peptide for recognition and, moreover, that only a limited number of peptides can activate the TCR of clone 121. In fact, we have screened 80,000 cDNA with CTL 121 and only two different cDNAs were found to encode a peptide presented to CTL 121 on allo-B*3501 molecules. We are fully aware that the two HLA alleles are closely related and that one should be circumspect about the value of a generalized conclusion. However, it has already been reported that a single amino acid mismatch between two HLA class I molecules can induce a T cell response (34). Taken together, our results might reinforce the notion that peptides recognized on allogeneic MHC molecules are processed from ubiquitous proteins (32, 35).

Acknowledgments

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14. Arden, B., S. P. Clark, D. Ketelbe, and T. W. Mak. 1995. Human T-cell receptor gamma gene segment usage in EBV-positive, but the production of TNF by the CTL was not very high (Fig. 4). A bladder carcinoma line, transiently transfected with an allo-B*3501 cDNA, also stimulated the CTL to produce TNF (Fig. 4). Most probably, the number of peptide-HLA complexes was too low to result in maximal stimulation. This is in accordance with the 10–15% lysis of the .221 cells transfected with allo-B*3501 that was observed in absence of synthetic peptide (Fig. 2). Whether or not the recognition of allogeneic HLA molecules by CTL is dependent on the presence of a peptide remains a subject of debate (3, 4, 32, 33). Here we show that CTL 121 clearly required the presence of a peptide for recognition and, moreover, that only a limited number of peptides can activate the TCR of clone 121. In fact, we have screened 80,000 cDNA with CTL 121 and only two different cDNAs were found to encode a peptide presented to CTL 121 on allo-B*3501 molecules. We are fully aware that the two HLA alleles are closely related and that one should be circumspect about the value of a generalized conclusion. However, it has already been reported that a single amino acid mismatch between two HLA class I molecules can induce a T cell response (34). Taken together, our results might reinforce the notion that peptides recognized on allogeneic MHC molecules are processed from ubiquitous proteins (32, 35).