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Peptide Presentation to an Alloreactive CTL Clone Is Modulated Through Multiple Mechanisms Involving Polymorphic and Conserved Residues in HLA-B27

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This study addressed the mechanisms by which HLA class I polymorphism modulates allorecognition. CTL 27S69 is an alloreactive clone raised against HLA-B*2705, with a known peptide epitope. This CTL cross-reacts with B*2702, which differs from B*2705 in the D77N, T80I, and L81A changes, but not with B*2701, which has D74Y, D77N, and L81A changes. To explain this differential recognition, B*2705 mutants mimicking subtype changes were used. The A81 mutant was not recognized, despite binding the natural epitope in vivo, suggesting that, when bound to this mutant, this peptide adopts an inappropriate conformation. The N77 and I80 mutations restored recognition in the N77A81 or I80A81 mutants. These compensatory effects explain the cross-reaction with B*2702. The Y74 and Y74N77 mutants were weakly recognized or not recognized by CTL 27S69. This correlated with the absence or marginal presence of the peptide epitope in the Y74N77-bound pool. As with B*2701, exogenous addition of the peptide epitope sensitized Y74 and Y74N77 targets for lysis, indicating that failure to cross-react with B*2701 or these mutants was due to poor binding of the peptide in vivo and not to inappropriate presentation. The abrogating effect of Y74 was critically dependent upon the K70 residue, conserved among subtypes, as demonstrated with mutants at this position. Thus, HLA polymorphism affects allorecognition by modulating peptide binding or the conformation of bound peptides. Compensatory mutations and indirect effects of a polymorphic residue on residues conserved play a critical role.

The differential presentation of a peptide epitope to an alloreactive CTL by closely related class I alloantigens provided the opportunity to analyze the mechanisms by which single changes in...
the MHC molecule modulate allorrecognition, and the interactive effects of multiple changes on peptide binding and presentation. Thus, the present work determined the basis for the differential recognition of B*2701 and B*2705 by CTL 27S69 by analyzing the recognition of single and double mutants mimicking subtype changes and the effects of these mutations on the binding and presentation of the natural peptide epitope.

Materials and Methods

**CTL 27S69**

CTL 27S69 was raised against B*2705. Its culture conditions and fine specificity with other HLA-B27 subtypes have been described previously (26). The natural peptide epitope recognized by this clone in the context of B*2705 and other subtypes has also been determined (14).

**HLA-B27 mutants and transfectant cells**

Hmy2.C1R (C1R) transfectants expressing B*2701, B*2702, B*2705, and various HLA-B27 mutants were used (Fig. 1). B*2705 mutants were designated with the one-letter code of the amino acid(s) introduced followed by the number(s) of the position(s) changed. A B*2701 mutant carrying the K70A mutation was designated as A70-B*2701. The generation and expression levels of these mutants on transfected cells have been described previously for most of them (27–30). The I80A81 construct was generated by site-directed mutagenesis from I80 genomic DNA and transfected into C1R cells using previously described procedures (27, 31). HLA-B27 subtypes and mutants were expressed at high and similar levels on C1R transfectants (27). RMA-S is a murine TAP-deficient cell line (32).

**Isolation and analysis of HLA-B27 mutant-bound peptides**

The peptide pools bound to the Y74N77 and A81 mutants were purified from 1–1.5 × 10⁶ C1R transfectant cells and fractionated by HPLC as described for B*2705 (14). The peptide composition of individual HPLC fractions was determined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF) (MS), using a Reflex instrument (Bruker-Franzen Analytik, Bremen, Germany) as described previously (14). Peptide sequencing was conducted with an LCQ electrospray/ion trap mass spectrometer (Finnigan, MAT, San Jose, CA), as described previously (14).

**Peptide synthesis**

The RRFFPPYYV octamer was synthesized using standard fluorenylmethoxycarbonyl chemistry and purified by HPLC. Its quantification was conducted by amino acid analysis and its correct composition was verified by MALDI-TOF and electrospray/ion trap MS.

**Epitope stabilization assay**

The quantitative procedure used has been described previously (34). Briefly, RMA-S transfected cells were incubated at 26°C for 24 h. Next, the synthetic peptide was added at 10⁻⁴ to 10⁻⁹ M. Cells were incubated for 1 h at 26°C, transferred to 37°C, and collected for flow microcytometry analysis after 2 h. Binding of the RRFFPPYYV octamer was expressed as the C50, which is the molar concentration of peptide at 50% of the maximum fluorescence obtained in the concentration range used. Values of C50 of ≤10 μM were considered to reflect high affinity. C50 values of between 10 and 50 μM were considered as intermediate affinity. C50 values of ≥50 μM indicated low affinity.

**Cytotoxicity assays**

When exogenous peptide was not added, a standard 4-h ⁵¹Cr release assay was conducted. In peptide sensitization assays, labeled targets were incubated with 1% FCS. Effector cells were then added and incubation at 37°C was conducted for 5 h. The procedures have been described in detail elsewhere (14).

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*Abbreviations used in this paper: C1R, Hmy2.C1R; MALDI-TOF, matrix-assisted laser desorption/ionization mass spectrometry.*
Table 1. Binding of RRFFPYVV to HLA-B27 mutants on RMA-S transfectant cellsa

<table>
<thead>
<tr>
<th>RMA-S Transfectants</th>
<th>C50</th>
</tr>
</thead>
<tbody>
<tr>
<td>B*2705</td>
<td>5</td>
</tr>
<tr>
<td>Q70</td>
<td>7</td>
</tr>
<tr>
<td>Y74</td>
<td>7</td>
</tr>
<tr>
<td>N77I80</td>
<td>4</td>
</tr>
<tr>
<td>A81</td>
<td>6</td>
</tr>
<tr>
<td>I80A81</td>
<td>4</td>
</tr>
</tbody>
</table>

a Binding to each HLA-B27 variant is expressed as the micromolar concentration of peptide required to obtain half the maximum fluorescence in the concentration range used (C50; see Materials and Methods). Data are the mean of two experiments.

K70, we tested two mutants at this position for recognition by CTL 27S69. One was Q70, a B*2705 mutant carrying the K70Q change. The second was a B*2701 mutant carrying the K70Q change, designated A70-B*2701 (Fig. 1B). Q70 targets were lysed with low efficiency (27% relative lysis), and similarly to Y74 targets. In contrast, the K70A mutation on the B*2701 background had the opposite effect, because A70-B*2701 targets were lysed similarly to B*2705.

These results demonstrate that K70 plays a key role in the allospecificity of CTL 27S69, and strongly suggest that the effect of the D74Y change on recognition by this CTL is mediated through K70.

The RRFFPYVV epitope binds in vitro HLA-B27 mutants not recognized by CTL 27S69

To test whether lack of recognition by CTL 27S69 could be explained by lack of binding of the corresponding peptide epitope, we tested the RRFFPYVV octamer with some cell surface-expressed mutants that abrogated or did not abrogate recognition by this CTL. As shown in Table I, this peptide efficiently bound (C50 of <10 μM) the mutants tested, regardless of whether they were recognized or not by CTL 27S69. These results indicate that there is no correlation between the binding efficiency of the octamer to these mutants in vitro and their recognition by CTL 27S69.

Differential presentation of exogenous peptide epitope by HLA-B27 mutants

Because four mutants abrogated or significantly reduced the recognition by CTL 27S69 (Fig. 1), the octamer epitope was tested for its capacity to sensitize C1R transfectants expressing these mutants for lysis (Fig. 2). The clone efficiently killed Y74 and Y74N77 targets in the presence of exogenously added octamer (half-maximal lysis was 2.3 × 10^{-13} M for Y74 and 4.4 × 10^{-13} M for Y74N77). This behavior is similar to that observed for B*2701 (14) and strongly suggests that the weak cross-reaction with Y74-C1R in the absence of exogenous peptide is due to a low amount of the peptide epitope bound to this mutant in vivo. The results also suggest that failure to kill Y74N77 targets in the absence of exogenous peptide is due to lack of constitutive binding of the octamer to this mutant in vivo.

The octamer also sensitized Q70-C1R targets, but the peptide was recognized much less efficiently (half-maximal lysis at 1.3 × 10^{-9} M) in the context of this mutant. This was not due to lower binding, relative to Y74 or Y74N77 (Table I). Because Q70 differs from Y74 at two positions (K70Q and Y74D), its less efficient presentation of the octamer was probably due to direct involvement of these residues in the alloantigenic epitope or to effects on peptide conformation.

Significant lysis of A81-C1R cells was only obtained when the concentration of this octamer was ≥1 μM, indicating inefficient presentation of the exogenous peptide epitope to CTL 27S69 by this mutant.

A81, but not Y74N77, binds the CTL 27S69 epitope in vivo

Because Y74N77 and A81-C1R cells were differentially sensitized by the octamer, the presence of this peptide in the corresponding mutant-bound peptide pools was analyzed. These pools were obtained from immunopurified mutant molecules on C1R transfectants and fractionated by HPLC; next, the fractions corresponding to the elution position of the octamer were analyzed by MALDI-TOF MS (Table II). Fractions 209 and 210 from A81-bound peptides revealed a species with the appropriate molecular mass of this peptide (mass/charge: 1149 Da). Its correct amino acid sequence was confirmed by electrospray/ion trap MS. This finding demonstrates that a lack of cross-reaction of CTL 27S69 with A81 is not due to absence of the octamer epitope in the peptide pool bound to this mutant in vivo.

The amount of the octamer relative to the related RRFFPYVVY nonamer was estimated to be 1:5.6 in A81 by MALDI-TOF analysis. This ratio is only slightly lower than the 1:4.5 ratio reported for B*2705 (14), further suggesting that the A81 mutation has little effect on the binding of the octamer epitope.

Analysis of the corresponding fractions from the Y74N77-bound peptide pool by MALDI-TOF MS revealed a species whose molecular mass was compatible with the octamer (mass/charge: 1148.44 Da) in HPLC fraction 206. The sequence of this peptide could not be determined. Other peptides coeluting with the octamer in both B*2705-bound (14) and A81-bound peptide pools were found and sequenced from the fractions analyzed from Y74N77 (Table II), demonstrating that fraction 206 is the one that should contain the octamer. This result is compatible with the presence of low amounts of the octamer bound to Y74N77 in vivo.

In an additional attempt to determine the presence of the octamer in the Y74N77-bound peptide pool, individual HPLC fractions from this pool were incubated with B*2701-C1R targets and tested for lysis by CTL 27S69 (Fig. 3). HPLC fractions 202–221 failed to sensitize these targets for lysis. In contrast, significant
lysis was detected in fraction 209 of the A81-bound peptide pool (used as a positive control). This result confirms that Y74N77 either fails to bind the octamer epitope in vivo or binds it in a very low amount. Thus, the lack of cross-reaction of CTL 27S69 with the Y74N77 mutant is probably due to a lack of or a marginal presence of the octamer in the mutant-bound peptide pool.

In the peptide pools used in these experiments, which were obtained from similar cell numbers, the total amount of peptides recovered from the A81 mutant was ~20% higher than for Y74N77, as estimated by the total absorbance of HPLC fractions at 210 nm. However, this was not reflected equally in individual peptide yields. For instance, among the peptides in Table II, RRFFPPYYV was obtained from both mutants with similar yields, GRHGVFLEL was 2.5-fold more abundant in A81, as estimated by MALDI-TOF analysis. It is obvious that different mutations may affect the binding in vivo of different peptides in distinct ways. Thus, peptide pools extracted from different mutants cannot be normalized for a given natural ligand. However, these data suggest that failure to detect the octamer epitope in Y74N77 is not simply due to a globally low yield of the peptide pool extract relative to A81.

### Discussion

A molecular understanding of how alloreactivity is modulated by HLA class I polymorphism can be achieved with CTL clones of well-characterized fine specificity and known peptide epitopes. In a previous report (14), identification of the peptide epitope recognized by CTL 27S69 allowed explanation of its fine specificity with HLA-B27 subtypes on the basis of three mechanisms. First, cross-reaction of this CTL with B*2705, B*2702, and B*2703 was through recognition of the same peptide in the context of the three subtypes. Second, lack of cross-reaction with B*2704, B*2706, and B*2710 occurred despite efficient binding of the epitope to these subtypes, and was very likely due to direct impairment of the V152E change, common to these three subtypes, on TCR interaction. Third, failure to cross-react with B*2701 was due to absence of the peptide epitope in the B*2701-bound pool in vivo, but not to an inability of the subtype to present the peptide, as shown by exogenous peptide sensitization of B*2701 targets.

Our results now explain the role of HLA-B27 polymorphism in the differential recognition of B*2701 and B*2702. A striking result was that the L81A change, common to B*2701 and B*2702, abrogated allorecognition, but not binding of the peptide epitope both in vitro and in vivo. A direct impairment of TCR interactions, as proposed for the V152E change (14, 36), is unlikely in this case because residue 81 is pointing into the F pocket of HLA-B27 (35, 37). The most likely explanation for the effect of the L81A change is that the conformation of the RRFFPPYYV epitope bound to this mutant is different from that in B*2705. The decreased hydrophobicity of the F pocket and its enlarged size as a result of the L81A change should destabilize the interaction with the C-terminal Val residue of the peptide and promote side chain rearrangements to compensate for this effect. Recognition of B*2702, despite the A81 change, is explained by the compensatory effects of the two other changes in this subtype, D77N and T80I. The latter one is likely to compensate, especially in terms of increased hydrophobicity, the corresponding decrease due to the L81A change. The mechanism by which the D77N change restores peptide presentation is not obvious.

Lack of recognition of B*2701 is not accounted for by the L81A change, due to the compensatory effect of the concurrent D77N change. Rather, it is explained by the Y74 mutation, unique to this subtype, especially together with N77. In contrast to A81, the effect of Y74 and Y74N77, like that of B*2701 (14), was to impair the binding of the peptide epitope in vivo, but not its presentation when added exogenously. These effects are probably not limited to the particular peptide epitope in this study, because multiple alloreactive CTL clones recognizing B*2702 but not B*2701 were affected by the Y74 and/or Y74N77 changes (27).

A previous study demonstrated that the influence of the D74Y change on the peptide specificity of HLA-B27 is largely mediated through the K70 residue (30). That this was also the case for the RRFFPPYYV epitope reveals a novel mechanism by which HLA class I polymorphism affects allorecognition: the long-range effect of a polymorphic residue on the conformation of a conserved one.

### Table II. Molecular species detected in HPLC fractions from the A81- and Y74N77-bound peptide pools by MALDI-TOF MS

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>m/z</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>A81</td>
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<tr>
<td>209</td>
<td>1084.24</td>
<td>RRFFPYYV</td>
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<td></td>
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<td></td>
<td>1148.84</td>
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<tr>
<td></td>
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<tr>
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</tr>
<tr>
<td></td>
<td>1051</td>
<td></td>
</tr>
<tr>
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<tr>
<td>206</td>
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</tr>
<tr>
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<td>1148.44</td>
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</tr>
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<tr>
<td></td>
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<tr>
<td></td>
<td>1071</td>
<td></td>
</tr>
<tr>
<td>208</td>
<td>1028</td>
<td>GRHGVFLEL</td>
</tr>
<tr>
<td></td>
<td>1059</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1311</td>
<td>RRLPIFSRL</td>
</tr>
<tr>
<td></td>
<td>1348</td>
<td>RRFFPYVV</td>
</tr>
</tbody>
</table>

* Those peptides whose amino acid sequence was determined by electrospray/ion trap MS are indicated. m/z, mass/charge.

[FIGURE 3. Lysis of B*2701-C1R target cells sensitized with aliquots of HPLC fractions of the Y74N77-bound (○) and A81-bound (●) peptide pools by CTL 27S69. Specific lysis of the control B*2705-C1R cells at the E:T ratio used (1:1) was 79%.]
In conclusion, modulation of allorrecognition by HLA-B27 class I polymorphism occurs through various mechanisms, including direct impairment of TCR interactions (14, 36), impairment of peptide binding, inappropriate peptide presentation (presumably through conformational alteration of the bound peptide), and long-range effects affecting a residue conserved among differentially recognized alloantigens. In addition, interactive effects among concomitant mutations allow recognition of an HLA alloantigen carrying a change that by itself would be abrogating.

The multiple mechanisms by which HLA class I polymorphism modulates allorrecognition and the subtleties of the interactive effects involving polymorphic residues have implications concerning the relationship between the structure and peptide-presentation properties of the class I molecules. First, there is no strict correlation between structural and peptide-presentation similarity. For HLA-B27, the best example is B*2710, a subtype differing from B*2705 by a single amino acid residue, but showing very low cross-reaction at the T cell level (36). This was well illustrated in the present study by the compensatory effects of additional mutations on A81. Second, there is no strict correlation between peptide-binding specificity and antigenic similarity. T cells specific for a given peptide in a particular HLA context may not recognize that peptide in complex with a different restriction element, as seen in this study with A81. Thus, when considering the role of HLA-B27 subtypes as restriction elements in T cell responses or as risk factors for spondyloarthropathy (38), their peptide-binding properties are not sufficient to understand their differential behavior, as their structural differences may modulate in various ways the antigenicity of bound peptides.

Acknowledgments
We thank Alicia Prieto (Centro de Investigaciones Biológicas, Madrid, Spain) and our colleagues at the Protein Chemistry Laboratory (Jesús Vázquez, Anabel Marina, and Samuel Oguea) for their help in MS. We also thank Juana Bustos for technical assistance in tissue culture and Stefan Krebs for the A70-B*2701 mutant.

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