Cutting Edge: Identification of Novel T Cell Epitopes in Lol p5a by Computational Prediction

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Although atopic allergy affects ≤20% of the population, the relationship between the protein structure and immunogenic activity of the allergens is still largely unknown. We observed that group 5 grass allergens are characterized by repeated structural motifs. Using a new algorithm, TEPITOPE, we predicted promiscuous HLA-DR ligands within the repeated motifs of the Lol p5a allergen from rye grass. In vitro binding studies confirmed the promiscuous binding characteristics of these peptides. Moreover, most of the predicted ligands were novel T cell epitopes that were able to stimulate T cells from atopic patients. We generated a panel of Lol p5a-specific T cell clones, the majority of which recognized the peptides in a cross-reactive fashion. The computational prediction of DR ligands might thus allow the design of T cell epitopes with potential useful application in novel immunotherapy strategies. 


A topic allergy affects ≤20% of the population in industrialized countries. Allergic patients exposed to environmental Ags (allergens) develop allergen-specific IgE Abs, the production of which is triggered by the cytokine IL-4 (1–3). IgE play a major role in the induction of the allergic inflammatory cascade because of their ability to bind FcεRI-bearing cells (mast cells, basophils, eosinophils, macrophages, and Langerhans cells). FcεRI-bound IgE can be cross-linked by allergens, thus triggering the release of inflammatory mediators (4).

Lolium perenne (rye grass) is one of the major sources of seasonal allergens worldwide (5–7). Grass allergens, like allergens from a wide variety of sources, are divided into groups based on sequence and biochemical similarities (8). Lol p5, belongs to group 5 grass allergens and is one of the most clinically relevant allergens in rye grass pollen (9–13). Indeed, 85% of sera from rye grass-allergic patients recognize Lol p5 (14). The two known Lol p5 isoforms, Lol p5a and Lol p5b, are characterized by an 80% amino acid sequence similarity and common IgE epitopes (12).

The administration of peptides representing T cell epitopes of allergens has been reported recently to impair T cell responsiveness to allergens in murine models and atopic patients (15–20). The T cell epitopes present in group 5 allergens have been analyzed to better understand the initiation of the atopic immune response and to design peptide-based protocols of immunotherapy (21–25). However, a relationship between the structure homology and the T cell reactivity of group 5 allergens has not been envisaged so far.

The ability of proteins to act as immunogens stems from the binding of peptides, derived from Ag processing, to MHC molecules. Peptides derived from exogenous proteins are presented mainly by MHC class II molecules to TCRs on CD4+ T cells (26). Recently, the rules governing MHC class II/peptide interaction have been extensively characterized (27). The anchoring, inhibitory, or neutral effects of peptide side chains on HLA-DR binding seem to be strictly dependent upon the position of the residue within a particular peptide frame (28). The combination of multiple peptide synthesis technology and in vitro HLA-DR/ligand binding assays led to the definition of the effects of each residue as a function of its position within the peptide frame and resulted in the development of matrices (28–30). These matrices define MHC class II ligand specificity in quantitative terms and provide a powerful tool for MHC class II ligand prediction (27).

We found that the protein structure of group 5 grass allergens is organized in repeated structural motifs. In an attempt to clarify the relationship between the structure homology and the T cell reactivity of group 5 allergens, and in particular the Lol p5 molecule, we used a new matrix-based algorithm, TEPITOPE (27, 28, 43).

Our results demonstrate that a systematic computational approach can be used to design HLA-DR ligands that stimulate T cell clones (TCCs)2 derived from rye grass allergic patients.

Materials and Methods

**Allergens and peptides**

Aqueous allergic extracts from *L. perenne* and *Artemisia vulgaris* pollen were a kind gift of Lopharma (Milan, Italy). Peptides were synthesized with a multiple peptide synthesizer (model 396, Advanced Chem Tech, Louisville, KY) using fluorenyl methoxycarbonyl chemistry and solid phase synthesis.
Allergen sequences were loaded into new HLA-DR ligand prediction software (TEPITOPE) to predict promiscuous HLA-DR ligands. TEPITOPE is based on 25 virtual matrices (27, 43) that cover a significant part of human HLA class II peptide binding specificity. We selected the HLA-DR alleles most frequent in the Caucasian population (i.e., DRB1*0101 (DR1), DRB1*0301 (DR3), DRB1*0401 (DR4), DRB1*0701 (DR7), DRB1*0801 (DR8), DRB1*1101 (DR5), and DRB1*1501 (DR2)) and set the TEPITOPE prediction threshold at 5% (27, 43). To identify DR ligands within Lol p5a repeated motifs, we selected peptides that were predicted to bind to at least three different allotypes.

### Results and Discussion

**Group 5 allergens contain repeated 32-aa sequence motifs**

In an attempt to correlate the protein structure and allergenic activity of group 5 allergens, we first analyzed the amino acid sequence of Lol p5a, a major allergen of *L. perenne*, using a computer-assisted analysis of protein sequence and alignment (MACAW) (34–36). As shown in Fig. 1, four repeats of a 32-aa sequence motif, which occupied ~50% of the Lol p5a sequence, were aligned in a homology block. A similar structural organization is shared by other major group 5 allergens; however, the number of repeats varies depending upon the size of the protein. These results suggest that the repeated 32-aa motif may be a molecular signature of group 5 allergens.

**Computational prediction of HLA-DR binding peptides within the Lol p5a sequence**

To determine whether the presence of the repeated sequence motifs had an impact on the antigenic activity of group 5 allergens, we used the software TEPITOPE to predict HLA-DR ligands. The same approach was recently successful in identifying a promiscuous melanoma-associated peptide capable at eliciting a specific cytotoxic response (37).

**Predicted Lol p5-derived epitopes activate T cells from Lol p5 allergic patients**

We subsequently asked whether the predicted HLA-DR ligands identified by TEPITOPE in Lol p5a mirrored the T cell epitopes
derived from natural processing of this allergen. For this purpose, unfractionated PBMCs from two Lol p5-allergic patients (ET: DRB1*07/*11 (DR7/5); CDL: DRB1*02/*07 (DR2/7)) were stimulated at micromolar concentrations with a pool of peptides (7, 20, 50, and 52) or with the malaria peptide CS380–396 as a control. Table II shows that indeed the selected peptides, but not the control peptide (data not shown), induced a vigorous proliferative response, the intensity of which was comparable with that triggered by native L. perenne. These results suggest that the peptides in the pool are functional T cell epitopes.

TCCs 30 and 25 from patients ET and CDL, respectively, were generated against the pooled peptides and tested for their ability to proliferate in response to individual peptides or native Lol p5. Fig. 2 shows the results obtained with a representative panel of TCCs. The majority of TCCs (e.g., CDL 5.73 and ET 5.21) were promiscuous, because they responded to peptides 7, 50, and 52, although not to peptide 20. In contrast, peptide 20-specific TCCs (e.g. CDL 5.73 and ET 5.21) proliferated in response to the natural allergen, peptide 20-specific TCCs (e.g., CDL 5.107 and ET 5.20) did not respond significantly to any of the other peptides. Interestingly, although a number of the promiscuous TCCs (e.g., CDL 5.73 and ET 5.21) proliferated in response to the natural allergen, peptide 20-specific TCCs (e.g., CDL 5.107 and ET 5.20) did not. None of the TCCs proliferated in response to an unrelated allergen extract (A. vulgaris). These results suggest that at least one of the peptides (either 7, 50, or 52) derives from natural Lol p5a processing, whereas peptide 20 does not.

Allergen-derived T cell epitopes have been traditionally identified by synthesizing overlapping peptides that encompass the whole antigenic sequence (reviewed in ref. 24). This approach, although often informative, has two disadvantages: a large number of peptides need to be synthesized and tested, and the constraints regulating peptide/MHC class II binding are often ignored when designing the peptides. Thus, epitopes with prominent antigenic properties might be missed because of inappropriate design.

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Table I. DR binding assay of Lol p5a-derived peptidesa

<table>
<thead>
<tr>
<th>Peptides</th>
<th>DRB1 * 0101 (DR1)</th>
<th>DRB1 * 1501 (DR2)</th>
<th>DRB1 * 0301 (DR3)</th>
<th>DRB1 * 0401 (DR4)</th>
<th>DRB1 * 1101 (DR5)</th>
<th>DRB1 * 0701 (DR7)</th>
<th>DRB1 * 0801 (DR8)</th>
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<tr>
<td>No. 7 (aa56-68)</td>
<td>3</td>
<td>0.4</td>
<td>100</td>
<td>22</td>
<td>1.6</td>
<td>0.5</td>
<td>40</td>
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<tr>
<td>dkFKIFEEAFAKses</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. 20 (aa109-121)</td>
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<td>4</td>
<td>1.2</td>
<td>30</td>
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<td>0.04</td>
<td>100</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>No. 50 (aa219-231)</td>
<td>0.5</td>
<td>1.6</td>
<td>20</td>
<td>15</td>
<td>1.6</td>
<td>1.5</td>
<td>15</td>
</tr>
<tr>
<td>vKYAVFEAALKta</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>No. 52 (aa224-236)</td>
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<td>8</td>
<td>100</td>
<td>55</td>
<td>0.9</td>
<td>0.03</td>
<td>10</td>
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</tr>
<tr>
<td>HA (aa307-319)</td>
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<td>2.9</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>0.22</td>
<td>15</td>
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</tbody>
</table>

*Synthesized Lol p5a-derived peptides are listed: 2 amino acids at the N and C terminus (lowercase letter) were appended to the peptide frame indicated by TEPITOPE (uppercase letter). The DR binding is expressed as the micromolar concentration of the biotinylated indicator peptide by 50% (IC50 M).

TEPITOPE analysis led to the identification of novel T cell epitopes. In a previous study based on traditional techniques of epitope mapping, two Lol p5a epitopes (amino acids 105–116 and 193–204) were presented by DRB1*0401/0408 molecules to T cells from an allergic subject have been described (22). Epitope amino acids 193–204 exactly overlap the most promiscuous ligand predicted by TEPITOPE not contained in Lol p5a motifs (data not shown). T cell epitopes within Lol p5a motifs, predicted by TEPITOPE, were not identified in this previous study because, as suggested by the results of the in vitro HLA-DR binding assay, they associate to DRB1*04 with a low binding affinity.

Cytokine secretion by peptide-specific TCCs

We subsequently analyzed the pattern of cytokines (IFN-γ, IL-4) secreted by peptide-specific TCCs in response to stimulation with allergenic peptides. As shown in Fig. 3a, peptide-specific TCCs were found to be heterogeneous in their IL-4- and IFN-γ-secreting abilities when triggered with a pool of peptides (7, 50, and 52) (6 µM each). In particular, we focused our attention on TCCs that recognize peptides 7, 50, and 52 as well as native Lol p5. TCCs were then stimulated with individual peptides (concentrations ranging from 0.006 to 6 µM) presented by autologous APCs. Fig. 3b shows that the TCCs ET 5.17 (Th1, IFN-γ−, IL-4−), CDL 5.33 (Th2, IFN-γ−, IL-4+), and CDL 5.73 (Th0, IFN-γ+, IL-4−) exhibited a dose-dependent cytokine response that was similar for the three peptides tested. Another Th0 clone, ET 5.21, was peculiar in that it responded to peptide 50 ≥100-fold more efficiently than to the other peptides. However, peptide 50, as well as the other peptides, induced both IFN-γ and/or IL-4 to a comparable extent; this was true for all TCCs. Thus, none of the tested peptides had the ability to determine a polarization of the cytokine secretion pattern. This observation might be explained by either intrinsic properties of the peptide sequence or an in vitro cytokine environment, which might not mediate the prominent Th2 cytokine profile, typical of an in vivo atopic immune response (38).

Molecular basis of multiple epitope recognition by Lol p5-specific TCCs

To investigate the molecular basis of the ability of individual Lol p5-reactive TCCs to recognize multiple epitopes, we initially asked which MHC class II molecule presented the peptides to the isolated TCCs. Inhibition studies with specific mAbs demonstrated that HLA-DR molecules, but not DP or DQ molecules, presented Lol p5 peptides (data not shown). Allergen-specific TCCs were stimulated with peptide 7, 50, or 52 using EBV B cell lines homozygous for the DRB1*02 (LD2B), DRB1*11 (SWEIG), or...
DRB1*07 (EKR) allele as APCs. As expected from the DR binding assay, all TCCs generated from a DRB1*07/11 (DR7/5) (ET) and DRB1*02/07 (DR2/7) (CDL) subject, respectively, were activated by each of the three peptides presented by DRB1*07 (data not shown). Fig. 4 shows the results obtained in one representative experiment with two TCCs, ET 5.21 and CDL 5.73. In particular, DRB1*07 was the only restriction element for the presentation of peptides 7 and 52 to clones CDL 5.73 and ET 5.21. In contrast, peptide 50 was recognized by clone CDL 5.73 when presented in association with DRB1*02 and was recognized by clone ET 5.21 when presented in association with DRB1*11 and DRB1*02. These results indicate that peptide 50, but not peptides 7 and 52, is able to be recognized by the same TCC in diverse DR contexts. Moreover, clone ET 5.21 is a promiscuous TCC, as it recognizes peptide 50 when bound to different DR alleles (DRB1*02 or DRB1*11), as already described for the recognition of other peptide Ags (39).

Because the DRB4*01 (w53) allele was expressed in association with DRB1*07 by both patients, we analyzed its role in the presentation of Lol p5a peptides. A heterologous EBV B cell line (DKB) expressing DRB4*01, but not DRB1*07, was not able to present the peptides and activate TCCs (data not shown). These data indicate that Lol p5a peptides are presented by DRB1*07 expressed by ET and CDL APCs.

Because the TCCs analyzed in our study shared common patterns of epitope recognition and HLA-DR restriction, we verified their clonal diversity through analysis of their TCR usage. The sequences coding for the Vα and Vβ chains of the TCR were amplified by RT-PCR using specific primers (40–42). All of the TCCs expressed different combinations of TCR Vα and Vβ chains, suggesting that no preferential Vα or Vβ usage is associated with the observed pattern of peptide recognition (data not shown).

Taken together, these data indicate that the multiple specificity of the isolated Lol p5-specific TCCs appears to result from the capacity of individual TCRs to respond to different peptides in the context of the same HLA-DR molecule (i.e., DRB1*07) as well as from the ability of selected peptides (e.g., peptide 50) to be presented to different TCRs in association with more than one HLA-DR allele.

**Conclusion**

The administration of allergen-derived T cell epitopes to atopic patients was observed to impair the in vivo T cell responsiveness to allergens (16, 18–20). Our results indicate that TEPITOPE may be used as a fast and reliable tool for the selection of natural T cell epitopes recognized by a broad variety of TCR molecules in diverse contexts.
HLA-DR contexts. The possibility of predicting promiscuous peptides encourages the use of TEPITOPE to design peptide-based vaccines that will be active in a large portion of the population.

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