Cutting Edge: Identification of Novel T Cell Epitopes in *Lol p*5a by Computational Prediction

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Although atopic allergy affects ≤20% of the total 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 p*5a 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 p*5a-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. ***The Journal of Immunology,*** **1999, 163: 1725–1729.**

Atopic 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 world-wide (5–7). Grass allergens, like allergens from a wide variety of sources, are divided into groups based on sequence and biochemical similarities (8). *Lol p*5, 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 p*5 (14). The two known *Lol p*5 isoforms, *Lol p*5a and *Lol p*5b, 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 p*5 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 allergenic 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 fluorenylmethoxycarbonyl chemistry and solid phase synthesis.

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[2] Abbreviation used in this paper: TCC, T cell clone.

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T cell epitope prediction

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 p*5a repeated motifs, we selected peptides that were predicted to bind to at least three different allotypes.

DR-peptide binding assays

Peptide interactions with detergent-solubilized HLA-DR molecules were measured using an ELISA-based high-flux competition assay (28). HLA-DR molecules were isolated from the following human lymphoblastoid cell lines: DRB1*0101 (DR1), HOM-2; DRB1*0301 (DR3), WT49; DRB1*0401 (DR4), PREISS; DRB1*1101 (DR5), SWEIG; DRB1*0701 (DR7), EKR; and DRB1*0801 (DR8), BM9. DRB1*1501 (DR2) was isolated from the L cell transfectant L466.1. The molecules were affinity purified using the mAb 1-1C4 (31) as described previously (28).

Peptide competition assays were conducted to measure the ability of unlabeled peptides (no.'s 7, 20, 50, and 52) to compete with a biotinylated indicator peptide for binding to purified HLA-DR molecules. The following biotinylated indicator peptides were used: $GFKA_7$ for DRB1*0101 (DR1) and DRB1*0701 (DR7), $GIRA_2YA_4$ for DRB1*1501 (DR2), $IAYDA_5$ for DRB1*0301 (DR3), UD4 for DRB1*0401 (DR4), TT 830–843 for DRB1*1101 (DR5), and $GYRA_6L$ for DRB1*0801 (DR8). The amount of biotinylated peptide specifically bound to DR molecules was revealed by the addition of phosphatase-labeled streptavidin and 4-*p*-nitrophenyl-phosphate. To determine relative peptide binding affinity, the promiscuous $HA_{307–319}$ peptide from influenza hemagglutinin (PKYVKQNTLKLAT) was included in each competition assay. The relative binding data for the unlabeled competitor peptides were expressed as IC_{50} (i.e., the concentration of competitor peptide required to inhibit binding of the biotinylated indicator peptide by 50%).

Generation of Lol p5-specific TCCs

PBMCs were isolated from two *Lol p*5-allergic patients identified on the basis of clinical and laboratory findings. The HLA typing of the two patients was: ET: DRB1*07/*11 (DR7/5), DRB3 (w52), DRB4*01 (w53), and DQB1*02/*0301 (DQ2/3); CDL: DRB1*02/*07 (DR2/7), DRB4*01 (w53), DRB5 (w51), and DQB1*02/*05202 (DQ2/5). PBMCs were resuspended in RPMI 1640 medium supplemented with 2 mM glutamine, sodium pyruvate, nonessential amino acids (Life Technologies, Grand Island, NY) and autologous plasma (5% v/v), seeded at 1×10^6 cells/ml and stimulated with pooled peptides 7, 20, 50, and 52 (6 μM each). The malaria peptide $CS_{380–396}$ (6 μM) was included as a control. After 6 days, human rIL-2 (Hoffmann-La Roche, Nutley, NJ) was added at a final concentration of 30 U/ml. TCCs were obtained from actively proliferating T cells as described previously (32) and cultured in medium supplemented with IL-2 (50 U/ml).

Analysis of antigenic specificity and MHC class II restriction of TCCs

TCCs were stimulated for 48 h with *Lol p*5-derived peptides (6 μM each), with aqueous extracts from *L. perenne*, or, as negative control, with *A. vulgaris* pollen (final concentration, 2–10 μg/ml of protein), in the presence of irradiated (5,000 or 10,000 rad) PBMCs or autologous EBV-transformed B cells. [^{3}H]thymidine incorporation was then assessed by scintillation counting. TCCs that responded to peptides or pollen extracts with a proliferation index of >3 were considered to be significantly stimulated. *Lol p*5a epitope mapping was performed following the same procedure, but stimulating TCCs with individual peptides.

The HLA-DR restriction analysis of TCCs was determined using PBMCs or autologous EBV-transformed B cells as APCs and blocking Abs: anti-DR (L243, 1:4 culture supernatant), anti-DQ (SVLP3) or anti-DP (B7.21) both as 1:1000 ascites. To identify the DR-restricting alleles DR homozygous EBV B cell lines were used as APCs: LD2B, DRB1*1501 (DR2); SWEIG, DRB1*1101 (DR5); EKR, DRB1*07 (DR7); DKB, DRB1*09012 (DR9).

Cytokine secretion by TCCs

TCCs were stimulated for 48 h with individual peptides in the presence of autologous PBMCs or EBV-transformed B cells as APCs. Culture media were then harvested and analyzed by specific sandwich ELISA for IL-4 (PharMingen, San Diego, CA) and IFN-γ (33).

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Lol p5a aa 44-75   AAVAAAANAPPADKFKIFEAAFSESSKGLLAT
        aa 93-124  VAYKAAEGATPEAKYDAFVTALTEALRVIAGA
        aa159-190  IAATAANAAPTNDKFTVFESAFNKALNECTGG
        aa207-238  QAYAATVAAAPEVKYAVFEAALTKAITAMTQA
Lol p5b aa 81-112  AAVAAAAVVPPADKYKTFVETFGTATNKAFVE
        aa136-167  LAYEAAQGATPEAKYDAYVATLTEALRVIAGT
        aa202-233  TAATAANAAPANDKFTVFENTFNNAIKVSLGA
        aa250-281  QAYAAKQATAPEVKYTVSETALKKAVTAMSEA
Phl p5a aa 50- 81  KAALAGAGVQPADKYRTFVATFGPASNKAFAE
        aa109-140  LAYKTAEGATPEAKYDAYVATLSEALRIIAGT
        aa173-204  VAATAANAAPANDKFTVFEAAFNDEIKASTGG
        aa221-252  QAYAATVATAPEVKYTVFETALKKAITAMSEA
Poa p9  aa 10- 41  TALKAASAVAPAEKFPVFQATFDKNLKEGLSG
KBG 31  aa 57- 88  TSYLSTKAAEPKEKFDLFVLSLTEVLRFMAGA
        aa137-168  AAVAAAAGVPAASKYKTFVATFGAASNKAFAE
        aa196-227  LAYKSAEGATPEAKYDAYVATLSEALRIIAGT
        aa260-291  VAATAANAAPANDKFTVFEAAFNDAIKASTGG
        aa308-339  QSYAATVATAPAVKYTVFETALKKAITAMSQA
                          5         15         25
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FIGURE 1. Homology block of domains from group 5 grass pollen allergens as predicted by MACAW software. *Lol p*5a and *Lol p*5b are isoforms of *Lol p*5 from *L. perenne; Phl p*5a, a representative isoform of *Phl p*5, is from *Phleum pratense; Poa p*9 KBG 31 is a representative isoform of *Poa p*9 from *Poa pratense.* The amino acid numbering on the left refers to each allergen domain sequence (*Lol p*5a numbering starts from the first amino acid of the mature protein; the putative signal peptide is 25 aa long). The numbering below the domains refers to the homology block amino acid frame. MACAW software averaged similarity scores over all possible pairs of residues with identical position within the homology block frame. Best matches are evidenced by boldface letters. These sequence data are available from GenBank/EMBL/DDBJ under the following accession numbers: *Lol p*5a, AF128442; *Lol p*5b, L13083; *Phl p*5a, X70942; *Poa p*9, M38342.

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 p*5a, 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 p*5a 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).

Most ligands were clustered at the C-terminal part of the 32-aa homology block, mainly between position 16 and 26. We focused our attention on ligands predicted within *Lol p*5a domains for further functional studies (see *Materials and Methods* for TEPITOPE prediction criteria). In particular, *Lol p*5a-derived peptides (peptides 7, 20, 50, and 52) were synthesized and assayed in vitro for their binding to HLA-DR molecules. Table I shows that these peptides exhibited a broad capability to bind diverse DR molecules.

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 p*5a mirrored the T cell epitopes

Table I. *DR binding assay of* Lol p5*a-derived peptides*[a]

Peptides	DRB1 * 0101 (DR1)	DRB1 * 1501 (DR2)	DRB1 * 0301 (DR3)	DRB1 * 0401 (DR4)	DRB1 * 1101 (DR5)	DRB1 * 0701 (DR7)	DRB1 * 0801 (DR8)
No. 7 (aa56-68) dkFKIFEAAFSes	3	0.4	100	22	1.6	0.5	40
No. 20 (aa109-121) daFVTALTEALrv	4	4	1.2	30	100	0.04	100
No. 50 (aa219-231) vkYAVFEAALTka	0.5	1.6	20	15	1.6	1.5	15
No. 52 (aa224-236) avFEAALTKAIta	3	8	100	55	0.9	0.03	10
HA (aa307-319) pkYVKQNTLKLat	0.2	2.9	3	2	0.6	0.22	15

[a] Synthesized *Lol p*5a-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). Peptides were tested in an ELISA competition assay for the binding to DR molecules, and compared to a known promiscuous HLA-DR ligand ($HA_{307–319}$). The DR binding is expressed as the micromolar concentration of the competitor peptide able to inhibit the binding of the biotinylated indicator peptide by 50% (IC_{50} μM).

derived from natural processing of this allergen. For this purpose, unfractionated PBMCs from two *Lol p*5-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 $CS_{380–396}$ 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 p*5. 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 5.107 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 p*5a 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.

Table II. *PBMC proliferation upon stimulation with* Lol p5*a-derived peptides and a natural allergen stimulus*[a]

Stimulus	Patient CDL	Patient ET
—	1,040 (±110)	13,304 (±622)
Peptides	9,152 (±250)	57,132 (±1,943)
Natural allergen	9,451 (±310)	70,734 (±930)

[a] PBMCs (1×10^6/ml) were stimulated with pooled peptides 7, 20, 50, and 52 (each at a concentration of 6 μM) or with the natural allergen contained in *L. perenne* pollen extract (total protein concentration of 2 μg/ml). The proliferation is reported as incorporated [^{3}H]TdR (cpm) after a 48-h stimulation. Results with SD values are representative of 50 replicates.

TEPITOPE analysis led to the identification of novel T cell epitopes. In a previous study based on traditional techniques of epitope mapping, two *Lol p*5a epitopes (amino acids 105–116 and 193–204) 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 p*5a motifs (data not shown). T cell epitopes within *Lol p*5a 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. 3*a*, peptide-specific TCCs were found to be heterogeneous in their IL-4- and IFN-γ-secreting ability 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 p*5. TCCs were then stimulated with individual peptides (concentrations ranging from 0.006 to 6 μM) presented by autologous APCs. Fig. 3*b* 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 $\geq$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 p*5-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 p*5 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

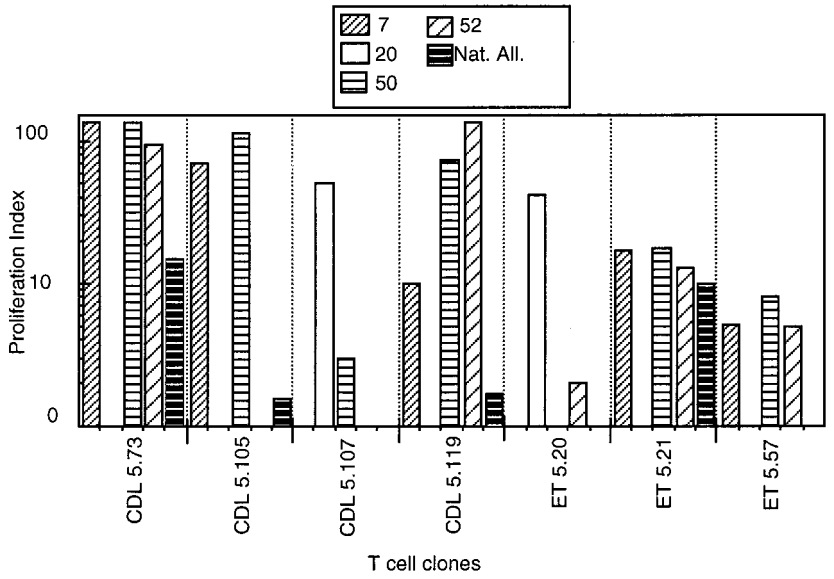


FIGURE 2. Epitope-recognition profile of TCCs. TCCs (4×10^5/ml) from ET and CDL patients were stimulated with peptides 7, 20, 50, and 52 (each peptide at a concentration of 6 μM) or with *L. perenne* pollen extract (natural allergen (Nat. All.)) (2 μg/ml total protein concentration) using autologous PBMCs (4×10^5/ml) as APCs. Cells were stimulated for 48 h, pulsed with [^{3}H]TdR, and harvested after 16 h. The TCC proliferation was calculated as the ratio of [^{3}H]TdR (cpm) in the presence of peptide stimulus to [^{3}H]TdR (cpm) in the absence of peptide stimulus.

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 ET5.21. 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 p*5a peptides. A heterologous EBV B cell line (DKB) expressing DRB4*01 (w53), but not DRB1*07, was not able to present the peptides and activate TCCs (data not shown). These data indicate that *Lol p*5a 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 and/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 p*5-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

a

TCC	IL-4 (pg/ml)	IFN-γ (pg/ml)
CDL 5.33	800	0
CDL 5.73	1500	4232
CDL 5.105	0	1194
CDL 5.107	176	0
CDL 5.109	400	2000
CDL 5.119	400	1129
ET 5.17	0	1869
ET 5.20	0	2000
ET 5.21	4600	1560
ET 5.22	0	1400
ET 5.57	0	250

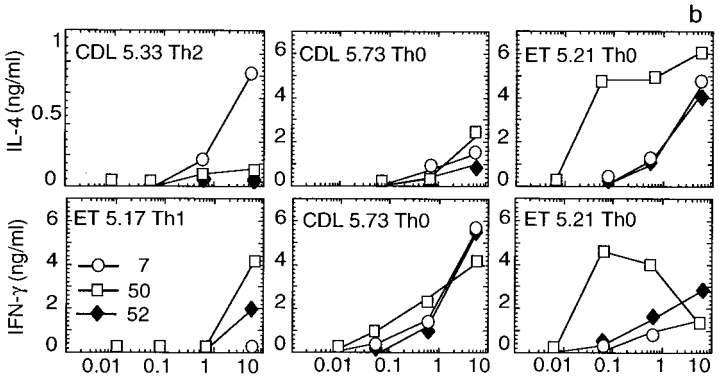


FIGURE 3. Determination of secreted IL-4 and IFN-γ. TCCs (4×10^5/ml) were stimulated with peptides 7, 50, and 52 that were either pooled (6 μM each) (*a*) or individually tested (from 0.006 μM to 6 μM) (*b*) in the presence of autologous PBMCs (4×10^5/ml) as APCs. The cytokines secreted in the culture supernatants after a 48-h stimulation were quantified by a specific sandwich ELISA.

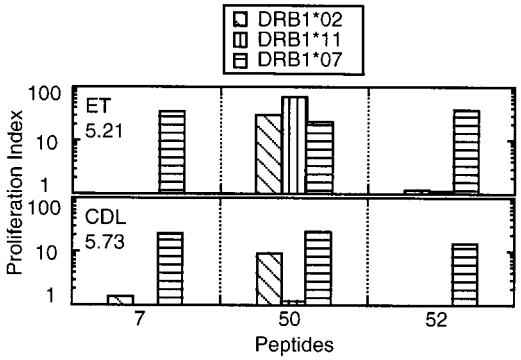


FIGURE 4. DR restriction analysis of TCCs. TCC ET 5.21 and TCC CDL 5.73 (4×10^5/ml) were stimulated with peptide 7, 50, or 52 (6 μM each peptide) using homozygous heterologous EBV-transformed B cell lines as APCs (2×10^5/ml). The EKR, SWEIG, and LD2B cell lines, respectively, express the DRB1*07, DRB1*11, and DRB1*02 molecules.

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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