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T Cell Epitope-Containing Peptides of the Major Dog Allergen Can f 1 as Candidates for Allergen Immunotherapy

Anu Immonen,²* Sandrine Farci,‡ Antti Taivainen,§ Jukka Partanen,¶ Sandra Pouvelle-Moratille,‡ Ale Närvänä,† Tuure Kinnunen,* Soili Saarelainen,* Marja Rytkönen-Nissinen,* Bernard Maillere,‡ and Tuomas Virtanen*

One prerequisite for developing peptide-based allergen immunotherapy is knowing the T cell epitopes of an allergen. In this study, human T cell reactivity against the major dog allergen Can f 1 was investigated to determine peptides suitable for immunotherapy. Seven T cell epitope regions (A–G) were found in Can f 1 with specific T cell lines and clones. The localization of the epitope regions shows similarities with those of the epitopes found in Bos d 2 and Rat n 1. On average, individuals recognized three epitopes in Can f 1. Our results suggest that seven 16-mer peptides (p15–30, p33–48, p49–64, p73–88, p107–122, p123–138, and p141–156), each from one of the epitope regions, show widespread T cell reactivity in the population studied, and they bind efficiently to seven HLA-DRB1 molecules (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*1101, DRB1*1301, and DRB1*1501) predominant in Caucasian populations. Therefore, these peptides are potential candidates for immunotherapy of dog allergy. The Journal of Immunology, 2005, 175: 3614–3620.

The problems associated with peptide-based allergen immunotherapy include the multitude of HLA class II alleles present in a population and the inherent capacity of these alleles to bind allergen peptides that are irrelevant for T cell recognition. Therefore, it is important to identify the T cell epitopes in the allergen and to assess which of them are suitable for treating a population of individuals with different HLA types. In this study, we have mapped the antigenic determinants of Can f 1, the major dog allergen, using specific T cell lines and clones and measured the binding of Can f 1 peptides to several MHC class II molecules. We observed that the epitopes of Can f 1 are clustered in few regions in the molecule. Consequently, we are able to suggest seven peptides, one from each of the epitope regions, to be used in specific immunotherapy of dog allergy. These peptides were bound by a large number of MHC class II alleles, and all subjects in the study recognized one to four of them.

Materials and Methods

Subjects

The study included 25 allergic patients whose allergy to dog was confirmed at the Pulmonary Clinic of Kuopio University Hospital, as described in detail elsewhere (12). For a person to be classified as dog-allergic, the following criteria were adopted: specific IgE by the dog UniCAP fluoroenzyme-immunometric assay (Pharmacia) of >0.7 kU/l and a skin prick test (SPT)³ with dog allergen (epithelial preparation from ALK Abello) of ≥3 mm. One of the subjects who had clinical symptoms upon exposure to dog and a positive SPT with dog allergen had no measurable IgE Ab to dog. Thirteen of the dog-allergic subjects were found to be sensitized to Can f 1 by SPTs with rCan f 1, as described previously (12). Twelve randomly selected healthy nonatopic dog owners served as control subjects. HLA class II genotyping for DRB1, DQB1, and DPB1 loci was performed at the Department of Tissue Typing, Finnish Red Cross Blood Service, Helsinki, Finland.

Can f 1 and the synthetic peptides

rCan f 1 was produced in Pichia pastoris, as described previously (12). Its 16-mer peptides, overlapping by 14 aa and covering the entire sequence, were synthesized using PerSeptive 9050 Plus automated peptide synthesizer (Millipore) with Fmoc strategy. The peptides were purified by HPLC...

³ Abbreviations used in this paper: SPT, skin prick test; SI, stimulation index; VEGP, von Ebner’s gland protein.

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Lymphocyte proliferation assays

The responses of T cell lines (5 × 10^4 cells/well) and CD4^+ T cells (2.5 × 10^5 cells/well) were tested in triplicate on 96-well round-bottom microtiter plates (Corning Scientific) with the peptides (10 μg/ml) and rCan f 1 (100 μg/ml), as described previously (10, 13). Gamma-irradiated (3000 rad) autologous PBMCs were feeder cells (3 × 10^5 cells/well) and rIL-2 (25 U/ml). From day 5 onward, the cultures were refed with fresh medium and rIL-2 (25 U/ml) at 2-3 day intervals and on day 7 with feeder cells (2–3 × 10^5 cells/well) and rIL-2. When the growth of the clones became visible (day 12–20), the cells were tested for specificity in the proliferation assay.

HLA class II peptide-binding assays

Purification of HLA-DR and HLA-DP4 molecules and peptide binding assays were performed as described previously (14–16). Briefly, HLA-DR and HLA-DP4 molecules were purified from EBV homozygous cell lines (kindly provided by Dr. C. de Toma, Centre d’Etude du Polymorphisme Humain, Paris, France) by affinity chromatography using L243 and B7/21 Abs, respectively. They were incubated with different concentrations of the competitor peptide and an appropriate biotinylated peptide as described previously. The bound peptide was eluted using L243 and B7/21 Abs, respectively. They were incubated with 50% of the labeled peptide (IC50) was calculated. Means were deduced from two to three independent experiments. To assess the validity of each experiment, unlabeled forms of the biotinylated peptides were used as reference peptides. Their IC50 variation did not exceed a factor of 3. These reference peptides were also used to take into account the disparity of the binding sensitivity between the different HLA class II molecules. For DRB1*0101, a very good binder displayed an IC50 of 1 nM, although for DRB1*0301, a very good IC50 is 300 nM. To facilitate the comparison of binding activity from one HLA class II molecule to another, we therefore expressed the data as the ratio between the IC50 of the tested peptides and that of the reference. A ratio < 100 identifies peptides with a good affinity for a given MHC class II molecule. Specifically, HA 306–318 (PKKYVKNTKLAT) was used as a reference peptide for DRB1*0101, DRB1*0401, DRB1*1101, and DRB5*0101. YKL (AAAYAKAKAAALAA), A3 152–166 (EAEQLRAY LDGTGVE), MT 2-16 (AKTIAYDEARRGLE), BI 21-36 (TERVR LVTRHYNREE), LOL 191–210 (ESWAVVRDIPDKLTGPT), and E2/E168 (AGDLLAETDKat) were used for the DRB1*0701, DRB1*1501, DRB1*0301, DRB1*1301, DRB3*0101, and DRB4*0101 alleles, respectively. Ox2 271–286 (EKKYFAATQFEPLAARL) was used as a reference peptide for HLA-DPB1*0401 and HLA-DPB1*0402 molecules.

Sequence alignments and the prediction of HLA-DR-binding ligands

For the alignment analyses, the sequential data of Can f 1 (accession no. O18875) (17), Bos d 2 (Q28133) (18), β-lactoglobulin (P02754), and Rat n 1 (P02761) were obtained from the Prosite database of the Swiss Institute of Bioinformatics (19). The amino acid sequences were aligned by the multiple sequence alignment program of the Baylor College of Medicine Search Launcher with the method ClustalW 1.8 (http://searchlauncher.bcm.tmc.edu/multi-align/Options/clustalw.html). The DR-binding peptide motifs in Can f 1 were predicted using the ProPred (20) and SYFPEITHI (21) programs.

Results

T cell epitopes of Can f 1 are clustered in few regions

As the reactivity of Can f 1-allergic and nonallergic subjects’ PBMCs to Can f 1 was weak, to obtain specific T cell lines (22), two stimulation cycles with rCan f 1 were required. To identify the epitopes of the allergen, 13 Can f 1-specific T cell lines (from 13 subjects) and 10 clones (from 7 subjects) with a sustained expansion capacity were selected. The proliferative responses of four Can f 1-specific T cell lines are shown as representative results of epitope mapping (Fig. 1). Proliferation tests with five Can f 1-specific T cell clones showed that their responses were restricted by HLA-DR (data not shown).

Results compiled in Fig. 2 show that Can f 1 contains few T cell epitopes in the molecule. The number of antigenic determinants recognized by a T cell line ranged from one to seven and was, on average, three. The length of the core sequences (the amino acids within a particular region which were shared by two to five consecutive stimulatory peptides) was 11.2 ± 0.35 (SE). The epitopes appeared to be clustered in certain regions of the molecule (Fig. 2, regions A–G). When the sequences of Can f 1 and Bos d 2 were aligned (Fig. 3), the epitope regions A, B, and F of Can f 1 were observed to colocalize with those we previously identified in Can f 1-allergic and nonallergic subjects. A summary of the epitope characteristics is given in Table I.
detected in Bos d 2 (13). In the analysis between Can f 1 and Rat n 1, the epitope regions A, C, D, and F of Can f 1 colocalized with those reported for Rat n 1 (23) (data not shown). In contrast, the epitopes recognized by human T cells on /H9252-lactoglobulin (24) did not show obvious colocalization with those of Can f 1 (data not shown).

An HLA class II allele can bind several Can f 1 peptides and one peptide can be bound by several alleles

To verify the data obtained from the epitope mapping with T cell lines and clones, the binding of Can f 1 peptides to HLA class II molecules was determined. To conform the peptide binding motifs of HLA-DR (25, 26) and HLA-DP4 molecules (16), we selected 25 peptides in the Can f 1 sequence based on the presence of an aliphatic or aromatic residue in position 3 or 4. These residues are found to be preferentially accommodated in the P1 pocket of the HLA class II binding groove and constitute the main anchor residue. Each DRB1 allele was observed to bind five to sixteen peptides strongly, while the other DRB alleles bound two to six peptides (Table I). Only two peptides bound to HLA-DP4 molecules. One of the peptides, p73–88 (in region D), was efficiently bound by all DRB alleles and by DRB5 (Table I). Almost equally broad binding capacity was seen with p9–24 (in region A), as six of seven DRB1 alleles bound it efficiently. In addition, three peptides, p15–30 (in region A), p81–96 (in region D), and p123–138 (in region F) were bound by five DRB1 alleles each. The first of these three peptides, p15–30, was also bound by DRB4 and DRB5, the second one, p81–96, by DRB5, and the last one, p123–138, by DRB3 and DP402.

Candidate peptides of Can f 1 for allergen immunotherapy

A peptide or a few peptides that are recognized by a number of individuals with different MHC class II alleles can be considered ideal for peptide-based allergen immunotherapy. In this respect, the pool of seven 16-aa long peptides of Can f 1 (p15–30, p33–48, p49–64, p73–88, p107–122, p123–138, and p141–156) appears promising because the T cells of all 15 people with various HLA genotypes recognized one to four of the peptides (Table II). At least one of the peptides elicited >90% of the maximal response in 10 of the 13 individual T cell lines. The response was 52 and 56% of the maximum in two T cell lines (data not shown). Moreover, the responses of the T cell clones from persons with T cell lines showing no reactivity to the candidate peptides were strong (SI 79 – 643) to a candidate peptide. Table II identifies the individuals showing their T cell responses to the candidate peptides and their MHC class II alleles able (or predicted) to bind the peptides. The number of candidate peptides, which the DRB1 alleles of an individual could bind, varied from three to six (Table II). For 10 of the 15 individuals in the study, the number of peptides with a potential to be bound by DRB1 alleles was at least five. These DRB1 alleles are representative of Caucasian populations because DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*1101,
Discussion

Recent studies have revealed that almost all important mammal-derived respiratory allergens are lipocalins, the one exception being Fel d 1 of cat (27, 28). Despite this, human T cell epitopes of lipocalin allergens are largely unknown. Information on their location is only available for Bos d 2, the major bovine respiratory allergen (13), Rat n 1, the major rat urinary allergen (23), and β-lactoglobulin, a milk allergen (24, 29, 30). In the present study, we have mapped the antigenic determinants of the major dog allergen, Can f 1, because knowing these determinants is a prerequisite for the development of peptide-based modes of allergen immunotherapy.

Can f 1 resembles other animal allergens in that it contains few T cell epitopes (31). These epitopes appear not to be distributed randomly in the molecule but are clustered in seven regions (A–G). Interestingly, the localization of epitope regions in Can f 1 shows similarities with those in Bos d 2 and Rat n 1. In particular, the epitope regions A, B, and F of Can f 1 colocalized with those of Rat n 1 (data not shown). However, we did not observe this kind of association between Can f 1 and β-lactoglobulin. The difference may be partly due to the fact that Inoue et al. (24) examined β-lactoglobulin with long peptides, which had a short overlap. This approach does not allow an exact determination of the epitope cores. In fact, the seven long T cell epitopes of β-lactoglobulin detected by Inoue et al. (24) cover the molecule almost completely. Another possibility for the difference between these two allergens is the route of sensitization. It is reasonable to assume that the environments in the airways and in the gastrointestinal tract differ to such an extent that it affects, for example, the way the Ag is processed. Our present results suggest that one starting point for mapping the T cell epitopes of other respiratory lipocalin allergens can be the known epitope regions of Bos d 2, Rat n 1, and Can f 1.

It is of interest that the first epitope region in Bos d 2, Rat n 1, and Can f 1 colocalizes within the structurally conserved region of lipocalins containing the signature motif G-X-W because this sequence is also present in human endogenous lipocalins (32). The sequential similarity between lipocalins is not limited to this region because, for example, Can f 1 and human tear lipocalin (von Ebner’s gland protein (VEGP)) share an overall amino acid identity of 57% (the SIB BLAST network service at the Swiss Institute of Bioinformatics, October 19, 2004). If the epitope regions A–G of Can f 1 are analyzed with ProPred at a threshold level of 10%, in 22 of the predicted HLA-binding sequences the amino acids in P1 positions are identical with those in VEGP (data not shown). Moreover, the identity between Can f 1 and VEGP along these 9-mer sequences could be up to 8 aa. These observations lead us to ask whether the similarity between endogenous and exogenous lipocalins could contribute to the quality of immune response against exogenous lipocalin allergens (33), especially as the human PBMCs and murine spleen cell responses to lipocalin allergens are weak (13, 23, 34). However, our results (Fig. 2) and the data of others (35–37) indicate that the amino acid sequence per se cannot determine the allergenicity of a protein because allergic and nonallergic people often recognize the same epitopes or regions in the molecule. One possibility to explain the Th2-deviated immune response by atopic individuals to a lipocalin allergen could be the way the allergen peptide is recognized by Th cells. For example, our present results show that the subjects did not recognize the cores of the epitopes identically, even though they could react to the same epitope region. Our previous study has shown that although two T cell clones restricted by HLA-DR4 recognized the same 16-mer peptide containing the immunodominant epitope of Bos d 2 the fine specificity of the clones was completely different (10). Interestingly, the clones recognized the epitope in a suboptimal way; when they were stimulated with a good agonist peptide, they favored the production of IFN-γ. As the weak T cell recognition has been observed to favor Th2-type responses (38–40), it is conceivable that the recognition of an epitope by the Ag TCR can contribute to the allergenic potential of a protein. Collectively, these observations suggest that the amino acid sequence of an epitope as such is not a valuable tool for predicting the qualitative outcome of a T cell response.

An attractive prospect would be to use a single peptide for the allergen immunotherapy of individuals with a variety of MHC haplotypes. To be feasible, the peptide should be promiscuous, i.e., able to bind to several MHC class II molecules (41, 42). In Can f 1, p73–88 fulfills this requirement because all seven DRB1 alleles analyzed bound it efficiently (Table I). Other peptides with almost as good binding capacity as p73–88 are p9–24, p15–30, p81–96, and p123–138. However, as observed in other forms of allergy, such as Japanese cedar pollen allergy (43), bee venom allergy (44), or cat allergy (45), a factor undermining the usability of a single peptide in allergen immunotherapy is that individual reactivity to the epitope regions of the allergen can vary considerably (Table II). Moreover, the peptides of Can f 1 bound or predicted to be bound by a MHC allele did not elicit a T cell response in all persons bearing the allele (Table II). This latter phenomenon can result from “a hole in the T cell repertoire” (46) or from the immunodominance of some epitopes of an Ag over the other upon T cell recognition.
cell response. Factors contributing to the immunodominance comprise, for example, differences in the TCR affinity for peptide-MHC complexes (47) and steps involved in the Ag processing (48) and presentation (49), including competition between peptides for binding to MHC molecules (49, 50). To overcome the problems, a reasonable presentation (49), including competition between peptides for binding at the maximum concentration of 100,000 nM.

### Table I. Binding capacities of the 16mer Can f1 peptides to HLA class II molecules

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</table>

*Binding capacity of Can f1 peptides to HLA class II molecules was determined as described in Materials and Methods. IC50 ratios are expressed as compared with IC50 values obtained with reference peptides. These peptides corresponded to the unlabeled form of the biotinylated peptides used in the assays. They exhibited the following IC50 values: DRB1*0101, 1 nM; DRB1*0301, 288 nM; DRB1*0401, 29 nM; DRB1*0701, 4 nM; DRB1*1101, 13 nM; DRB1*1301, 418 nM; DRB1*1501, 7 nM; DRB3*0101, 26 nM; DRB4*0101, 8 nM; DRB5*0101, 7 nM; DPB1*0401, 6 nM; and DPB1*0402, 11 nM. The ratios inferior to 100 are in bold; a dash means that no binding was observed at the maximum concentration of 100,000 nM.

### Table II. HLA-DRB alleles associated with a T cell response to the candidate peptides

<table>
<thead>
<tr>
<th>HLA-DRB Allele</th>
<th>Binding or Predicted to Bind the Peptide</th>
<th>HLA-DRB Allele</th>
<th>Persons with the Allele</th>
<th>Candidate Peptides with T Cell Response (Persons Responding Indicated by Capital Letters)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*0401</td>
<td>C, O</td>
<td>1*0404</td>
<td>L, N</td>
<td>O</td>
</tr>
<tr>
<td>1*1101</td>
<td>D, I, M</td>
<td>1*1301</td>
<td>F, M</td>
<td>I</td>
</tr>
<tr>
<td>4*0101</td>
<td>B, I, E, L, N, O</td>
<td>5*0101</td>
<td>A, D, G, H, J, K, N, O</td>
<td>B, E</td>
</tr>
</tbody>
</table>

* A–O, persons showing DR binding and a T cell response; +, DR binding or positive prediction for binding; –, no binding.

**Alleles were determined to bind the peptides at IC50 ratio < 100 in comparison with control peptides. For the alleles with no binding data available, binding was predicted by ProPred at a 3% threshold (B1*0401, B1*0801) or SYFPEITHI (B1*0901).
covering ~60% of Caucasians (14). The frequencies of the alleles vary from 53% (in Spain) to 82% (in Denmark). In the United States and Canada, they represent 58 and 55% of the population, respectively (51). Moreover, the T cells of two subjects who had only DRB1 alleles 0404, 0801, and/or 0901 responded to two and three of the candidate peptides, respectively. Because these alleles were predicted to bind the candidate peptides (Table II), the candidate peptide pool could cover ~85% of the Caucasian populations (51). The less polymorphic HLA-DRB molecules (DRB3, DRB4, and DRB5) are also present with high allelic frequencies in the Caucasian populations covering, on their own, 45% of the alleles (51). In the present study, their capacity to bind three of the candidate peptides suggest their involvement in the T cell responses (Table II).

In this report, we have identified T cell epitopes of the major dog allergen, Can f 1. To date, it is the third lipocalin allergen with known antigenic determinants, after bovine Bos d 2 (13) and rat Fel d 1 (10). The peptides exhibit a verified T cell reactivity, and they bind efficiently to the HLA-DRB1 molecules most commonly expressed in Caucasian populations.

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Disclosures
A. Immonen, T. Virtanen, S. Pouvelle-Moratille, and B. Maillere are authors of a patent on the use of the Can f 1 sequences described herein.

References


2. References


