

The immunodominant epitope of lipocalin allergen Bos d 2 is suboptimal for human T cells

Tuure Kinnunen¹, Cecile Buhot², Ale Närvänen³, Marja Rytkönen-Nissinen¹, Soili Saarelainen¹, Sandra Pouvelle-Moratille², Jaakko Rautiainen¹, Antti Taivainen⁴, Bernard Maillère², Rauno Mäntyjärvi¹ and Tuomas Virtanen¹

¹ Department of Clinical Microbiology, University of Kuopio, Kuopio, Finland

² Département d'Ingénierie et d'Etudes des Protéines, Commissariat à l'Energie Atomique-Saclay, Gif sur Yvette, France

³ Department of Chemistry, University of Kuopio, Kuopio, Finland

⁴ Department of Pulmonary Diseases, Kuopio University Hospital, Kuopio, Finland

We have proposed earlier that the poor capacity of the lipocalin allergen Bos d 2 to stimulate highly allergic subjects' peripheral blood mononuclear cells could be ascribed to endogenous lipocalins and could be related to the allergenic potential of the molecule. Here, we have characterized the proliferative and cytokine responses of human T cell clones against the immunodominant epitope of Bos d 2. We observed, for clone F1-9, that a substitution of aspartic acid for asparagine in the core region of the epitope increased the stimulatory capacity of the peptide about 100-fold in comparison with the natural peptide. For clone K3-2, from a different patient, the substitution of lysine for glutamine or isoleucine for leucine in the core region resulted in about 30-fold and 10-fold increases in the stimulatory capacity of the peptides, respectively. The clones also recognized self-protein-derived peptides but not the peptides derived from other lipocalins. We suggest that the poor recognition of the immunodominant epitope of Bos d 2 can be a factor accounting for Bos d 2-allergic subjects' weak cellular responses. Suboptimal recognition of self and allergen epitopes by T cells may be of significance for the allergenicity of proteins.

Key words: Allergen / Hypersensitivity / T lymphocyte / Immunity / Self tolerance

Received	12/3/02
Revised	17/2/03
Accepted	7/4/03

1 Introduction

The basis for the allergenicity of proteins has remained elusive despite the increasing amount of information on the characteristics of allergenic molecules (<http://www.expasy.ch/cgi-bin/lists?allergen.txt>). Diverse properties, such as enzymatic activity [1], have been proposed to account for allergenicity. Considering the vast repertoire of different molecules able to elicit IgE production in humans, it is unlikely that a single physicochemical factor could explain the allergenic potential of all these proteins. However, it seems obvious that proteins differ in their capacity to induce IgE production, since the human IgE response is more frequently directed to some of the proteins, *i.e.* major allergens, in an allergen source, and less frequently to minor allergens, whereas responses to other proteins are seldom seen [2]. Moreover, data suggest that the immunological characteristics of major and minor allergens differ: the immune

response against a minor allergen exhibited less the features of Th2 deviation than did the response against a major allergen [3].

Almost all animal-derived major allergens have recently been shown to belong to the lipocalin family of proteins [4]. Lipocalins are present both in humans and in animals and they mainly function as carriers of small hydrophobic molecules such as retinoids, pheromones, or odorants [5]. We have proposed that the presence of endogenous lipocalins could be a factor favoring the Th2 deviation of the immune response against exogenous lipocalin allergens [6]. We have previously observed that PBMC from patients with cow-dander-derived asthma proliferated very weakly upon stimulation with the major allergen of cow dander, Bos d 2, the stimulation indices being mainly below two [7]. The weak proliferation of human PBMC also seems to be characteristic of the responses against other animal-derived (nonlipocalin) allergens, such as cat Fel d 1 [8, 9].

One possibility accounting for the weak responsiveness is that the allergen-specific T cell repertoires do not contain T cells with high-avidity TCR, as a result of negative

[DOI 10.1002/eji.200322952]

Abbreviation: cpm: Counts per minute

selection by self peptides. Lipocalins can exhibit considerable amino acid identity between species, e.g. 57% identity between the dog major allergen Can f 1 and human tear lipocalin, 61% between the horse major allergen Equ c 1 and pig salivary lipocalin, and 43% between the food allergen Bos d 5 (β -lactoglobulin) and human glycodelin (the BLAST network service at the Swiss Institute of Bioinformatics, November 8, 2002 [10]).

We suggest here that the weak proliferation of cow-dander-asthmatic patients' PBMC upon stimulation with Bos d 2 may be affected by the low stimulatory capacity of the immunodominant epitope. To our knowledge, this is the first time it has been shown that the immunodominant epitope of an allergen is a suboptimal ligand for human T cell clones. Moreover, the T cell clones were able to recognize self-protein-derived peptides that may further modify the immune response in the periphery. These factors may be associated with the allergenicity of Bos d 2.

2 Results

2.1 The T cell response to the immunodominant epitope of Bos d 2

The proliferative PBMC responses of cow-dust-asthmatic patients with positive skin-prick-test reactions to Bos d 2 were very weak against the allergen whereas the isolated T cell clones were able to proliferate vigorously upon stimulation with Bos d 2 [7]. Fig. 1 shows the typical dose-response curves of the clones specific for the peptide 127–142 (p127–142) of Bos d 2 containing the immunodominant T cell epitope. The concentration of the peptide inducing the half-maximal response (EC_{50}) varied between 1 μ g/ml and 10 μ g/ml. As Bos d 2-specific T cell clones in general exhibit limited capacity to expand and survive over time, clones F1-9 and K3-2 with sustained expansion capacity from two patients were used in the subsequent experiments.

2.2 T cell clones F1-9 and K3-2

The T cell clone F1-9 expressed TCR-AV2, analyzed by flow cytometry [7]. In this study, characterizing the clones F1-9 and K3-2 with a panel of 24 V β -specific mAb showed that all cells expressed TCR-BV7S2 and TCR-BV13S1, respectively (data not shown). The TCR genes expressed by the clones were identified by PCR. The PCR amplification was done using a panel of TCR-subfamily-specific primers [11]. In-frame TCR- β rearrangements corresponding to V7S2DJ2S7C1 and

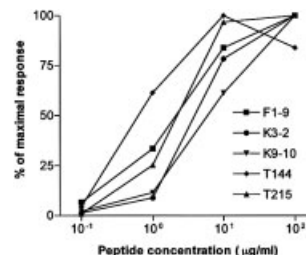


Fig. 1. Proliferative responses of Bos d 2-specific T cell clones from three cow-dust-asthmatic patients upon stimulation with p127–142 containing the immunodominant epitope of Bos d 2. The maximal responses of the clones ranged between 9200 and 34000 cpm and the medium background values between 175 and 870 cpm.

V13S1DJ1S4C2 were identified for F1-9 and K3-2, respectively (Fig. 2). For clone F1-9, an in-frame rearrangement corresponding to TCR- α V2S1J8 was identified. The clone also had an additional out-of-frame rearrangement for TCR- α . The TCR- α rearrangement of clone K3-2 could not be identified with the primer panel used. The results are in keeping with those of flow-cytometric analyses and confirmed the clonality of the clones.

2.3 The MHC restriction of clones F1-9 and K3-2

The proliferative responses of the clones F1-9 and K3-2 upon stimulation with p127–142 was inhibited by anti-HLA-DR mAb, which was not the case with anti-HLA-DP or -DQ mAb (data not shown). The genotyping of patient F's and K's DR locus revealed three alleles, DRB1*0401, DRB1*0403 and DRB4*0101. Subsequent proliferation tests using EBV-transformed homozygous cell lines for antigen presentation suggested that the response of both clones against p127–142 was restricted by DRB1*0401 (stimulation indices were >15 at 100 μ g/ml with the cell line BOLETH, and with other cell lines were <2 in two independent experiments). The finding was confirmed using the cell line T2.DR4 expressing the MHC molecule (Table 1).

Clone F1-9		V β	N β -D β -N β	J β
TCR- β	V7S2DJ2S7	LCASSQ	DPRANPR	EQYFGPGTRLTVT
Clone K3-2		V β	N β -D β -N β	J β
TCR- β	V13S1DJ1S4	VYFCAS	RARDP	EKLFFGSGTQLSVL

Fig. 2. Predicted complementarity-determining region 3 amino acid sequences of the TCR- β chains from T cell clones F1-9 and K3-2.

Table 1. Proliferation of T cell clones F1-9 and K3-2 using T2.DR4 (DRB1*0401) for antigen presentation^{a)}

Clones	Proliferation (cpm)	
	p127–142	Background
F1-9	28,046±1,134	2,380±465
K3-2	13,571±31	4,565±158

^{a)} The clones were stimulated with p127–142 at 100 µg/ml. The results are presented as mean cpm ± SEM of triplicate wells and they are representative of two independent experiments.

The binding of p127–142 and its 48 analogues to DRB1*0401 was determined (Table 2). A strong decrease in binding by the analogues Y131A and S136Y was observed, which is in keeping with the known peptide-binding motif of DRB1*0401 (Database Syfpeithi,

Table 2. Binding of p127–142 and its 48 analogues to DRB1*0401^{a)}

Peptide	IC ₅₀ ratio	IC ₅₀
p127–142	1.0	8.5±5.1×10 ⁻⁸
pY131A	82.4	7.0±2.8×10 ⁻⁶
pS136Y	70.6	6.0±1.4×10 ⁻⁶
pN135D	0.5	4.3±1.1×10 ⁻⁸
pQ133K	0.8	7.0±0.0×10 ⁻⁸
pL134I	0.8	7.0±0.0×10 ⁻⁸
All, except	0.6±0.3 ^{b)}	–
pY131A and		
pS136Y		

^{a)} The binding capacity of p127–142 and its 48 analogues to DRB1*0401 was determined as described in Sect. 4. IC₅₀ values are expressed in nM (mean±SD) and have been calculated from at least two independent experiments. IC₅₀ ratios are expressed as compared with p127–142.

^{b)} Mean±SD.

<http://www.syfpeithi.de/>), and indicates that Y131 and S136 are the MHC-binding anchor amino acids in positions P1 and P6, respectively [12]. Other amino acid substitutions did not induce great changes in binding (Table 2), the highest concentration of a peptide preventing the binding of 50% of the labeled peptide (IC₅₀) ratio (as compared with p127–142) being 1.8 (pG139Y) and the lowest 0.1 (pN142 W).

2.4 The immunodominant T cell epitope of Bos d 2 is suboptimal for clones F1-9 and K3-2

Analyses with the alanine-substituted peptide analogues of p127–142 showed that clones F1-9 and K3-2 recognized the immunodominant peptide in different ways (Fig. 3). Although the residues in the central part of the peptide (Q132, L134, N135, E137 and R138) were crucial for clone F1-9, N-terminal residues were also important for clone K3-2. A set of truncated derivatives of p127–142 was employed to determine more precisely the core regions recognized by the clones (Fig. 4). Clone F1-9 mounted a proliferative response even when the first four N-terminal or last four C-terminal amino acids were removed, suggesting that the epitope core was between Y131 and R138. Clone K3-2 was very sensitive to the removal of N-terminal amino acids, whereas three C-terminal residues could be removed without affecting the response.

In keeping with the differential recognition of the epitope by the T cell clones, it was possible to stimulate clone F1-9 with peptides p130–139 and p129–140 truncated at both termini whereas this was not the case with clone K3-2 (Fig. 4). We also observed that the response of clone F1-9 decreased as the length of these peptides decreased. Most probably the phenomenon was associated with the capacity of the peptides to bind to MHC (Fig. 4).

The proliferative responses of clones F1-9 and K3-2 were further analyzed with additional analogues of p127–142 containing conservative and nonconservative amino acid substitutions. It was observed with clone F1-9 that the substitutions for Q132, E137 and R138 were not tolerated whereas one to three peptides with substitutions for the MHC anchor amino acids Y131 and S136 and for Q133, L134 and N135 stimulated the clone (Fig. 5). Clone K3-2 resembled clone F1-9 in that it did not tolerate at all substitutions for E137 and R138, and tolerated substitutions for Y131, Q133, L134 and S136 (data not shown). Clone K3-2 differed from F1-9 in that it did not tolerate substitutions for E129 and N135, and tolerated substitutions for Q132.

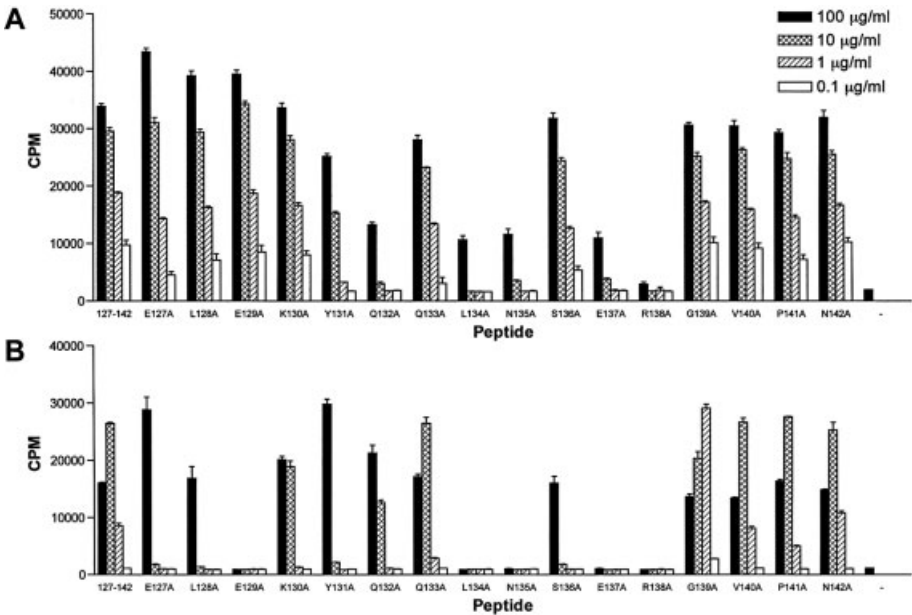


Fig. 3. Proliferative responses of the T cell clones F1-9 (A) and K3-2 (B) to the alanine-substituted peptide analogues of p127–142. The peptide concentrations from left to right are 100, 10, 1 and 0.1 µg/ml. The results are presented as mean cpm ± SEM of triplicate wells and they are representative of three independent experiments. –, medium background.

For clone F1-9, one of the peptide analogues, pN135D, was discovered to be a strong superagonist (Fig. 6). The substitution of aspartic acid for asparagine resulted in an about 100-fold decrease in the EC₅₀ of the peptide (Fig. 6). Two peptide analogues, pQ133K and pL134I, were discovered to exhibit enhanced stimulatory capacity for clone K3-2. Their EC₅₀ values were about 30-fold and 10-fold lower than that of p127–142, respectively. Because these peptide analogues bound to DRB1*0401 with similar affinities as p127–142 (Table 2), it is probable that the heteroclitic activity of the peptides was due to a more efficient recognition by the clones.

The enhanced stimulatory capacity of the superagonists was also observable in the production of IFN-γ and IL-4 by the clones (Fig. 6). Clone F1-9, which can be regarded as a Th0 clone, secreted clearly more IFN-γ when stimulated with pN135D than it did when stimulated with the natural ligand. A change in the IFN-γ:IL-4 ratio reflected this phenomenon: e.g. at a concentration of 10 µg/ml of p127–142, the ratio was 0.9 whereas at the same concentration of pN135D it was 3.7 (Fig. 6). Clone K3-2, which did not produce detectable levels of IFN-γ upon stimulation with the natural ligand at 10 µg/ml, secreted the cytokine when stimulated with the superagonists pQ133K or pL134I at the same concentration. In all experiments, the IFN-γ:IL-4 ratio was slightly higher when K3-2 was stimulated with the superagonists at

100 µg/ml compared with the ratio upon stimulation with p127–142 at the same concentration (data not shown).

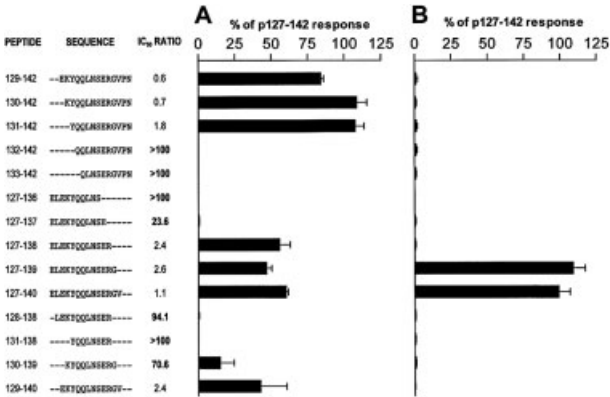


Fig. 4. Proliferative responses of the T cell clones F1-9 (A) and K3-2 (B) to the truncated analogues of p127–142. The results are presented as mean percentages ± SEM of the response against p127–142 of three independent experiments. The peptide concentration was 100 µg/ml. The proliferative response of F1-9 against p127–142 in all tests was >16200 cpm and the background value <1500 cpm. The respective values for K3-2 were >13800 cpm and <1200 cpm. The binding capacity of the analogues to DRB1*0401 was expressed as an IC₅₀ ratio in comparison with p127–142.

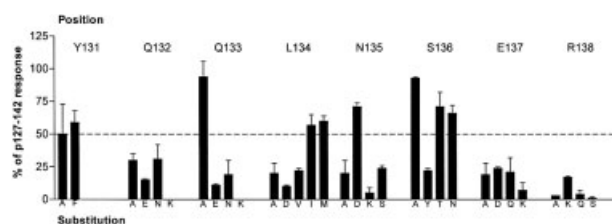


Fig. 5. Proliferative responses of the T cell clone F1-9 to the analogues of p127–142 with conservative or nonconservative amino acid substitutions. The results are presented as mean percentages $\pm$ SEM of the response against p127–142 of three independent experiments. The peptide concentration was 100 μ g/ml. The proliferative response of F1-9 against p127–142 in all tests was >20900 cpm and the background value <1700 cpm.

2.5 Allergen-specific T cell clones react against self-derived peptides

A database search motif for stimulatory ligands was formulated for clone F1-9 (Table 3) according to the results obtained with the peptide analogues (Fig. 5). The substituted amino acids in positions that were tolerated, *i.e.* allowing a proliferative response ($>50\%$ of the response against p127–142 at 100 μ g/ml) were included in the search motif whereas nontolerated substitutions were excluded. In addition, the known amino acid preferences at the two major HLA-DRB1*0401 anchor positions (P1 and P6) were included in the search motif. Based on the

Table 3. Database search motif for stimulatory ligands for the clone F1-9 (DRB1*0401)^{a)}

p127–142	E	L	E	K	Y	Q	Q	L	N	S	E	R	G	V	P	N
Search motif	X	X	X	X	<u>Y</u>	<u>Q</u>	<u>Q</u>	<u>L</u>	<u>N</u>	<u>S</u>	<u>E</u>	<u>R</u>	X	X	X	X
					<u>E</u>	<u>A</u>	<u>I</u>	<u>D</u>	<u>N</u>							
					<u>W</u>		<u>M</u>		<u>T</u>							
					<u>I</u>				<u>Q</u>							
					<u>A</u>				<u>A</u>							
Nontolerated substitutions						<u>e</u>	<u>e</u>	<u>a</u>	<u>a</u>	<u>y</u>	<u>a</u>	<u>a</u>				
						<u>a</u>	<u>n</u>	<u>d</u>	<u>k</u>		<u>d</u>	<u>k</u>				
						<u>n</u>	<u>k</u>	<u>v</u>	<u>s</u>		<u>q</u>	<u>q</u>				
						<u>k</u>					<u>k</u>	<u>s</u>				

a) Underlined upper-case letters represent the amino acids substitutions tested to be tolerated by the clone. Bold upper case letters represent known amino acid preferences at the two major HLA-DRB1*0401 anchor positions P1 and P6 (Database SYFPEITHI, <http://www.syfpeithi.de/>). Letters in lower case represent the substitutions that were not tolerated.

search in the Swiss-Prot protein sequence database, nine peptides, SP1–9, were synthesized. Peptides SP10–20, corresponding to p127–142 of Bos d 2 in other lipocalin proteins, were selected after sequence homology alignment analyses. The results are shown in Table 4.

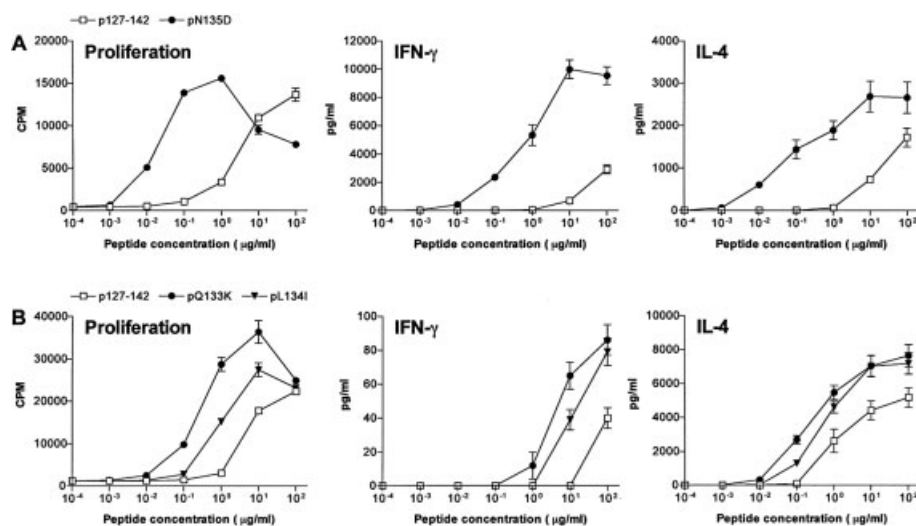


Fig. 6. Proliferative and cytokine responses of the T cell clones F1-9 (A) and K3-2 (B) upon stimulation with the natural Bos d 2 peptide p127–142 and the superagonists. The proliferative responses are presented as mean cpm $\pm$ SEM of triplicate wells and the cytokine responses as mean concentrations $\pm$ SEM of duplicate measurements. The results are representative of three independent experiments.

Table 4. Proliferative responses of the Bos d 2 (p127–142)-specific T cell clone F1–9 against the peptides obtained through database searches^{a)}

Peptide ^{b)}	Source ^{c)}	Sequence												Results (cpm)					
														100 µg/ml	10 µg/ml				
127–142	Native peptide	E	L	E	K	Y	Q	Q	L	N	S	E	R	G	V	P	N	38301±1995	14811±650
SP1	KIAA1224 (Human)	D	L	E	S	Y	L	Q	L	N	C	E	R	G	T	W	R	5893±360	1766±22
SP2	RBBP-1 (Human)	S	N	Q	C	I	Q	Q	L	T	S	E	R	F	D	S	P	1259±52	1554±94
SP3	YTRE (<i>Bacillus subtilis</i>)	V	L	E	L	I	Q	Q	L	N	R	E	R	G	I	T	F	1611±91	1632±40
SP4	5-HT-2C (Human)	P	R	G	T	M	Q	A	I	N	N	E	R	K	A	S	K	2095±13	1632±58
SP5	Nestin (Human)	V	R	L	E	L	Q	Q	L	Q	A	E	R	G	G	L	L	1430±33	1570±82
SP6	DNAE (<i>Chlamydia pneumoniae</i>)	G	K	K	D	F	Q	Q	M	E	Q	E	R	E	K	F	C	1636±111	1533±44
SP7	SPV1 ORF14 (<i>Spiroplasma citri</i>)	H	V	I	E	V	Q	Q	I	N	S	E	R	S	W	F	F	3222±104	1680±152
SP8	NF-H (Human)	Y	Q	E	A	I	Q	Q	L	D	A	E	L	R	N	T	K	2299±52	1484±44
SP9	R51H2 (Human)	T	R	L	I	L	Q	Y	L	D	S	E	R	R	Q	I	L	14175±1825	1411±30
SP10	Can f 1 (Dog)	A	L	E	D	F	R	e	F	s	R	a	k	G	L	N	Q	1383±86	1483±40
SP11	Can f 2 (Dog)	D	F	L	P	A	F	e	S	V	C	E	D	I	G	L	H	1245±11	1297±52
SP12	Equ c 1 (Horse)	I	K	E	E	F	V	k	I	V	Q	k	R	G	I	V	K	1234±95	1423±64
SP13	Bos d 5 (Cow)	A	L	E	K	F	D	k	a	L	K	a	L	P	M	H	I	1314±83	1213±75
SP14	Bla g 4 (Cockroach)	Y	N	D	K	G	k	A	F	s	A	P	Y	S	V	L	A	1196±17	1395±42
SP15	Mus m 1 (Mouse)	I	K	E	R	F	a	Q	L	C	E	E	H	G	I	L	R	1342±51	1490±39
SP16	Rat n 1 (Rat)	I	K	E	K	F	a	k	L	C	E	a	H	G	I	T	R	1446±58	1510±79
SP17	A1AG (Human)	L	G	E	F	Y	e	A	L	D	C	L	R	I	P	K	S	1397±35	1515±70
SP18	VEGP (Human)	A	L	E	D	F	e	k	a	a	G	a	R	G	L	S	T	1415±20	1422±62
SP19	APD (Mouse)	T	I	T	Y	L	k	D	I	L	T	S	N	G	I	D	I	1027±30	1414±28
SP20	NGAL (Mouse)	L	K	E	R	F	T	R	F	a	K	S	L	G	K	L	D	1514±28	1517±35

^{a)} Letters in bold represent the amino acids that fit the search motif (Table 3). Normal uppercase letters represent the amino acid substitutions in the core region that have not been tested. Letters in lower case represent the substitutions that were not tolerated by the clone. The results are presented as mean cpm ± SEM of triplicate wells. The background value was 1633±31 cpm. The results are representative of three independent experiments.

^{b)} The IC₅₀ ratios (see Sect. 4) were 26.5 for SP1, 11.2 for SP7, >100 for SP8, and 20.6 for SP9 as compared with p127–142.

^{c)} The source sequences are accessible in the SWISS-PROT database through <http://www.expasy.ch/sprot/> or <http://www.expasy.ch/cgi-bin/lists?allergen.txt>.

Five of the nine peptides (SP1, SP4, SP7, SP8 and SP9) obtained through the database search with the search motif (Table 3) induced the proliferation of F1-9 (Table 4). Four of the peptides were of human origin. Of these, the strongest stimulator was SP9, containing the superagonist substitution N135D, whereas the other human-protein-derived peptide, SP8, containing the same substitution, was only weakly stimulatory. Interestingly, clone K3-2 also recognized one of the ligands, SP4 [6084±207 counts per minute (cpm) at 100 µg/ml, medium background 899±11 cpm; representative of two independent experiments] although the search motif was based on clone F1-9. The weak proliferation induced by the self-protein-derived peptides could partly be due to their weak binding to MHC (Table 4). The lipocalin-

derived peptides (SP10–20) failed to induce proliferation of the clones (data for K3-2 not shown). This was probably due to the low overall homology of the peptides with p127–142 and the fact that they contained nontolerated amino acid substitutions.

3 Discussion

The natural immunodominant epitope of Bos d 2 allergen was found to be suboptimal for human T cell clones. Conservative amino acid changes at P5 for F1-9 and at P3 and P4 for K3-2 transformed p127–142 to a superagonist able to induce the clones to proliferate and to produce cytokines at considerably lower concentrations of

peptide compared with p127–142 (Fig. 6). Moreover, the ratio between IFN- γ and IL-4 secreted by F1-9, a Th0 clone, changed in favor of IFN- γ and K3-2, a Th2 clone, increased IFN- γ production. These phenomena are to be attributed to the recognition of the peptides by the TCR, since p127–142 and the superagonists bound with equal affinities to MHC (Table 2). This concept is also supported by the observation that a glutamine of a human collagen II peptide at P5 projected out of the peptide-binding site of DR4 (DRB1*0401) and the amino acids at P3 and P4 were also exposed [13].

Peptide analogues able to induce heteroclitic responses (stronger than by the natural ligand) have been described for T cell clones reactive against self antigens such as myelin basic protein and proteolipid protein [14, 15]. In contrast, little enhancement over the natural ligand has been seen for peptide analogues with clones specific for true foreign antigens, such as determinants in influenza virus [16]. Accordingly, it was recently reported that, for autoreactive T cell clones, the natural ligand is often suboptimal whereas for T cell clones specific for foreign antigens the natural ligand is much closer to the optimal one [17].

In fact, it has been suggested that the heteroclitic ligands would be the “real” agonists for the self-reactive T cells, whereas the self ligands would represent suboptimal partial agonists [16]. Provided that the heteroclitic response does not result from the higher affinity of the peptide for MHC, it would primarily depend on TCR. Indeed, at the level of signaling, it was observed that heteroclitic ligands induced high levels of the phosphorylated p38 form of TCR ζ and of phosphorylated ZAP-70, a pattern characteristic for a true agonist, whereas self ligands induced a predominance of p32 phospho- ζ with low or absent levels of ZAP-70 phosphorylation [16, 18].

In our previous study, the proliferative responses of cow-dander-asthmatic patients' PBMC were in general weak against Bos d 2, and the T cell lines of all six patients recognized the immunodominant epitope in p127–142 (for five patients inducing the strongest proliferative response among the Bos d 2 epitopes; two T cell lines did not recognize any other epitope) [7]. We have shown here that the EC₅₀ values of isolated Bos d 2-specific T cell clones upon stimulation with p127–142 are typically between 1 μ g/ml and 10 μ g/ml (0.5–5.0 μ M) in the proliferation assays. In this respect Bos d 2-specific clones resemble human myelin basic protein-specific T cell clones [18, 19]. Some myelin basic protein-specific clones need even higher concentrations of the peptide (EC₅₀ 8–80 μ M) for the half-maximal proliferative response [14] although lower values (EC₅₀ about 0.01 μ M) have also been reported [20]. In contrast to self

antigens, the EC₅₀ values for influenza virus hemagglutinin-specific T cell clones appear to be at the level of 0.1 μ M or below [21].

Taking into account these considerations, it can be speculated that the poor recognition of the immunodominant Bos d 2 epitope, shown to be suboptimal for clones F1-9 and K3-2, might account for the weak cellular reactivity against Bos d 2. We have previously suggested that during thymic maturation, endogenous lipocalins could have led to the elimination of the majority of T cells able to react efficiently against exogenous lipocalin allergens [6]. The remaining T cell repertoire could contain T cells that recognize lipocalin allergen epitopes in a deviant way. For example, the extent of TCR ligation by a peptide can be a factor influencing the differentiation of naive T cells into polarized phenotypes: weak stimulation was observed to favor Th2-type responses, whereas stronger stimulation favored Th1-type responses [22, 23]. In parallel with this concept, a T cell repertoire lacking high-avidity T cells for a peptide has been suggested to contribute to the Th2 predominance of the immune response [24].

Our results show that self-protein-derived peptides were able to induce proliferative responses in F1-9 and K3-2, albeit more weakly than the natural Bos d 2-derived peptide. The phenomenon was especially apparent with clone F1-9 (Table 4), for which the search motif was designed (Table 3); the clone recognized four human nonlipocalin-derived peptides. Neither one of the clones recognized the two human-lipocalin-derived peptides (A1AG and VEGP) or the nonhuman-lipocalin-derived peptides. The finding probably reflects the fact that the lipocalin-derived peptides showed lower homology with p127–142 than the nonlipocalin-derived peptides and that they contained nontolerated amino acid substitutions. Three of the stimulatory self-protein-derived peptides (KIAA1224, 5-HT-2C and NF-H) were of nervous-system origin, whereas the most stimulatory of the peptides (R51H2) originated from the DNA-repair protein RAD51 homolog 2 expressed in various human tissues, including thymus [25]. The peptide contains an aspartic acid in the same position that turned p127–142 into a superagonist. Partial agonists like the R51H2-derived peptide could be hypothesized to be involved in the positive selection of Bos d 2-specific T cells in the thymus [26].

Although it is not actually known whether the self-protein-derived peptides provoking responses in F1-9 and K3-2 are accessible to peripheral T cells *in vivo*, considering that T cell recognition has been shown to be degenerate, up to the point that no sequential homology with the original ligand is needed to activate a clone [27], it is conceivable that a number of peptides able to inter-

act with the lipocalin-allergen-specific T cell clones could be present in humans.

In fact, it seems that T cell crossreactivity between self-protein-derived and Bos d 2-allergen-derived peptides is not a unique phenomenon. It has been reported previously that the fragments and peptides of hornet venom allergen, Dol m 5, induced the proliferation of spleen cells from mice immunized with a mouse testis protein [28]. In another study, the crossreactivity between two homologous peptides, a peptide derived from the major allergen of house dust mite Der p 1 and the MHC-binding CLIP peptide, was studied in mice immunized with Der p 1 [29]. The murine CLIP peptide was observed to act as a weak agonist for the Der p 1-specific T cell lines and clones.

Although the significance of crossreactivity between exogenous allergens and endogenous proteins is not clear, the recognition of self is considered to be an essential part of immune regulation [30, 31]. Whether allergy (Th2 deviation) represents a form of a protective immune response or immune regulation upon pressure of agents resembling immunological self is not known at present [6].

We observed in this study that the immunodominant epitope of Bos d 2 allergen is a suboptimal ligand for human T cell clones, and that the T cell clones were able to recognize self-protein-derived peptides. Although the characteristics of the clones examined are parallel with the other clones we have isolated, the validity of the observations remains to be confirmed with a greater number of allergen (lipocalin)-specific clones. In this respect, studies exploiting combinatorial peptide libraries [17] most probably will provide more comprehensive data. In all, our observations raise the important question of whether the way the T cells recognize foreign proteins is involved in determining their allergenicity, *i.e.* which proteins are major or minor allergens, or nonallergenic. If the existence of heteroclitic ligands for allergen-specific T cells turns out to be a more general phenomenon, new prospects for peptide-based allergen immunotherapy may open.

4 Materials and methods

4.1 Synthetic peptides

The Bos d 2 peptide p127–142 (ELEKYQQLNSERGVNP), its 16-mer analogues and truncated derivatives were synthesized using PerSeptive 9050 Plus automated peptide synthesizer (Millipore, Bedford, MA, USA) with Fmoc strategy. The peptides were purified by HPLC and verified by mass

spectrometry. The single amino acid substitutions (conservative and nonconservative) in the peptide analogues were introduced in all 16 positions.

4.2 Allergen-specific T cell clones

The Bos d 2-specific T cell clones F1-9, T144, T215, K3-2 and K9-10 from three cow-dust-asthmatic farmers with positive skin-prick-test reactions to the allergen were isolated and propagated as described previously [7].

The TCR V β expression of the clones F1-9 and K3-2 was examined by flow cytometry as described previously [7] with a panel of 24 V β -specific mAb (IOtest Beta Mark, Beckman Coulter, Marseille, France) in combination with the CD3–PerCP mAb (Becton Dickinson, San Jose, USA).

The clonality of F1-9 and K3-2 was determined by identifying the TCR genes expressed. In brief, total RNA was isolated from the clone by the RNagents® total RNA isolation system (Promega, Madison, WI, USA) following the manufacturer's instructions. The first-strand cDNA was synthesized using TCR C α or TCR C β -specific primers (a kind gift from Dr Bernhard Hemmer, Philipps University, Marburg, Germany) following amplification with a set of primers specific for the TCR V α and TCR V β sequences as described previously [11]. The amplification products were fractionated on a 2.0% agarose gel. The amplified products were purified from agarose gels using an Ultrafree-DA Centrifugal Filter device (Millipore) and sequenced using Thermo Sequenase CY5 Dye Terminator Kit and A.L.F. Express DNA Sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden). The nomenclature for TCR V α and TCR V β sequences were used according to Arden et al. [32] and for TCR J α and J β sequences according to Rowen et al. [33].

4.3 T cell proliferation assay

T cell proliferation assays were set up in triplicates in 96-well round-bottomed microtiter plates with 2.5×10^4 T cells and 5×10^4 APC per well as described previously [7] with some modifications. An autologous EBV-transformed B cell line or other cell lines specified below were γ -irradiated (6000 rad) and were used as sources for APC. Peptides were added in cultures at several concentrations. After 72 h of incubation, [^{3} H]thymidine was added (1 μ Ci per well). After additional 16 h, the cells were harvested onto glass fiber filters and radioactivity was measured by scintillation counting.

4.4. Cytokine analysis

T cells were stimulated (5×10^5 cells/ml, 0.5 ml) with peptides in the presence of autologous EBV-transformed B cells (5×10^5 /ml). After a 24 h incubation, the supernatants were collected. Cultures without a stimulant served as negative

controls. The concentrations of IFN- γ and IL-4 were measured in duplicate using commercial ELISA kits (DuoSet, R&D Systems, Minneapolis, MN, USA) with AMDEX amplification (Amersham Pharmacia Biotech, Little Chalfont, GB) as described by the manufacturers. The detection limits of the ELISA experiments were 10 pg/ml for IFN- γ and 20 pg/ml for IL-4.

4.5 Determination of MHC restriction

The MHC element restricting the response of F1-9 and K3-2 upon stimulation with p127–142 was determined using anti-HLA-DR, -DP and -DQ monoclonal antibodies (Becton Dickinson) at several dilutions in the proliferation assay. The DR genotyping was performed in the tissue-typing laboratory of the Finnish Red Cross, Helsinki, Finland. The specific DR allele was determined by proliferation assays with a panel of cell lines as sources for APC. The EBV-transformed homozygous cell lines PITOUT (DRB1*0701, DRB4*0101) and VEU (DRB1*0403) were from the European Collection for Biomedical Research, Essen, Germany and BOLETH (DRB1*0401, DRB4*0103) was kindly provided by Dr J. Choppin (Hôpital Cochin, Paris). A T2 cell line T2.DR4 expressing the DRB1*0401 allele, kindly provided by Dr H. Zarour (Pittsburgh, USA), was used for confirming the restriction element [34].

4.6 HLA-DRB1*0401 peptide-binding assay

The HLA-DRB1*0401 molecule was purified from EBV-transformed homozygous cell line BOLETH by affinity chromatography using mAb L243 [35]. Competitive binding assays were performed as previously described [35]. Briefly, an appropriate dilution of the HLA-DRB1*0401 preparation was incubated with a biotinylated HA 306–318 peptide (30 nM) and serial dilutions of competitor peptides. After a 24-h incubation, complexes were incubated on plates coated with 10 μ g/ml L243 mAb and revealed using streptavidin-alkaline-phosphatase conjugate (Amersham Pharmacia Biotech, Little Chalfont, UK) and 4-methylumbelliferyl phosphate substrate (Sigma, France). Results were expressed as IC₅₀ values. They were based on at least two independent experiments.

4.7 Database search

The sequences of the peptides SP1–SP9 were obtained from the Swiss-Prot protein sequence database by using the program FindPatterns with a search motif described in Sect. 2. The sequences for the peptides SP10–SP20 corresponding to the p127–142 of Bos d 2 were obtained by the sequence homology alignments of lipocalin protein sequences from the Swiss-Prot protein sequence database using the program BestFit. Both programs are a part of the Wisconsin Package of the Genetics Computer Group, Inc. The peptides were synthesized as described above.

Acknowledgements: We thank Dr Bernhard Hemmer (Philipps University, Marburg, Germany) for help in establishing the PCR method for determining TCR gene expression. The skilful technical assistance of Virpi Fisk is gratefully acknowledged. The work was financially supported by Kuopio University Hospital (Project # 5021605), the Academy of Finland (Contracts # 2939 and # 48657) and the Paulo Foundation.

References

- 1 Comoy, E. E., Pestel, J., Duez, C., Stewart, G. A., Vendeville, C., Fournier, C., Finkelman, F., Capron, A. and Thyphronitis, G., The house dust mite allergen, *Dermatophagoides pteronyssinus*, promotes type 2 responses by modulating the balance between IL-4 and IFN- γ . *J. Immunol.* 1998. **160**: 2456–2462.
- 2 Ipsen, H., Nedergaard Larsen, J., Niemeijer, N. R., Löwenstein, H., Schou, C. and Spangfort, M. D., Allergenic extracts. In Middleton, E. Jr., Reed, C. E., Ellis, E. F., Adkinson, N. F. Jr., Yunginger, J. W. and Busse, W. W. (Eds.) *Allergy: Principles and Practice*. Mosby-Year Book, Inc., St. Louis 1998, pp 404–416.
- 3 Vrtala, S., Mayer, P., Ferreira, F., Susani, M., Sehon, A. H., Kraft, D. and Valenta, R., Induction of IgE antibodies in mice and rhesus monkeys with recombinant birch pollen allergens: different allergenicity of Bet v 1 and Bet v 2. *J. Allergy Clin. Immunol.* 1996. **98**: 913–921.
- 4 Virtanen, T., Zeiler, T. and Mäntyjärvi, R., Important animal allergens are lipocalin proteins: Why are they allergenic? *Int. Arch. Allergy Immunol.* 1999. **120**: 247–258.
- 5 Åkerström, B., Flower, D. R. and Salier, J. P., Lipocalins: unity in diversity. *Biochim. Biophys. Acta* 2000. **1482**: 1–8.
- 6 Virtanen, T., Zeiler, T., Rautiainen, J. and Mäntyjärvi, R., Allergy to lipocalins: a consequence of misguided T-cell recognition of self and nonself? *Immunol. Today* 1999. **20**: 398–400.
- 7 Zeiler, T., Mäntyjärvi, R., Rautiainen, J., Rytkönen-Nissinen, M., Vilja, P., Taivainen, A., Kauppinen, J. and Virtanen, T., T cell epitopes of a lipocalin allergen colocalize with the conserved regions of the molecule. *J. Immunol.* 1999. **162**: 1415–1422.
- 8 Counsell, C. M., Bond, J. F., Ohman, J. L., Greenstein, J. L. and Garman, R. D., Definition of the human T-cell epitopes of Fel d 1, the major allergen of the domestic cat. *J. Allergy Clin. Immunol.* 1996. **98**: 884–894.
- 9 Marcotte, G. V., Braun, C. M., Norman, P. S., Nicodemus, C. F., Kagey-Sobotka, A., Lichtenstein, L. M. and Essayan, D. W., Effects of peptide therapy on *ex vivo* T-cell responses. *J. Allergy Clin. Immunol.* 1998. **101**: 506–513.
- 10 Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J., Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997. **25**: 3389–3402.
- 11 Genevee, C., Diu, A., Nierat, J., Caignard, A., Dietrich, P. Y., Ferradini, L., Roman-Roman, S., Triebel, F. and Hercend, T., An experimentally validated panel of subfamily-specific oligonucleotide primers (V alpha 1-w29/V beta 1-w24) for the study of human T cell receptor variable V gene segment usage by polymerase chain reaction. *Eur. J. Immunol.* 1992. **22**: 1261–1269.
- 12 Sette, A., Sidney, J., Oseroff, C., del Guercio, M. F., Southwood, S., Arrhenius, T., Powell, M. F., Colon, S. M., Gaeta, F. C. and Grey, H. M., HLA DR4w4-binding motifs illustrate the biochemical basis of degeneracy and specificity in peptide-DR interactions. *J. Immunol.* 1993. **151**: 3163–3170.

- 13 Dessen, A., Lawrence, C. M., Cupo, S., Zaller, D. M. and Wiley, D. C., X-ray crystal structure of HLA-DR4 (DRA*0101, DRB1*0401) complexed with a peptide from human collagen II. *Immunity* 1997. **7**: 473–481.
- 14 Hemmer, B., Fleckenstein, B. T., Vergelli, M., Jung, G., McFarland, H., Martin, R. and Wiesmuller, K. H., Identification of high potency microbial and self ligands for a human autoreactive class II-restricted T cell clone. *J. Exp. Med.* 1997. **185**: 1651–1659.
- 15 Nicholson, L. B., Waldner, H., Carrizosa, A. M., Sette, A., Collins, M. and Kuchroo, V. K., Heteroclitic proliferative responses and changes in cytokine profile induced by altered peptides: Implications for autoimmunity. *Proc. Natl. Acad. Sci. U S A* 1998. **95**: 264–269.
- 16 Germain, R. N. and Stefanova, I., The dynamics of T cell receptor signaling: Complex orchestration and the key roles of tempo and cooperation. *Annu. Rev. Immunol.* 1999. **17**: 467–522.
- 17 Zhao, Y., Gran, B., Pinilla, C., Markovic-Plese, S., Hemmer, B., Tzou, A., Whitney, L. W., Biddison, W. E., Martin, R. and Simon, R., Combinatorial peptide libraries and biometric score matrices permit the quantitative analysis of specific and degenerate interactions between clonotypic TCR and MHC peptide ligands. *J. Immunol.* 2001. **167**: 2130–2141.
- 18 Hemmer, B., Stefanova, I., Vergelli, M., Germain, R. N. and Martin, R., Relationships among TCR ligand potency, thresholds for effector function elicitation, and the quality of early signaling events in human T cells. *J. Immunol.* 1998. **160**: 5807–5814.
- 19 Hemmer, B., Pinilla, C., Gran, B., Vergelli, M., Ling, N., Conlon, P., McFarland, H. F., Houghten, R. and Martin, R., Contribution of individual amino acids within MHC molecule or antigenic peptide to TCR ligand potency. *J. Immunol.* 2000. **164**: 861–871.
- 20 Hausmann, S., Martin, M., Gauthier, L. and Wucherpfennig, K. W., Structural features of autoreactive TCR that determine the degree of degeneracy in peptide recognition. *J. Immunol.* 1999. **162**: 338–344.
- 21 Korb, L. C., Mirshahidi, S., Ramyar, K., Akha, A. A. S. and Sadegh-Nasseri, S., Induction of T cell anergy by low numbers of agonist ligands. *J. Immunol.* 1999. **162**: 6401–6409.
- 22 Janssen, E. M., van Oosterhout, A. J. M., van Rensen, A. J. M. L., van Eden, W., Nijkamp, F. P. and Wauben, M. H. M., Modulation of Th2 responses by peptide analogues in a murine model of allergic asthma: Amelioration or deterioration of the disease process depends on the Th1 or Th2 skewing characteristics of the therapeutic peptide. *J. Immunol.* 2000. **164**: 580–588.
- 23 Brogdon, J. L., Leitenberg, D. and Bottomly, K., The potency of TCR signaling differentially regulates NFATc/p activity and early IL-4 transcription in naive CD4+ T cells. *J. Immunol.* 2002. **168**: 3825–3832.
- 24 Murray, J. S., How the MHC selects Th1/Th2 immunity. *Immunol. Today* 1998. **19**: 157–163.
- 25 Rice, M. C., Smith, S. T., Bullrich, F., Havre, P. and Kmiec, E. B., Isolation of human and mouse genes based on homology to REC2, a recombinational repair gene from the fungus *Ustilago maydis*. *Proc. Natl. Acad. Sci. U S A* 1997. **94**: 7417–7422.
- 26 Evavold, B. D., Sloan-Lancaster, J. and Allen, P. M., Tickling the TCR: selective T-cell functions stimulated by altered peptide ligands. *Immunol. Today* 1993. **14**: 602–609.
- 27 Hemmer, B., Vergelli, M., Gran, B., Ling, N., Conlon, P., Pinilla, C., Houghten, R., McFarland, H. F. and Martin, R., Cutting edge: Predictable TCR antigen recognition based on peptide scans leads to the identification of agonist ligands with no sequence homology. *J. Immunol.* 1998. **160**: 3631–3636.
- 28 King, T. P. and Lu, G., Hornet venom allergen antigen 5, Dol m 5: Its T-cell epitopes in mice and its antigenic cross-reactivity with a mammalian testis protein. *J. Allergy Clin. Immunol.* 1997. **99**: 630–639.
- 29 Kristensen, N. M., Hoyne, G. F., Hayball, J. D., Hetzel, C., Bourne, T. and Lamb, J. R., Induction of T cell responses to the invariant chain derived peptide CLIP in mice immunized with the group 1 allergen of house dust mite. *Int. Immunol.* 1996. **8**: 1091–1098.
- 30 Cohen, I. R. and Young, D. B., Autoimmunity, microbial immunity and the immunological homunculus. *Immunol. Today* 1991. **12**: 105–110.
- 31 Janeway, C. A. Jr., How the immune system works to protect the host from infection: a personal view. *Proc. Natl. Acad. Sci. U S A* 2001. **98**: 7461–7468.
- 32 Arden, B., Clark, S. P., Kabelitz, D. and Mak, T. W., Human T-cell receptor variable gene segment families. *Immunogenetics* 1995. **42**: 455–500.
- 33 Rowen, L., Koop, B. F. and Hood, L., The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. *Science* 1996. **272**: 1755–1762.
- 34 Turvy, D. N. and Blum, J. S., Detection of biotinylated cell surface receptors and MHC molecules in a capture ELISA: a rapid assay to measure endocytosis. *J. Immunol. Methods* 1998. **212**: 9–18.
- 35 Texier, C., Pouvelle, S., Busson, M., Herve, M., Charron, D., Menez, A. and Maillere, B., HLA-DR restricted peptide candidates for bee venom immunotherapy. *J. Immunol.* 2000. **164**: 3177–3184.

Correspondence: Tuomas Virtanen, Department of Clinical Microbiology, University of Kuopio, P.O.B. 1627, FIN-70211 Kuopio, Finland
 Fax: +358-17-162705
 e-mail: tuomas.virtanen@uku.fi