

T Cell Epitopes of a Lipocalin Allergen Colocalize with the Conserved Regions of the Molecule¹

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In this study we characterized the human T cell-reactive sites of the major cow dander allergen, Bos d 2, a member of the lipocalin protein family. We showed that Bos d 2 contains only a limited number of epitopes. This is in contrast to many other allergens, which usually contain multiple T cell epitopes throughout the molecule. The epitopes of Bos d 2 were primarily concentrated in the conserved regions of the molecule. One of the epitopes was recognized by all the cow-asthmatic individuals regardless of their HLA phenotype. Computer-predicted T cell epitopes on Bos d 2, other lipocalin allergens, and human endogenous lipocalins were situated in similar locations on these molecules and corresponded to experimentally identified epitopes on Bos d 2. The results suggest that human endogenous lipocalins could be involved in the modulation of immune responses against exogenous lipocalin allergens. In addition, our findings are likely to facilitate the development of new forms of immunotherapy against allergies induced by the important group of lipocalin allergens. *The Journal of Immunology*, 1999, 162: 1415–1422.

The characterization of allergens at the molecular level is progressing rapidly (1). This progress has important practical ramifications because detailed information regarding the molecular structure of allergens is necessary for the systematic development of new preparations for allergen immunotherapy. Because T cells play a central role in regulation of the immune system (2, 3) it seems reasonable to select them as targets for immunomodulatory measures (4).

Several major animal allergens belong to the group of proteins called lipocalins. These include the major urinary proteins of mouse and rat, Mus m 1 (5, 6) and Rat n 1 (6, 7), the bovine allergen Bos d 2 (8, 9), the canine allergens Can f 1 and Can f 2 (10), and the food allergen β -lactoglobulin, Bos d 5 (11, 12). In addition, a cockroach allergen, Bla g 4, is known to be a lipocalin (calycin) (13). Lipocalins function as carrier molecules in the transport of hydrophobic ligands. They have been identified in the body fluids of numerous species, including humans (6, 14, 15). Molecular analyses have revealed varying degrees of sequence homology (10–20%) and structural similarity between lipocalins (14, 16). The kernel (or core) lipocalins possess three short structurally conserved regions, one of which contains the pattern -G-x-W shared by all lipocalins (14, 17, 18).

The finding that certain important (aero)allergens share a common molecular background may offer a way to approach the basic question of the molecular determinants of their allergenicity. However, knowledge on the immunological properties of lipocalin allergens is still limited. As triggers of immediate allergic reactions,

lipocalins are known to bind IgE both in vitro and in vivo (13, 19, 20). The regions in Bos d 2 and Bos d 5 most important for IgE binding appear to be located in the carboxy-terminal portion of the molecules (19, 21, 22).

Even less is known about the interactions of lipocalin allergens with the cellular compartment of the immune system than about their Ab binding. We have reported previously that in proliferation tests employing PBMCs, affinity-purified Bos d 2 distinguishes more accurately cow-asthmatic patients from healthy controls than does crude cow dander extract (23). We subsequently demonstrated that recombinant fragments of Bos d 2 with reduced IgE binding capacity are effective stimulators of Bos d 2-specific T cell clones (19). No information on other lipocalin allergens is available. To our knowledge, the only mammalian allergen against which cellular reactivity has been examined in detail is the major cat allergen, Fel d 1 (24, 25).

The purpose of this study was to characterize human T cell reactivity to the lipocalin allergen Bos d 2, the predominant allergen in cow dander (26). We observed that several T cell epitopes of Bos d 2 overlapped the structurally conserved regions of the molecule. Together with computer predictions for lipocalins, this points to the possibility that the response to exogenous allergenic lipocalins may be modulated by the presence of endogenous lipocalins.

Materials and Methods

Subjects

Twenty-three cow-asthmatic patients were included in the study. The bovine origin of their asthma was confirmed at the Pulmonary Clinic of Kuopio University Hospital as described in detail elsewhere (23). For a person to be classified as being asthmatic to cow-derived material, the inhalation test, skin prick test (cow allergen preparations from ALK, Denmark) and radioallergosorbent test (RAST; cow allergen; Pharmacia Biotech, Uppsala, Hørsholm, Sweden) had to be positive. Sensitization to Bos d 2 was confirmed by skin prick tests with the highly purified allergen, as described below. HLA-DR/DQ expression was determined by a standard complement-dependent microlympho-cytotoxicity test using commercial antisera (Biotest, Dreieich, Germany). HLA-DR-positive cells were enriched using microbead separation (Dynal, Oslo, Norway).

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Skin prick test

Skin prick tests were performed according to Nordic recommendations (27) in duplicate on the backs of cow-asthmatic patients, using five 10-fold dilutions of native (n)³ Bos d 2 in concentrations up to 100 µg/ml. Histamine (10 mg/ml) and diluent (PBS) were included as positive and negative controls. After 15 min, the wheals were marked and documented by direct tracing onto strips of tape. Wheal diameters were calculated using the formula: $(d_{\max} + d_{\min})/2 = d_{\text{mean}}$.

Allergen preparations and determination of amino acid sequences

nBos d 2 was purified from commercial raw material (Allergon, Ängelholm, Sweden) by affinity chromatography and gel filtration, as described previously (23).

The procedure for cDNA cloning and sequencing of major cow dander allergen has been described in detail elsewhere (8). In brief, the clone Pot12, corresponding to the Bos d 2 allergen, was isolated from the cDNA library of cow skin by immunoscreening with serum from a cow-asthmatic patient. Further screening of the cDNA library was done with a DNA probe obtained from a preliminary positive plasmid. Nucleotide sequencing was performed with the automated laser fluorescent (ALF) DNA sequencer using an Auto Read kit (Pharmacia Biotech).

rBos d 2 and its fragments were produced as fusion proteins in the *Escherichia coli* strain TG10B using the glutathione S-transferase (GST) Gene Fusion System according to the manufacturer's instructions (Pharmacia Biotech). The cloning vector for expression was pGEX2T, which expresses sequences fused to the carboxy terminus of the GST protein from *Schistosoma japonicum* (28). Numbering of the amino acids was initiated at the first amino acid of the mature protein, excluding the 16-amino acid leader sequence (our unpublished results). The rBos d 2 (1–156) expression plasmid (pGEX2T-POT) was constructed as described previously (8). The expression plasmid of the fragment rBos d 2 (1–115) was produced using the *StuI* and *EcoRI* restriction sites of the pGEX2T-POT plasmid according to standard procedures (29). The rBos d 2 (65–156) insert was generated using the PCR technique with Bos d 2-specific primers (the 5' primer, TCTGGATCCCTGTTGCTCACAGAAGTGG, and the 3' primer, CGATGAATCTTATGGAGGACAATTGTCTG). The primers, which included the 5' (*BamHI*) and 3' (*EcoRI*) cloning sites for the pGEX2T expression vector, were used in PCR with the clone Pot12 to produce a DNA fragment encoding the sequence of the carboxy-terminal fragment of Bos d 2 (corresponding amino acids 65–156).

All protein concentrations were determined by the method of Bradford (30) using the commercial Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Sterile-filtered preparations were stored either at 4°C or frozen at –70°C.

Synthetic peptides

The 16-mer peptides overlapping by 14 amino acid residues and covering the Bos d 2 sequence were synthesized by Fmoc (*N*-[9-fluorenyl]methoxycarbonyl) chemistry on a simultaneous multiple-peptide synthesizer (SMPS 350; Zinsser Analytic, Frankfurt, Germany). The peptides were desalted by gel filtration and purified by reversed-phase HPLC. The correct sequences were confirmed by mass spectrometry. The lyophilized peptides were reconstituted in PBS and sterile-filtered or γ -sterilized before storage at –70°C. Thirteen of the 71 synthesized peptides could not be tested because they proved to be insoluble in PBS. Therefore, in two cases the overlap was 8 amino acids, in two other cases 10 amino acids, and in three cases 12 amino acids.

Lymphocyte proliferation assays

PBMC were separated from the heparinized peripheral blood of 23 cow-asthmatic patients by Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient centrifugation, as described elsewhere (23). The Ag-specific proliferation tests using PBMCs, T cell lines, and clones were performed as follows. The cells were seeded out and stimulated in triplicate at densities of 10^5 cells/well (PBMC) or 5×10^4 cells/well (T cell lines or clones) in the wells (0.2 ml) of round bottomed 96-well microtiter plates (Corning Glass, Corning, NY). For the Ag stimulants used in the study, nBos d 2, tetanus toxoid (TET), rBos d 2, and rBos d 2 fragments, the optimal concentrations were predetermined to be 50 µg/ml. Peptides were used at a concentration of 10 µg/ml. Culture medium was RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 2 mM L-glutamine, 20 µM

2-ME, sodium pyruvate (Life Technologies), nonessential amino acids (Life Technologies), 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES (Life Technologies), and 5% inactivated human AB serum (Finnish Red Cross, Helsinki, Finland). For testing the T cell lines and clones, γ -irradiated (60 Gy) autologous PBMCs were added as APCs at a density of 10^5 cells/well. Cultures were incubated for 5 days (PBMCs) or 3 days (T cell lines and clones) in a humidified 5% CO₂ incubator at 37°C, then pulsed for 16 h with 0.5 mCi of [³H]thymidine per well (specific activity, 2.0 Ci/mmol; Amersham Pharmacia Biotech, Little Chalfont, U.K.). Radioisotope uptake was measured by scintillation counting, and the results expressed as a stimulation index (SI: ratio between the mean cpm in cultures stimulated with APCs plus Ag, and the mean cpm in cultures without Ag). T cell lines and clones were allowed to rest a period of 2 wk or longer before testing with Ag. Stimulation indices of ≥ 2 and ≥ 5 in T cell lines and cloned T cells, respectively, were regarded as positive responses.

T cell lines and clones

For the generation of Bos d 2-specific T cell lines, the PBMCs were cultivated in 24-well plates (Corning) at a density of 1.5×10^6 cells/well in complete RPMI 1640 medium containing nBos d 2 (50 µg/ml). On day 6, human rIL-2 (CLB, Amsterdam, The Netherlands) was added to a final concentration of 5 IU/ml. The rIL-2 concentration was optimized to 25 IU/ml on day 9. On day 14, blasts were separated by density gradient centrifugation and restimulated with nBos d 2 at a density of 10^6 cells/well in 24-well plates. Autologous γ -irradiated PBMCs were added as APCs at a density of 2×10^6 cells/well. The cycle was repeated as described above. The established T cell lines were expanded and restimulated at 2-wk intervals with PHA (11.3 µg/ml) plus rIL-2 (25 IU/ml) or with Bos d 2 in the presence of γ -irradiated autologous PBMCs, as described above. The rIL-2 concentration was adjusted to 25 IU/ml at 3-day intervals.

T cell clones were isolated from Ag-reactive T cell lines by the limiting dilution method, as reported previously (31). The T cells were seeded out into the wells of round bottomed 96-well microtiter plates (Corning) at concentrations of 0.3 and 1 cells/well, with γ -irradiated allogeneic PBMCs as feeder cells (3×10^5 cells/well), PHA (11.3 µg/ml), and rIL-2 (25 IU/ml). Cultures were then refed weekly with 2×10^5 cells/well plus rIL-2 (25 IU/ml), and twice a week with rIL-2 (25 IU/ml) alone. When the growth of the clones became visible (days 12–20), the cells were expanded with PHA, feeder cells, and rIL-2.

The phenotype of the T cell clones and lines was examined by flow cytometry on a FACScan machine (Becton Dickinson, Mountain View, CA) using the CD4FITC + CD8PE + CD3PerCP reagent (Becton Dickinson). The TCR V α /V β elements were stained using the α /V screening panel (T Cell Diagnostics, Woburn, MA), that contains FITC-conjugated murine Abs to the TCR elements V β 3.1, V β 5.2/5.3, V β 5.3, V β 5.1, V β 6.7, V β 8, V β 12, V β 13.1/13.3, V α 2, and V α 12.1. The staining was combined with the CD4PE or CD4PerCP reagents (Becton Dickinson).

Induction and measurement of cytokine production

Thirty-eight Bos d 2-specific T cell clones were stimulated with PMA (10 ng/ml; Sigma, St. Louis, MO) in the wells of anti-CD3 mAb-coated flat-bottom 96-well microtiter plates (Corning) at a density of 2×10^5 cells/well in a volume of 200 µl. The culture medium was supplemented with 10% FCS (Biological Industries, Beit Haemek, Israel) instead of AB serum. Wells were precoated with the anti-CD3 mAb by incubation for 1 h at room temperature with mouse hybridoma (OKT3, ATCC, CRL-8001) ascites fluid (kind gift of Dr. Matti Kaartinen, Helsinki, Finland), at the predetermined dilution of 1:20,000 in serum-free culture medium. For negative controls, cells were incubated in uncoated wells without PMA. After an incubation period of 24 h (for the production of IL-2) or 40 h (for the production of IL-4, IL-5, and IFN- γ), culture supernatants were collected and stored in aliquots at –70°C until examined.

To measure the IL-2 produced by the T cell clones, 0.2 ml of supernatant was added at different dilutions (1:2 to 1:8) to 4×10^3 indicator cells (CTLL-2 murine cell line) as previously described (31, 32). A semiquantitative estimate of IL-2 production was determined using a standard curve of rIL-2 (CLB). For the measurement of IL-5, the murine LyH7.B13 cell line was used as a source of indicator cells (kind gift of Dr. R. Palacios, Basel, Switzerland). A semiquantitative estimate of IL-5 production was obtained using a standard curve of human IL-5 (Immugenex, Los Angeles, CA) (31, 33). The quantitative determinations of IFN- γ and IL-4 (pg/ml) were performed in duplicate by commercial ELISA kits (Duoset human IL-4; Genzyme, Cambridge, MA, and PeliKine-compact human IFN- γ , CLB) according to the manufacturers' instructions.

As virtually all the clones produced at least low levels of different cytokines upon stimulation, an arbitrary cut-off level was set at 15% of the highest measured level of each cytokine. This cut-off level allowed the

³ Abbreviations used in this paper: n, native; TET, tetanus toxoid; SI, stimulation index; Q, quartile deviation.

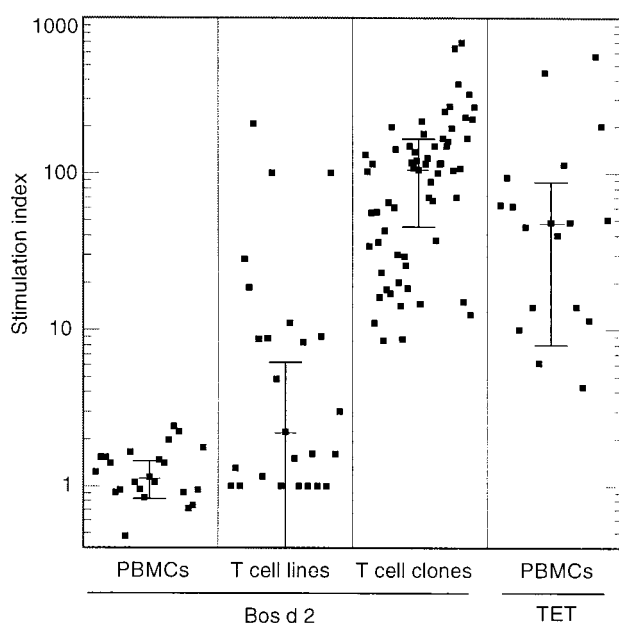


FIGURE 1. Proliferative responses of PBMCs and T cell lines of cow-asthmatic farmers against Bos d 2 or TET Ag. The median and quartile deviations are shown.

most clear-cut classification of the Th subsets. Thus, the clones producing IL-4 and/or IL-5 above the cut-off level and IL-2 and IFN- γ below the cut-off were classified as “Th2-like” clones, whereas the clones producing IL-2 and/or IFN- γ above the cut-off level and IL-4 and IL-5 below the cut-off were classified as “Th1-like” clones. Clones producing both types of cytokines above the cut-off levels were classified as “Th0-like” clones.

Sequence data and the prediction of T cell epitopes

Sequence data for the lipocalin proteins Mus m 1, Rat n 2, Equ c 1, von Ebner gland protein (VEGP), apolipoprotein D (Apo D), α -1 acid glycoprotein precursor (A1AG), and retinol-binding protein (RBP) were obtained from the Prosite database of the ExPASy molecular biology server of the University of Geneva (34). The sequences of Can f 1 and Can f 2 have been reported by Konieczny et al. (10). The amino acid sequences were aligned using the multiple sequence alignment program of the Baylor College of Medicine Search Launcher with the method ClustalW 1.7 (DNA protein). The locations of possible T cell epitopes were predicted for different lipocalins using the T-site program (35) for Macintosh computer with the searching algorithm for α -helical periodicity and amphipathicity and a window size of 7 amino acids.

Results

Cellular response to Bos d 2

In the first set of experiments, PBMCs from 23 patients with clinically verified cow asthma were tested in proliferation assays employing highly purified nBos d 2. Proliferative responses of PBMCs were generally low, with SI values ranging from 0.5 to 2.4 (median, 1.14 ± 0.3 quartile deviation (Q); Fig. 1). The cpm values ranged from 165 to 1832 (539 ± 423 , mean $\pm$ SD) and background values from 135 to 1758 (464 ± 381). This finding was in contrast to the positive results of the skin prick tests (mean diameter, 6.5 ± 1.9 mm; SD) with the nBos d 2 allergen (100 μ g/ml). Nonresponsiveness in the proliferation tests was characteristic of Bos d 2. Proliferative responses to the control Ag TET were vigorous (Fig. 1).

To further analyze the Bos d 2-specific T cell reactivity, 25 T cell lines were generated from the peripheral blood of 18 cow-asthmatic patients. The proliferative responses of T cell lines against Bos d 2 were measured after two to three enrichment cycles. A substantial increase in responsiveness was achieved, but

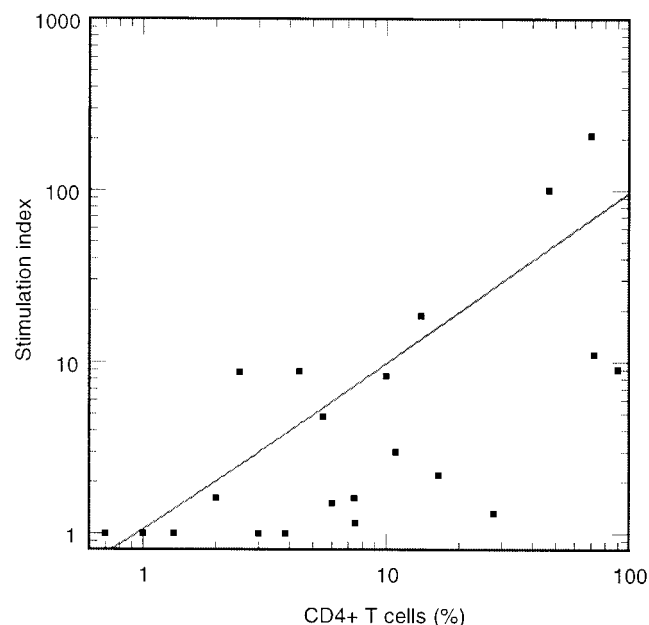


FIGURE 2. Proliferation response of Bos d 2-induced T cell lines from cow-asthmatic farmers plotted against the percentage of viable CD4⁺ T cells. The regression line is shown (Spearman rank correlation, $r = 0.742$, $p < 0.001$).

the majority of T cell lines (56%) still exhibited low reactivity against Bos d 2, with SI values below 3 (median, 2.2 ± 3.95 Q; Fig. 1). Repeated stimulation with Bos d 2 was observed to favor the accumulation of CD8⁺ T cells in the cultures. As shown in Fig. 2, proliferative responsiveness correlated with the proportion of CD4⁺ T cells in the lines (Spearman rank correlation, $r = 0.742$, $p < 0.001$).

Sixty-three Bos d 2-specific T cell clones were isolated from the T cell lines of five patients. These clones were of the CD4⁺ phenotype and responded vigorously upon stimulation with nBos d 2 (median, 105 ± 60 Q; range, 8.5–692; Fig. 1). Five additional T cell clones of the CD8⁺ phenotype were derived from the T cell lines of two patients and analyzed for specificity. They proved unresponsive to Bos d 2 in the proliferation assays (data not shown) and were not characterized further.

Cytokine production by Bos d 2-specific T cell clones

Cytokine production was measured from 38 Bos d 2-specific T cell clones. Almost all of them produced measurable amounts of the cytokines IL-4 (10.6 ± 12.0 ng/ml; mean $\pm$ SD), IL-5 (39.6 ± 25.1 pg/ml), IL-2 (2.5 ± 9.4 IU/ml), and IFN- γ (18.6 ± 29.6 ng/ml) upon stimulation with anti-CD3 Ab plus PMA. Unstimulated clones did not produce any detectable cytokine (data not shown). Fifty-five percent of the clones (21/38) were classified as

Table 1. Th phenotypes of the Bos d 2-specific T cell clones according to their cytokine production pattern

Clones	Th1-like	Th0-like	Th2-like
Patient 1 (n = 5)	0	2	3
Patient 2 (n = 5)	1	2	2
Patient 3 (n = 7)	1	3	3
Patient 4 (n = 13)	1	6	6
Patient 5 (n = 8)	0	1	7
Total (n = 38)	3	14	21

Table II. *Th phenotypes of the T cell clones with known epitope specificities according to their cytokine production pattern*

Clones	Th1-like	Th0-like	Th2-like
Reactive to epitope G ^a (n = 17)	0	7	10
Reactive to other epitopes (n = 6)	3	2	1
Total (n = 23)	3	9	11

^a Epitope regions mentioned are shown in Fig. 3.

Th2-like clones because of a predominant IL-4 and/or IL-5 production exceeding the cut-off values of 6.9 ng/ml and 18.2 pg/ml, respectively, and of low IL-2 and IFN- γ production below the cut-off values of 8.6 IU/ml and 16.9 ng/ml (Table I). A considerable number of the clones secreted all of these cytokines above the cut-off values and were therefore classified as Th0-like clones (14/38; 37%). A minority of the clones (3/38; 7.9%) produced predominantly the Th1-related cytokines IL-2 and/or IFN- γ .

Table II shows that all the 17 T cell clones reactive to epitope G (see Fig. 3) were classified as Th2-like or Th0-like clones while four of the six clones reactive to the other epitopes were Th1-like. There was no difference between the subsets in the magnitude of the proliferative responses (data not shown).

T cell epitopes of Bos d 2

As a first step for localizing the T cell reactive regions in Bos d 2, 42 Ag-specific T cell clones were tested with the two overlapping recombinant fragments of the allergen. All the clones with the exception of one clone from patient no. 1 responded exclusively to only one or the other of the fragments. In the case of positive responses, the SI values for the fragment (1–105) ranged from 13 to 375 (median, 65 ± 32 Q), and the values for the fragment (65–156) from 11 to 82 (median, 26 ± 20 Q). When the responses were negative, the SI values remained below 1.5. This finding suggested that the overlapping region (amino acids 66–104) played a minor role as a T cell epitope region. Two regions, one at the amino-terminal portion (amino acids 1–65) and the other at the carboxy-terminal portion (amino acids 105–156), seemed to contain the most important T cell epitopes of Bos d 2.

A detailed epitope mapping of Bos d 2 was performed using a set of overlapping 16-mer peptides as described in *Materials and Methods*. The results obtained with the T cell lines of six patients are compiled in Fig. 3. The core sequences of the epitopes, defined as those amino acids within a particular region shared by two to five consecutive peptides capable of stimulating a T cell line, are shown as bars above the Bos d 2 sequence. A total of seven different epitopes, designated from A to G, were detected. The lengths of their core sequences ranged from 8 to 14 amino acids. Each individual T cell line reacted against from one to five epitopes. The epitopes recognized by the T cell clones corresponded to those recognized by the T cell lines (Table III).

Epitope G at the carboxy-terminal end was recognized by the T cell lines of all six patients and by the majority of the tested T cell clones (17/23). Peptides localizing within this region provoked the highest stimulation indices of the T cell lines of five patients (data not shown). The T cell lines of two patients did not detect any other epitope (Fig. 3). One single peptide with the sequence ELEKYQQLNSERGVN was recognized by all the T cell lines as well as by the epitope G-specific T cell clones.

The core sequences of epitope B were identical for all three of the T cell lines and all four of the T cell clones reacting to it. In other epitopes, the location of the core sequences varied by a few

Table III. *Reactivity of T cell clones against recombinant fragments and peptides of Bos d 2*

Patient	No. of Clones	Fragment Reactivity	Core Sequence of Reactive Peptides	Corresponding T Cell Line Epitope ^a
1	1	[1–105]	[41–48]	B
	7	[1–105]	NT ^b	A, B, C, or E ^c
	1	Both	NT	E ^c
2	2	[1–105]	[41–48]	B
	1	[65–156]	[131–138]	G
	2	[65–156]	[127–140]	G
3	1	[1–105]	[41–48]	B
	1	[65–156]	[131–140]	G
	1	[65–156]	NT	G ^c
4	1	[1–105]	[13–24]	A
	12	[65–156]	[131–138]	G
	12	[65–156]	NT	F or G ^c

^a See Fig. 3.

^b NT, not tested.

^c Deduced from fragment reactivity.

amino acids between individuals and also to some extent between the clones of an individual patient.

Prediction and structural association of T cell epitopes in lipocalins

Bos d 2, some other lipocalin allergens, and randomly selected human endogenous lipocalins were analyzed for T cell epitopes by the computer program T-site (Fig. 3). Five of the epitopes predicted by the program for Bos d 2 colocalized with the empirically verified epitopes A, B, E, F, and G. Correspondence was best with epitopes B, F, and G. Epitopes A, E, F, and G were roughly localized within the structurally conserved regions of the lipocalins as defined by Flower (14). Epitope G, the dominant epitope of Bos d 2, was especially interesting. The empirically identified Bos d 2 epitopes, the computer-predicted epitopes for the lipocalin allergens and for four human endogenous lipocalins, as well as the carboxy-terminally situated conserved region of lipocalins were all found overlapping or adjacent to each other within this site.

TCR V α /V β analysis of Bos d 2-specific T cells

To examine whether the T cell reactivity, which was weak and focused against a few regions in Bos d 2, would reflect the oligoclonality of the immune response against Bos d 2, the usage of TCR V α and V β elements of the Bos d 2-specific T cell lines was determined. In most T cell lines, the frequency of CD4⁺ T cells bearing particular TCR V α /V β elements was 2- to 8-fold higher than in T cells from peripheral blood (Table IV). This was most obvious in those T cell lines highly responsive to Bos d 2 (patients 1, 2, and 4; SI, 28, 100, and 9, respectively). However, the dominant TCR elements showed considerable interindividual variation. The T cell lines of two patients with the HLA-DR4/53 phenotype contained elevated numbers of V β 6.7-positive T cells.

Each T cell clone expressed a single type of TCR V α /V β element, indicating pure clonality of the cultures, with the exception of a few clones (6/23) that could not be stained with any of the mAbs used.

HLA-DR/DQ phenotype of the patients

HLA-DR/DQ phenotype analysis was performed for those patients included in the epitope mapping study. As shown in Table V, the patients expressed different HLA-DR/DQ alleles. In four of the six patients examined, only one HLA allele was detected, suggesting

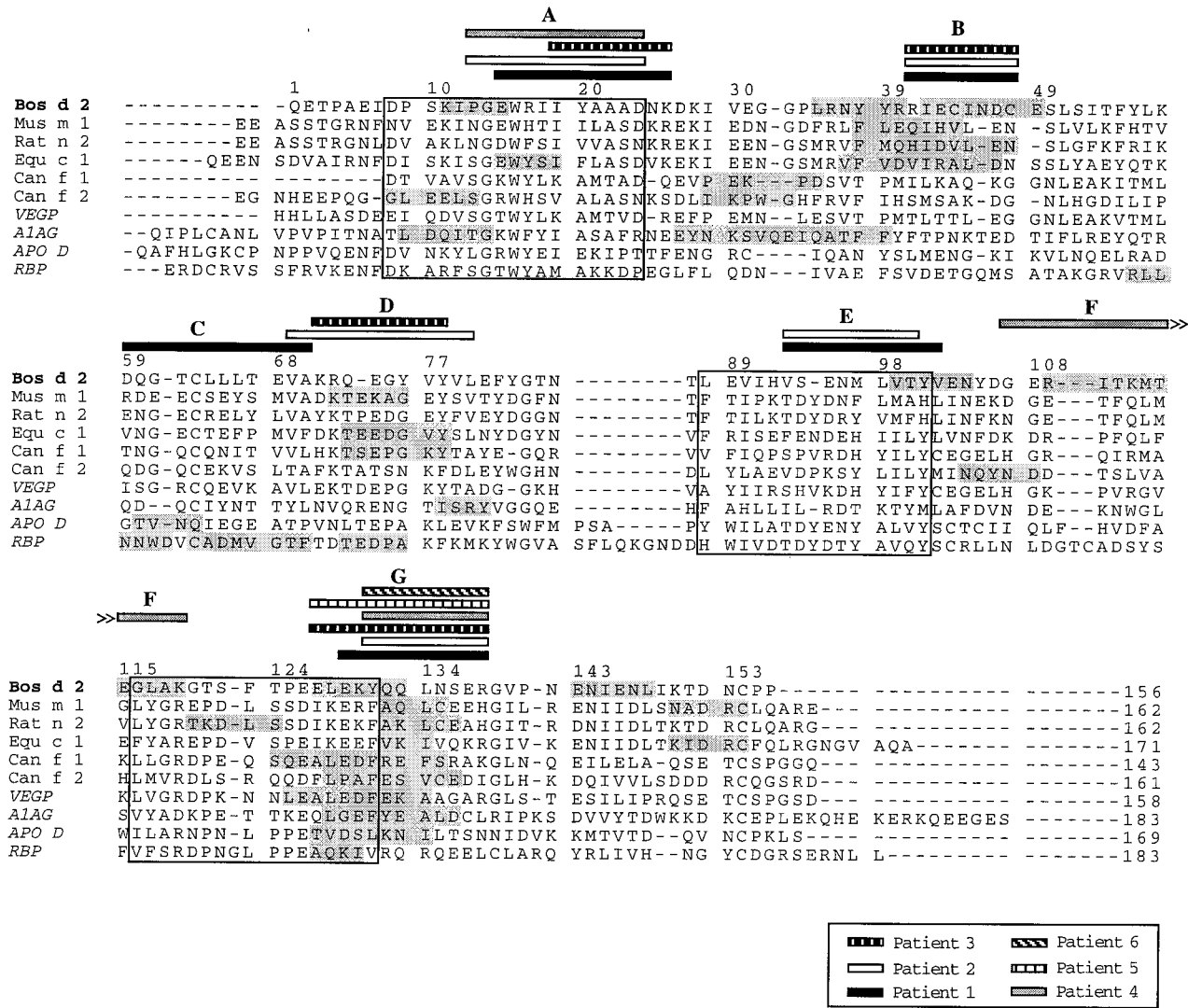


FIGURE 3. Sequence alignment of Bos d 2, other lipocalin allergens, and human lipocalin proteins. The three structurally conserved regions for the kernel lipocalins described by Flower (14) are boxed. The computer-predicted T cell epitopes are in grey. The experimentally verified T cell epitopes of Bos d 2 determined with T cell lines are indicated as bars above the sequence of Bos d 2.

homozygosity of their HLA-DR/DQ repertoire. HLA-DR4/53, normally found in 25% of the population (36), was detected in three patients (50%). HLA-DR4/53 was associated with DQ8 (3) in two cases and with DQ3 in one case. HLA-DR1 and DR2 were each expressed by two patients. Interestingly, those patients who responded uniformly to epitope B (patients 1, 2, and 3) all expressed the same HLA-DR4/53 phenotype, suggesting that the epitope B represents a single HLA-DR4/53-restricted T cell epitope.

Discussion

The T lymphocytes of the Th2 phenotype are important in the pathophysiology of allergic diseases (2, 37, 38). Through the secretion of cytokines such as IL-4 or IL-13 (3), this subset of lymphocytes is able to direct the Ig synthesis toward the production of IgE. On the other hand, it is still basically unclear which factors or circumstances favor the selection of Th2 lymphocytes during

Table IV. TCR Vα/β expression in Bos d 2-specific T cell lines^a

Patient	Vα/β elements								
	α2	α12.1	β5.2/5.3	β5.3	β5.1	β6.7	β8	β12	β13
1	~	~	~	~	~	20.5 (4)	~	5.7 (2.2)	~
2	~	~	~	~	~	24 (7.4)	~	~	17 (6.6)
3	~	7 (2.6)	19.7 (2.2)	14 (0.6)	~	~	~	~	~
4	~	5.2 (1.6)	~	~	~	~	~	~	~
5	7.6 (2)	4 (1.6)	14 (2.3)	~	~	~	~	10 (1.8)	~
6	~	4 (1.9)	~	~	~	~	~	~	~

^a Values are percentages of positive cells among CD4⁺ T cells. In parenthesis, the frequency of positive cells among PBMCs are given. Only values more than 2-fold the PBMC background are shown.

Table V. HLA phenotype of the cow-asthmatic patients included in the T cell epitope mapping with T cell lines and clones

Patient	HLA DR							HLA DQ						
	1	2	3	4	10	52	53	1	2	3	5	6 (1)	8 (3)	
1				+			+						+	
2				+									+	
3				+			+			+				
4		+										+		
5	+	+									+	+		
6			+		+	+		+	+					

allergic sensitization in atopic people. For example, it has been observed that the amount of immunizing agent (4, 39, 40), adjuvants (41–43), as well as Ag processing within the microenvironment of the Ag contact site (4, 44) all influence the quality of the Th response.

Recently, increased interest has focused on the role of the Ag itself and its molecular properties in evoking qualitatively different immune responses. There are several reports indicating that different Ags induce either Th1 or Th2-like responses (45–47). Minor amino acid substitutions in the epitope sequence of an allergen have been shown to modify the repertoire of cytokines secreted by T-lymphocytes (48–50).

We have previously described the molecular and immunological characteristics of Bos d 2, the predominant allergen in bovine dander and a member of the lipocalin group of proteins (8, 9, 26). In this study, the PBMCs of clinically verified cow-asthmatic patients with skin reactivity against nBos d 2 were observed to exhibit poor Bos d 2-specific proliferative responses. A general defect in the proliferative capacity of lymphocytes in these patients could be excluded, because the TET control Ag induced good proliferative responses (Fig. 1). Although it has been suggested that Th2 cells have a reduced proliferative capacity (51), this does not seem apparent at the clonal level, because our isolated Bos d 2-specific T cell clones exhibited excellent proliferative responses upon Ag stimulation, with SI values ranging between 10 and several hundred, regardless of their Th phenotype. Therefore, the low cellular responsiveness of PBMCs might be better explained as resulting from a low frequency of responding T cells in the peripheral blood, or from suppression mediated by immunoregulatory mechanisms. The latter alternative is supported by the observation that most of the Bos d 2-specific T cell lines exhibited a clear tendency to accumulate CD8⁺ T cells after repeated stimulations with Ag in vitro (Fig. 2). In agreement with this view are the results from animal studies suggesting that inhaled or ingested protein Ags may induce a transient recruitment of CD8⁺ Ag-specific regulatory T cells, which are able to mediate hyporesponsiveness in adoptive transfer studies (52, 53). Supporting this view, Nakajima et al. (54) have shown that CD8⁺ T cells accumulated in more than half of their casein-specific T cell lines. In another study, it was demonstrated that casein was recognized by CD8⁺ T cells in association with MHC class I molecules, and that these T cells could suppress in vitro IgE synthesis via IFN- γ production (55). Whether the development of CD8⁺ T cells in in vitro cultures is a phenomenon associated with certain characteristics of proteins, such as the allergenic capacity, is not known at the moment. Moreover, it should be mentioned that Nakajima et al. (54) made another finding with casein which is similar to our observations with Bos d 2. They reported that casein only weakly stimulated the PBMCs of milk-allergic patients. In this context, it is of interest to pay attention

to the recent finding according to which cat allergen Fel d 1 was not capable of inducing consistent proliferative responses of the PBMCs of cat-allergic people (56).

Bos d 2 was discovered to contain a limited number of T cell epitopes, with surprisingly little variation in core sequences recognized by different individuals. The highest number of epitopes to which a single individual could react was five, and the total number of epitopes detected was seven. The T cell clones of four patients were able to detect four distinct epitopes (Table II). The variation in the epitope core sequences suggested that these epitopes consist of clusters of slightly overlapping determinants. In accordance with this observation was the finding that certain epitopes were recognized by T cells from patients with different HLA-DR/DQ alleles (data not shown). An exception seems to be region B, which was recognized by T cell lines and T cell clones from three patients in a uniform manner. All these patients shared the same HLA-DR4/53 allele. Whether epitope B is HLA-DR4/53-restricted remains to be verified in further studies.

An important observation was that the carboxy-terminal portion of the molecule contained an epitope recognized by the T cell lines of all the patients as well as by the majority of the T cell clones (epitope G, Fig. 3). This epitope evoked the most intense proliferative responses by the T cell lines of five patients (patients 2, 3, 4, 5, and 6; data not shown). T cell lines from two of these patients did not recognize any other epitope. T cell clones reactive to this epitope were predominantly of the Th0/Th2-like phenotype, in contrast to the T cell clones reactive to the other epitopes (Table II). Because experimental immunotherapeutic studies with peptides have shown that a single immunodominant epitope may induce hyporeactivity against the entire molecule (57), epitope G may offer a starting point for the development of allergen derivatives for immunotherapy.

The T cell lines enriched by Bos d 2 preferentially exhibited certain TCR V α / β elements (Table IV). The dominant TCR elements varied between individuals, including two who had identical HLA-DR/DQ phenotypes (patients 1 and 2; Table V). Likewise, there was no correlation between specific V α / β elements and the particular epitopes recognized by the T cell lines or clones (data not shown). In fact, T cell clones with very similar peptide specificities have been shown to use different V α / β elements (58). Our observation of different V α / β elements (data not shown) in all four T cell clones responsive in an identical and possibly HLA-DR4/53-restricted manner to epitope B (patients 1, 2, and 3; Table III) is in agreement with this view.

To our knowledge, Fel d 1 is the only mammalian allergen so far that has been analyzed for T cell epitopes (24, 25). Fel d 1 and Bos d 2 seem to resemble each other in that they both contain only a few T cell epitopes concentrated in certain limited regions of the molecules (24). Other allergens, mainly those derived from plants,

characteristically contain multiple T cell epitopes located throughout the molecule (59–63). Whether animal allergens as a general rule contain fewer T cell epitopes than plant allergens remains to be verified.

Lipocalins are a large group of proteins with similar biological functions and varying degrees of homology (16). They possess structurally conserved regions (Fig. 3) that seem to contain T cell epitopes, according to our experimental results and the computer predictions. The carboxy-terminal epitope G of Bos d 2 was especially interesting because the corresponding regions in all the analyzed lipocalin allergens as well as in the human endogenous lipocalins were associated with a predicted T cell reactivity.

Allergy can be understood as a state of intolerance against non-harmful agents in the environment. It is interesting to note that aero-allergens seem to commonly cause sensitization and transient IgE production during infancy and childhood (64, 65). This usually subclinical and transient allergic stage is thought to represent a necessary counterregulatory mechanism for preventing potential autodestructive immune reactions triggered by self-mimicking exogenous Ags (51). According to this view, a primary immune response mediated by inflammatory Th1 lymphocytes during early infancy would be followed by the outgrowth of less hazardous Th2 lymphocytes, simultaneously establishing the first allergen-specific memory. As is suggested by studies of the autoimmune diseases, the switch to a Th2-like immune response is protective (66, 67). Therefore, one factor predisposing toward allergic sensitization would be an excessive degree of similarity between endogenous self-Ags and exogenous allergens at the level of epitope recognition, so that those potentially self-reactive T cells which have escaped thymic deletion and are under the regulatory mechanisms of peripheral tolerance might be able to recognize the epitopes of exogenous Ags resembling self, most probably less efficiently than their natural target epitopes. In turn, this would result in changes in the pattern of secreted cytokines, as suggested by studies with altered peptide ligands (48, 68). This hypothesis is especially conceivable with animal-derived allergens because of the close biological relationship between mammals, but may also be adapted to plant allergens. It has been observed that the members of the group 9 pollen allergens share a common, highly conserved T cell epitope the sequence of which is very close to those found in several human cell adhesion molecules (69). Thus, the suboptimal TCR-mediated activation of the T cell compartment resulting in the deviated production of cytokines may also be caused by plant allergens even if there may be a difference in the number of T cell epitopes and therefore in the overall cellular response. In this context, it is interesting to notice that a significant number of plant and animal allergens with known sequences are evolutionary conserved proteins with important biological functions (1, 6).

To our knowledge, ours is the first report describing the localization of T cell-reactive sites of a lipocalin allergen. The weak response of PBMCs, the limited usage of TCR V α /V β elements at an individual level, and the relatively few epitopes detected within the allergen molecule suggest that the repertoire of T cells responding to Bos d 2 is limited. These findings, in association with the observation of T cell epitopes within the structurally conserved regions and of elevated numbers of CD8⁺ T cells in the allergen-specific T cell lines, point to the possibility that mechanisms of self tolerance are involved in the immune response against Bos d 2. In addition to these theoretical implications, the limited number of immunodominant epitopes is likely to facilitate the development of new modalities for immunotherapy of cow dander-associated allergic diseases.

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