

Lipocalin allergen Bos d 2 is a weak immunogen

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Keywords: allergenicity, animal allergen, cytokine production, epitope mapping, immune response, immunogenicity

Abstract

The immunological characteristics of an important group of animal-derived allergens, lipocalins, are poorly known. To explore the immunology of the lipocalin allergen Bos d 2, several mouse strains with different H-2 haplotypes were immunized with the allergen. Only the BALB/c mouse mounted a distinct humoral response against Bos d 2. The proliferative spleen cell responses of all mouse strains remained very weak. Further experiments with BALB/c mice confirmed that Bos d 2 is a weak inducer of both humoral and cellular responses, and that the responses were weaker than with the control antigens hen egg lysozyme (HEL) and tetanus toxoid. IgG subclass analyses showed that Bos d 2 was prone to favor the T_H2 response. Although s.c. immunization using complete Freund's adjuvant favored the T_H1-deviated immune response by lymph node cells, Bos d 2 was able to induce the production of IL-4 while the control antigen HEL did not. Epitope mapping revealed that BALB/c mice recognized one immunodominant epitope in Bos d 2, almost identical to that recognized by humans. The epitope was shown to be immunogenic in subsequent experiments. However, further studies are needed to clarify the significance of priming and stimulation doses of the immunodominant and other epitopes in Bos d 2 for the outcome of immune response against the allergen. The murine immune response against Bos d 2 closely resembled that observed in humans. The weak immunogenicity of Bos d 2 may be associated with its allergenicity.

Introduction

Several important animal-derived aeroallergens belong to the group of proteins called lipocalins (<http://www.expasy.ch/cgi-bin/nicesite.pl?PS00213>, 1 September 2001). These allergens include the major urinary proteins of mouse and rat, Mus m 1 (1,2) and Rat n 1 (2,3), the dog allergens Can f 1 and Can f 2 (4), the horse allergens Equ c 1 and Equ c 2 (5,6), the cockroach allergen Bla g 4 (7), the major *Triatoma protracta* allergen (8), and the bovine Bos d 2 (9), which is an occupational allergen causing asthma and other work-related allergic disorders among dairy farmers. We have recently shown that the human immune response against Bos d 2 is dichotomous in that cow-asthmatic patients have high levels of Bos d 2-specific IgE in their sera, in contrast to the predominantly very weak cellular responses against the allergen (10). The relationship between these two immunoparameters may in fact be a trait related to the allergenicity of lipocalins (11). However, apart from the fact that they induce IgE production,

the immunologic characteristics of lipocalin allergens are poorly known (12). To explore the immunology of Bos d 2, murine immune responses against Bos d 2 were characterized in detail, and compared with those induced by hen egg lysozyme (HEL) and tetanus toxoid (TT) control antigens. It turned out that Bos d 2 is a very weak immunogen by all the methods used and that it exhibited a tendency to deviate the immune response towards T_H2 dominance. These features may be related to the allergenicity of lipocalin allergens.

Methods

Antigens and peptides

The antigens used were natural (n) Bos d 2 (10,13), *Escherichia coli*-produced recombinant (r) Bos d 2, its N- and C-terminal fragments (amino acids 1–115 and 65–156

respectively), and the fusion part glutathione S-transferase (GST) (9,14), *Pichia pastoris*-produced (pp) rBos d 2 (15,16), HEL (Sigma, St Louis, MO; L-6876) and TT (National Public Health Institute, Helsinki, Finland). *In vitro* tests employed the same Bos d 2 preparation as was used for immunization. For the mapping of the epitopes of Bos d 2, 16mer peptides overlapping by 14 residues were prepared and verified as reported previously (10). 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 (10). For additional experiments, the peptide 64 (ELEKYQQLNSERGVNP) containing the immunodominant epitope of Bos d 2 was synthesized using PerSeptive 9050 Plus automated peptide synthesizer (Millipore, Bedford, MA) with Fmoc strategy. The peptides were purified by HPLC (Shimadzu, Tokyo, Japan) with a C₁₈ reverse phase column and acetonitrile as eluent (0.1% TFA in H₂O/0–60% acetonitrile gradient for 60 min) and verified with a MALDI-TOF mass spectrometer (Bruker, Bremen, Germany). All protein concentrations were determined by the method of Bradford (17) using the commercial BioRad (Hercules, CA) protein assay. Sterile-filtered or γ -irradiated preparations were stored either at 4°C or frozen at –70°C.

Immunization of mice

Female A.SW (H-2^s), A/J (H-2^a), BALB/c (H-2^d), B10.M (H-2^f), C57BL/6 (H-2^b) and CBA/s (H-2^k) mouse strains (6–8 weeks old) were obtained from the National Laboratory Animal Center (Kuopio, Finland). Mice were maintained under pathogen-free conditions throughout the study. They were injected i.p. or s.c. at the base of the tail at 2-week intervals 1–4 times, depending on the experiment, with the antigen in alum [5 mg, prepared as described elsewhere (18) or Imject Alum (Pierce, Rockford, IL)], incomplete Freund's adjuvant (IFA; Sigma; F-5506) or complete Freund's adjuvant (CFA; Sigma; F-5881) in a volume of 0.2 ml, as indicated in the text. Control mice were treated with PBS. In testing the Bos d 2-specific responses of different inbred mouse strains, TT-immunized mice served as controls. Samples were collected 10 days after each immunization. Experiments were performed in agreement with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg 18 March 1986, adopted in Finland 31 May 1990). The study was approved by the Animal Care and Use Committee of the University of Kuopio.

Measurement of antibody responses

Specific murine antibody responses were measured by indirect ELISA as described previously (13), with modifications. For measuring specific IgG levels of different inbred mouse strains, ELISA plates (Nunc, Roskilde, Denmark) were coated with nBos d 2 at a concentration of 1 µg/ml at 4°C overnight in the coating buffer. After the plates were blocked with diluent [0.1% (v/v) casein in PBS], they were stored frozen. Serum samples were added in duplicate (1:250, 1 h, room temperature). Bound IgG was detected by goat anti-mouse IgG (BioMakor, Israel; 1:1000, 0.5 h, room temperature). Next, biotinylated rabbit anti-goat IgG (Cappel, Turnhout, Belgium; 1:2000, 0.5 h, room temperature) was

added. The color reaction was developed using a Vectastain ABC Elite kit (Vector, Burlingame, CA) as described previously (13). The results were expressed as optical density (OD) values.

The levels of IgG subclasses were examined as described above with modifications. ELISA plates were coated with Bos d 2, HEL or TT at a concentration of 1 µg/ml (IgG1) or 50 µg/ml (IgG2a). Duplicate serum samples were incubated at 37°C for 1 h at 10-fold dilutions [1:100–1:1,000,000 (IgG1) and 1:10–1:100,000 (IgG2a)]. Bound antibodies were detected by biotinylated anti-IgG1 (1:1000) or anti-IgG2a (1:100) antibodies (Caltag, Burlingame, CA) respectively, incubating at 37°C for 1 h. After measuring the color reaction (see above), the results were expressed as OD values at an appropriate serum dilution.

Proliferation assays

After spleens or inguinal lymph nodes were removed from the mice, single-cell suspensions were prepared and the cells of each group of mice (3–4 mice) were combined. Red blood cells were removed from spleen cell suspensions by lysing with 0.017 M Tris–0.75% NH₄Cl (5 ml/spleen). Alternatively, the spleen cells were isolated by Lympholyte M (Cedarlane, Hornby, Ontario, Canada) density gradient centrifugation according to the protocol of the manufacturer. Finally, the cells were washed twice with the culture medium [DMEM with glutamax (Gibco, Grand Island, NY) supplemented with 20 µM 2-mercaptoethanol, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% inactivated FCS (Biological Industries, Beit Haemek, Israel)].

Spleen or lymph node cells were stimulated in triplicate at a density of 2×10^5 or 10^5 cells/well respectively in the wells (0.2 ml) of round-bottomed 96-well microtiter plates (Corning, Acton, MA). In each stimulation, the concentrations of antigens (Bos d 2, HEL and TT) ranged from 12.5 to 100 µg/ml and those of the peptide containing the immunodominant T cell epitope from 0.3 to 30 µg/ml. The positive control was concanavalin A (Con A; Pharmacia Biotech, Uppsala, Sweden) at 5 µg/ml and wells without a stimulant served as the negative control. For the mapping of epitopes in Bos d 2, the spleen cells were stimulated with each of the 16mer peptides of Bos d 2 at an optimal concentration of 10 µg/ml in 12 wells. The concentrations of *E. coli*-produced rBos d 2, its N- and C-terminal fragments (amino acids 1–115 and 65–156 respectively), and GST were 50 µg/ml.

After addition of the stimulants, the cells were incubated for 3 days in a humidified 5% CO₂ incubator at 37°C and then pulsed for 16 h with 0.5 µCi/well [³H]thymidine (sp. act. 2.0 Ci/mmol; Amersham, Little Chalfont, UK). Radionuclide uptake was measured by scintillation counting and the results expressed as a stimulation index (SI: ratio between the mean c.p.m. in cultures with stimulant and the mean c.p.m. in cultures without stimulant).

Induction and measurement of cytokine production

Spleen or lymph node cells were stimulated with Bos d 2, HEL or TT at a predetermined concentration of 100 µg/ml at an optimal density of 3×10^6 cells/ml in 4-ml test tubes in a volume of 1.5 ml for 48 h (IL-2 and IFN- γ) or 96 h (IL-4 and IL-5) in a humidified 5% CO₂ incubator at 37°C. Positive (Con A at 5

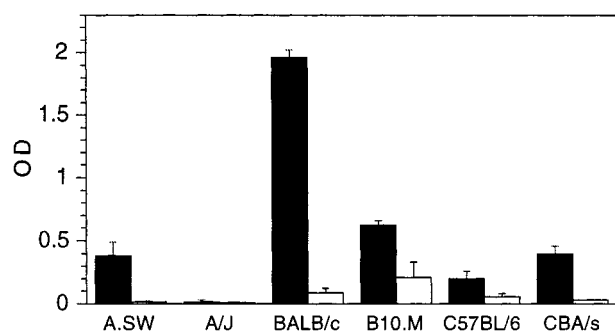


Fig. 1. Specific IgG levels of six inbred mouse strains [A.S.W (H-2^s), A/J (H-2^a), BALB/c (H-2^d), B10.M (H-2^f), C57BL/6 (H-2^b) and CBA/s (H-2^k)] against nBos d 2. Filled bars represent the responses of Bos d 2-immunized mice and open bars those of control mice. The results are expressed as mean OD values ($\pm$ SEM) of individual mice at a serum dilution of 1:250.

μ g/ml) and negative controls (cells with culture medium only) were included. Stimulation of the spleen or lymph node cells from mice immunized with the peptide were performed at two peptide concentrations, 3 and 30 μ g/ml. An irrelevant peptide served as an additional control. After stimulation, test tubes were centrifuged, and the culture supernatants were collected, aliquoted and stored at -70°C until examined. The levels of IL-4, IL-5 and IFN- γ in the supernatants were measured by ELISA in duplicate using commercial reagents (all reagents from PharMingen, San Diego, CA) according to the manufacturer's instructions. Color reactions were developed as described for antibody measurements. Results were calculated from the standard curve using Deltasoft 3 software with a four-parameter fit (BioMetallics, Princeton, NJ). The limits of detection were 5 pg/ml for IL-4, 30 pg/ml for IL-5 and 100 pg/ml for IFN- γ . For measuring IL-2, CTLL-2 cell line was used as previously described (10) with the exception that mAb against murine IL-4 (R & D Systems, Abingdon, UK; MAB404) at a neutralizing concentration (1 μ g/ml) was included in the assay. A semiquantitative estimate of IL-2 production was determined from a standard curve of rIL-2 (Strathmann Biotech, Hamburg, Germany). The limit of detection for IL-2 was 0.05 U/ml.

Statistical analysis

Statistical differences between groups were determined by the Kruskal-Wallis test and *post hoc* comparisons with the Mann-Whitney *U*-test. Values of $P < 0.05$ were considered significant.

Results

Mouse strains' immune responses to Bos d 2

Inbred mice with different H-2 haplotypes were immunized i.p. twice at a 2-week interval with nBos d 2 (25 μ g/mouse) in alum. As shown in Fig. 1, only BALB/c mice showed clearly elevated levels of specific IgG to Bos d 2 ($P = 0.02$ between BALB/c and the other strains), although the anti-Bos d 2 IgG levels of A.S.W, B10.M and CBA/s mice also exceeded the arbitrary cut-off absorbance of 0.3 (mean of all control mice plus 3 SD). When

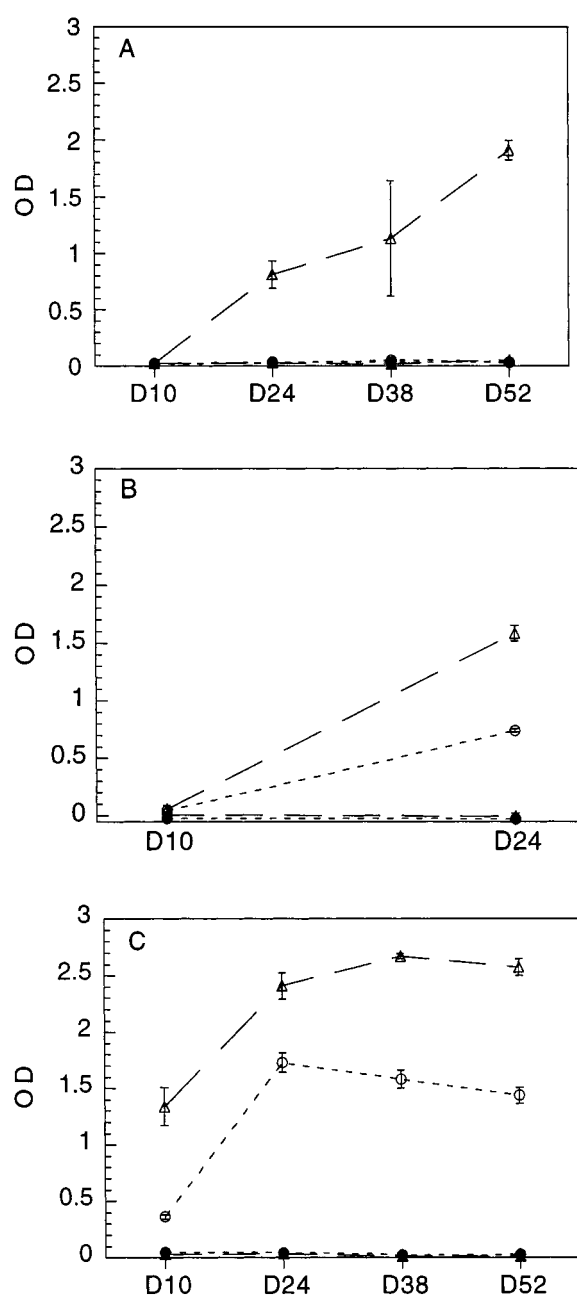


Fig. 2. Specific IgG subclass levels of BALB/c mice immunized with nBos d 2 (A), HEL (B) or TT (C). Specific IgG1 levels of immunized mice are indicated by open triangles and those of PBS-treated mice by closed triangles. Specific IgG2a levels of immunized mice are indicated by open circles and those of PBS-treated mice by closed circles. Mice were immunized i.p. at 2-week intervals with the antigens (50 μ g/mouse) in alum. Antibody levels were measured 10 days after each immunization (days 10, 24, 38 and 52). Results are expressed as mean OD values ($\pm$ SEM) of individual mice at serum dilutions of 1:1000 (IgG1) and 1:100 (IgG2a). On day 24, the specific IgG1 levels differed significantly between the immunization groups ($P < 0.05$).

the same mouse strains were immunized with TT (25 μ g/mouse) all of them developed high anti-TT IgG levels (OD values > 2 ; data not shown). The proliferative responses of the spleen cells of all mouse strains against nBos d 2 (determined

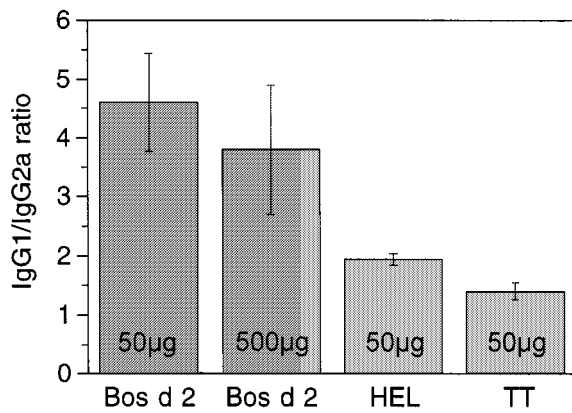


Fig. 3. The IgG1/IgG2a ratios of mice immunized with pprBos d 2, HEL or TT. Groups of mice were immunized i.p. twice at a 2-week interval with a low (50 µg/mouse) or high (500 µg/mouse) pprBos d 2 dose, or with HEL (50 µg/mouse) or TT (50 µg/mouse) in alum. The specific IgG1 and IgG2a levels were determined at serum dilutions of 1:100 and 1:10 respectively, and indicated as OD values. The results are represented as mean IgG1/IgG2a ratios ($\pm$ SEM) of individual mice. The differences between low-dose pprBos d 2, HEL and TT immunization groups were significant ($P < 0.05$).

at a concentration of 50 µg/ml) turned out to be very low (SI < 1.7 , the medium background range 600–2900 c.p.m.). An increase of the immunizing allergen (50 µg/mouse of pprBos d 2), the use of different adjuvants (IFA or CFA) and an increase in the allergen concentration upon stimulation *in vitro* (up to 100 µg/ml of pprBos d 2) did not enhance the proliferative responses of spleen cells of BALB/c, CBA/s or B57BL/6 mice (data not shown).

IgG subclass responses to Bos d 2, HEL and TT

To further characterize the immune responses of BALB/c mice to Bos d 2, groups of mice were immunized repeatedly at 2-week intervals with 50 µg/mouse of nBos d 2, HEL or TT in alum. Figure 2(A and B) show that the specific IgG1 response to nBos d 2 and HEL respectively started slowly, being clearly higher than in the PBS control mice after two immunizations on day 24 ($P < 0.05$), whereas the antibody level against TT was already elevated after the first immunization (Fig. 2C; $P < 0.05$). Immunization with nBos d 2 did not induce Bos d 2-specific IgG2a antibodies detectable at any serum dilution (Fig. 2A), whereas a response against HEL was apparent after the second immunization (Fig. 2B; $P < 0.05$) and against TT after the first immunization (Fig. 2C; $P < 0.05$). The experiment was repeated twice by immunizing twice at a 2-week interval. The results were similar in all three experiments with HEL and TT, and in the first two experiments with nBos d 2. In the third experiment, when pprBos d 2 was used instead of the natural preparation, low IgG2a production was observed after the second immunization when tested at a lower serum dilution of 1:10 [mean OD 0.17 ± 0.04 (SEM), $P = 0.02$]. When mice were immunized with a higher dose of pprBos d 2 (500 µg/mouse), the anti-pprBos d 2 IgG2a level further increased slightly [mean OD 0.28 ± 0.05 (SEM), $P = 0.02$]. However, the difference of the IgG2a levels between the groups immunized with high (500 µg/mouse) or low (50 µg/mouse) doses of

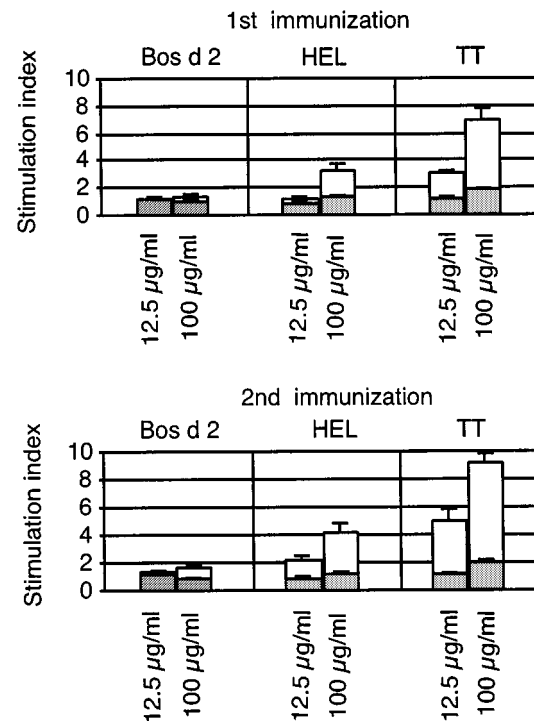


Fig. 4. Proliferative spleen cell responses of BALB/c mice to pprBos d 2, HEL and TT. Groups of mice were immunized i.p. twice at a 2-week interval with the antigens (50 µg/mouse) in alum. Ten days after each immunization, spleen cell responses were examined as described in Methods. Filled bars represent the responses of PBS-treated mice. The results are represented as mean SI ($\pm$ SEM) of three independent experiments. The medium background was in all cases < 3000 c.p.m. The differences between groups were statistically significant ($P < 0.05$) except after the first immunization upon stimulation *in vitro* at a concentration of 12.5 µg/ml of the antigens.

pprBos d 2 was not statistically significant ($P > 0.05$). This was also the case with IgG1 antibodies ($P > 0.05$), even though there was a tendency that the high dose of pprBos d 2 was more effective in inducing IgG1 synthesis than the low dose (data not shown). Figure 3 shows the IgG1/IgG2a ratios after immunization with a low or high pprBos d 2 dose, and with HEL and TT. The differences between groups immunized at 50 µg/mouse were significant ($P < 0.05$).

Cellular responses to Bos d 2, HEL and TT

As summarized in Fig. 4, the mice immunized i.p. with pprBos d 2 exhibited the weakest proliferative spleen cell responses *in vitro* in comparison to those immunized i.p. with HEL or TT. The second immunization increased the responses, but the effect was negligible with pprBos d 2. An experiment of four immunizations with nBos d 2 at 2-week intervals gave identical results with no enhancement of spleen cell proliferation *in vitro* after repeated immunizations (data not shown). The differences (Fig. 4) between groups were statistically significant ($P < 0.05$) except after the first immunization when tested *in vitro* at a concentration of 12.5 µg/ml. When mice were immunized with a higher dose of pprBos d 2 (500 µg/mouse) the proliferative responses were not enhanced (SI 1.2 after the first and 1.8 after the second immunization).

Table 1. Cytokine production by spleen cells of pprBos d 2, HEL and TT-immunized BALB/c mice after culture with the antigens^a

Antigen	IL-4 (pg/ml)		IL-5 (pg/ml)		IL-2 (U/ml)		IFN- γ (pg/ml)	
	First immunization	Second immunization ^b	First immunization	Second immunization	First immunization	Second immunization ^b	First immunization	Second immunization
pprBos d 2	1.8 $\pm$ 1.8	2.6 $\pm$ 2.6	160 $\pm$ 116	173 $\pm$ 71	0.88 $\pm$ 0.19	1.09 $\pm$ 0.20	<	<
HEL	36.1 $\pm$ 8.9	30.0 $\pm$ 3.6	518 $\pm$ 29	195 $\pm$ 50	1.82 $\pm$ 0.10	2.06 $\pm$ 0.38	<	<
TT	29.2 $\pm$ 4.9	177 $\pm$ 28	218 $\pm$ 84	658 $\pm$ 173	2.36 $\pm$ 0.36	2.92 $\pm$ 0.34	<	<
Medium	<	<	<	<	0.42 $\pm$ 0.05	0.47 $\pm$ 0.06	<	<

^aGroups of mice were immunized i.p. twice at a 2-week interval with the antigens (50 μ g/mouse) in alum. Ten days after each immunization, spleen cell responses were examined as described in Methods. The results are represented as mean concentrations $\pm$ SEM of three independent experiments. Medium is the combined medium background of all immunization groups, < below the detection limit (see Methods).

^b $P < 0.05$ between pprBos d 2 and other immunization groups.

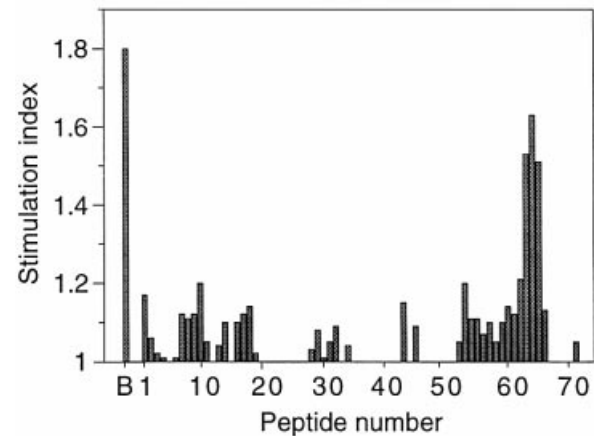


Fig. 5. Mapping of T cell epitopes of Bos d 2. BALB/c mice were immunized i.p. with nBos d 2 (50 μ g/mouse) in alum and the spleen cell responses were examined 10 days later as described in Methods. The results are expressed as SI [ratio between the mean c.p.m. in 12 cultures with a peptide (10 μ g/ml) and the mean c.p.m. in 12 cultures without a peptide] upon stimulation with the overlapping 16mer peptides of Bos d 2 (numbered consecutively). The medium background was 5900 c.p.m. $\pm$ 300 (SEM) of replicate wells. B indicates Bos d 2 (50 μ g/ml).

The cytokine production of spleen cells *in vitro* from mice immunized i.p. with pprBos d 2, HEL or TT once or twice at a 2-week interval is summarized in Table 1. Bos d 2 was observed to induce only a low-level production of IL-4, the differences between groups being significantly different after the second immunization ($P < 0.05$). TT was a stronger inducer of IL-4 than HEL ($P < 0.05$). The IL-5 production was not statistically different between the groups ($P > 0.05$). HEL and TT were stronger inducers of IL-2 production than Bos d 2 ($P < 0.05$), and HEL and TT did not differ in this respect ($P > 0.05$). IFN- γ production upon stimulation with the antigens was not detected. Con A stimulation induced an abundant production of the four cytokines measured in all groups (data not shown).

To verify whether the protocol of immunization influenced the outcome, the immune responses were examined by immunizing the BALB/c mice s.c. with pprBos d 2 or HEL (50 μ g/mouse) in CFA. While the proliferative responses of lymph node cells against both antigens were observed to be stronger than those of spleen cells in previous experiments, Bos d 2 tended to provoke weaker responses [SI 3.5 ± 0.7 (SEM)] than HEL [4.2 ± 1.4 (SEM)] at 100 μ g/ml (three independent experiments, $P > 0.05$). Interestingly, Bos d 2 induced the production of IL-4 (Table 2) which was not seen with HEL ($P < 0.05$). The production of IL-5 and IFN- γ did not differ between the immunization groups ($P > 0.05$).

T cell epitopes in Bos d 2

The mapping of T cell epitopes with peptides spanning the sequence of Bos d 2 revealed that the T cells of BALB/c mice recognized in Bos d 2 one immunodominant epitope (Fig. 5). It was localized in the C-terminal end of the molecule, almost identically to the epitope G recognized by human T cells (10). The core sequence, defined as those amino acids within the region shared by the three overlapping peptides (peptides 63–65) capable of inducing the strongest stimulation of spleen

Table 2. Cytokine production by lymph node cells of pprBos d 2- and HEL-immunized BALB/c mice after culture with the antigens^a

Antigen	IL-4 (pg/ml) ^b	IL-5 (pg/ml)	IFN- γ (pg/ml)
pprBos d 2	15.5 $\pm$ 5.0	79.7 $\pm$ 16.4	420.8 $\pm$ 76.0
HEL	<	60.0 $\pm$ 23.4	231.2 $\pm$ 82.5

^aMice were immunized s.c. at the base of a tail once with the antigens (50 μ g/mouse) in CFA. Ten days after the immunization, inguinal lymph node cell responses were examined as described in Methods. The results are represented as mean concentrations $\pm$ SEM of three independent experiments. Medium background values were below the detection limit (see Methods). <, below the detection limit.

^b $P < 0.05$.

Table 3. Cytokine production by spleen cells of peptide-immunized BALB/c mice^a

Adjuvant	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-2 (U/ml)	IFN- γ (pg/ml)
Alum	7.8 $\pm$ 1.5	332 $\pm$ 160	2.3 $\pm$ 1.0	<
IFA	15.1 $\pm$ 4.9	464 $\pm$ 51	2.6 $\pm$ 0.4	<
CFA	13.1 $\pm$ 5.5	227 $\pm$ 39	2.1 $\pm$ 0.2	<
Medium	<	<	0.6 $\pm$ 0.1	<

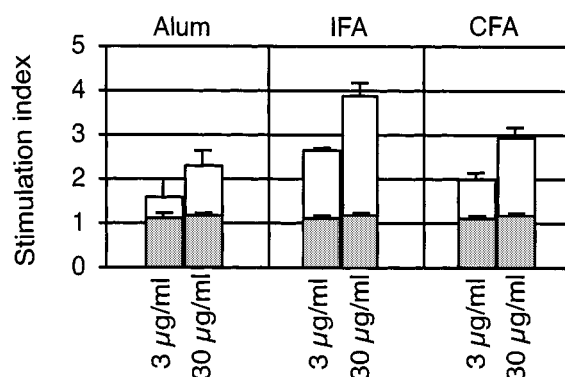
^aGroups of mice were immunized i.p. with 100 μ g/mouse of the peptide 64 containing the immunodominant T cell epitope in alum, IFA or CFA. Ten days after immunization, spleen cell responses were examined as described in Methods. The results are represented as mean concentrations $\pm$ SEM of three independent experiments upon stimulation with 30 μ g/ml of the peptide. Medium is the combined medium background of all immunization groups. <, below the detection limit (see Methods).

cells, was EKYQQLNSERGV. The importance of the C-terminal end of Bos d 2 for the T cell response was further corroborated by the results of an analysis with the two overlapping recombinant fragments of the allergen produced in *E. coli*. The stimulation indices induced by the N-terminal fragment (amino acids 1–115), C-terminal fragment (amino acids 65–156), complete rBos d 2 and GST control were 1.5, 2.0, 1.9 and 1.0 respectively [medium background 5900 $\pm$ 300 c.p.m. (SEM) of 12 replicate wells]. The analysis was repeated once with peptides 2, 46 and 53–71. The results were in accordance with those obtained in the previous experiment, verifying the immunodominance of the C-terminal epitope (data not shown).

Spleen and lymph node cell responses to the immunodominant epitope of Bos d 2

To verify the immunogenicity of the immunodominant epitope of Bos d 2 and to delineate its role in determining the quality of immune response against Bos d 2, BALB/c mice were immunized i.p. with 100 μ g/mouse of the peptide 64 (ELEKYQQLNSERGVNP) in alum, IFA or CFA. IFA and CFA tended to induce the stronger proliferative responses of spleen cells (Fig. 6), but the differences between the groups were not statistically significant ($P > 0.05$).

The immunodominant peptide induced the production of IL-5 from spleen cells (Table 3) in a similar manner as the complete pprBos d 2 (Table 1). IL-2 production was also elevated. IL-4 production was low and IFN- γ could not be

**Fig. 6.** Proliferative spleen cell responses of BALB/c mice to the peptide 64 containing the immunodominant T cell epitope. Groups of mice were immunized i.p. with the peptide (100 μ g/mouse) in alum, IFA or CFA. Spleen cell responses were examined 10 days later as described in Methods. Filled bars represent the responses of PBS-treated mice. The results are represented as mean SI ($\pm$ SEM) of three independent experiments. The medium background was in all cases <1300 c.p.m. The differences between groups were not statistically significant ($P > 0.05$).

detected. Con A stimulation induced an abundant production of the four cytokines measured in all groups (data not shown). The differences in the cytokine levels between groups immunized with different adjuvants (Table 3) were not statistically significant ($P > 0.05$).

When BALB/c mice were immunized s.c. with the immunodominant peptide in CFA the proliferative responses of lymph node cells upon stimulation with the peptide (Table 4) appeared to be stronger than in previous experiments when the spleen cell responses of mice immunized i.p. were studied (Fig. 6, CFA) even though the optimal immunization dose was lower (10 μ g/mouse s.c. or 100 μ g/mouse i.p.). Interestingly, the s.c. route of immunization resulted in the strong production of IFN- γ (Table 4), which was not the case when mice were immunized i.p. (Table 3). Pointing to the T_H1 dominance of the response (Table 4), no production of IL-4 was observed while IL-5 production was lower than in previous experiments (Table 3).

Discussion

We have shown previously that cow-dander-allergic asthmatics who show high IgE reactivity against the major allergen of cow, Bos d 2, have weak proliferative responses of peripheral blood mononuclear cells against the allergen (10). We have lately hypothesized that the seemingly paradoxical situation with high IgE and weak cellular responses against Bos d 2 may be associated with the allergenicity of the protein (11,12). The situation is very interesting because it has turned out that almost all important animal-derived aeroallergens belong to the protein family of lipocalins (<http://www.expasy.ch/cgi-bin/nicesite.pl?PS00213>, 1 September 2001). To circumvent the limitations associated with human studies, we conducted experiments in mice to delineate the immunologic properties of Bos d 2. It was observed that the murine immune response against Bos d 2 closely resembles that observed in humans.

Table 4. Response of lymph node cells of peptide-immunized BALB/c mice^a

	Proliferation (SI)	IL-4 (pg/ml)	IL-5 (pg/ml)	IFN- γ (pg/ml)
Peptide 64	7.0 $\pm$ 1.5	<	48.5 $\pm$ 11.6	734.8 $\pm$ 231.1

^aMice were immunized s.c. at the base of a tail once with 10 μ g/mouse of the peptide 64 containing the immunodominant T cell epitope in CFA. Ten days after the immunization, inguinal lymph node cell responses were examined as described in Methods. The results are represented as mean SI or cytokine concentrations $\pm$ SEM of three independent experiments upon stimulation with the peptide at 30 μ g/ml. In proliferation tests, medium background was in all cases <400 c.p.m. In cytokine measurements, medium background values were below the detection limit (see Methods). <, below the detection limit.

The conspicuous feature of Bos d 2 was its poor capacity to induce both humoral and cellular responses in mice. When mice with different MHC haplotypes were immunized i.p. with Bos d 2 in alum, only the BALB/c mouse mounted a distinct IgG response against the allergen. The proliferative responses of spleen cells of all mouse strains remained weak. Although additional mouse strains with the capacity to respond to Bos d 2 might have been found by screening a wider repertoire of strains (19), it is justified to say that Bos d 2 is a poor immunogen in several mouse strains.

For the assessment of the immunogenicity of a protein, the selection of proteins for comparison is important. HEL is being widely used in diverse immunological studies. In the immunization experiments of several syngenic mouse strains with different MHC haplotypes, HEL induced the lowest responses in the BALB/c mouse (19). In contrast, TT is known to be very immunogenic (10). Accordingly, TT induced strong IgG responses in all mouse strains used in this study (data not shown). Taking into account these considerations, our results show that Bos d 2 is a poor immunogen by several parameters, even in the responder strain BALB/c. The Bos d 2-specific IgG1 response started slowly and was lower than the antigen-specific responses of HEL- or TT-immunized mice on day 24. Although the difference in IgG2a responses was not significant ($P = 0.06$, Kruskal–Wallis test) it was clear that Bos d 2 also induced antibodies of this subclass very poorly. Moreover, both the proliferative responses and the capacity to induce cytokine production by spleen cells after i.p. immunizations indicated that Bos d 2 is a weaker immunogen than the control antigens HEL and TT.

Repeated i.p. immunizations with nBos d 2 in alum failed to induce IgG2a antibodies in contrast to HEL or TT. The finding suggests that the immune response against Bos d 2 was T_H2 biased because the synthesis of IgG2a is strongly dependent on IFN- γ (20,21). Although Bos d 2-specific IgG2a antibodies could be detected at a serum dilution of 1:10 after immunization with pprBos d 2, even in this case the IgG1/IgG2a ratio was significantly higher with pprBos d 2-immunized mice in comparison with HEL- or TT-immunized mice (immunizations at 50 μ g/mouse). The analysis of the cytokine responses of spleen cells revealed that none of the antigens was able to induce IFN- γ production at a detectable level. This can be attributed to the characteristics of the BALB/c mouse, which is known to be prone to T_H2-type responses (22,23), to alum adjuvant (24,25), to the immunization route used (25) and/or to the sensitivity of the cytokine assay. On the other hand, all the antigens induced the synthesis of IL-2, which suggests that a block in IL-2 production (26) does not explain the poor proliferative response against Bos d 2. Interestingly, when

mice were immunized s.c. with pprBos d 2 or HEL in CFA, lymph node cells produced IFN- γ which suggests, as mentioned above, that the choice of adjuvant and route of immunization influence the outcome of immune response. While the immune responses against both antigens exhibited the features of immune deviation to T_H1, the important point was that only pprBos d 2 also induced the synthesis of IL-4.

pprBos d 2 was weak in inducing the synthesis of IL-4 from spleen cells after i.p. immunization in alum, whereas the levels of IL-5 induced by the three antigens upon stimulation *in vitro* did not differ significantly (Table 1). Similarly, IL-5 levels secreted by lymph node cells did not differ significantly between pprBos d 2 and HEL after s.c. immunization in CFA, while HEL did not induce measurable levels of IL-4 ($P < 0.05$; Table 2). We have previously shown that the secretion of type 2 cytokines IL-4 and IL-5 can be dissociated. For example, a proportion of T cell clones derived from skin of atopic dermatitis patients produced IL-5 with no detectable IL-4 (27). This was also the case with some of the Bos d 2-specific clones isolated from the peripheral blood of cow-dust-asthmatic patients (10). Heeger *et al.* have shown that the opposite situation may also be possible. When mice were immunized with an autoantigen myelin oligodendrocyte glycoprotein the numbers of IFN- γ -, IL-2- and IL-4-secreting spleen cells were high, whereas the number of IL-5-secreting cells was low or none (28). Indeed, it seems that the synthesis of IL-4 and IL-5 can be independently regulated (29,30).

Our results suggest that human and murine immune systems recognize the lipocalin allergen Bos d 2 in a similar way. Allergic persons' immune responses to Bos d 2 appear to be mainly humoral, cellular responses remaining very weak; the same was true with mice. There are data suggesting that certain antigens or single T cell epitopes could account for the T_H1 or T_H2 dominance of the immune response. For example, mice made tolerant to a *Leishmania major*-derived antigen by transgenic expression in the thymus mounted a decreased T_H2-type response and a healing phenotype upon infection with the parasite (31). In BALB/c mice, respiratory syncytial virus-derived protein G primes for pulmonary eosinophilia when mice are challenged with the virus (32–34). Dust mite allergen Der p 1 has been reported to promote T_H2-type responses (24). In an autoimmune model of uveoretinitis, one epitope induced a T_H1-type response, while the other induced a T_H2-type response (35). In another study, the injection of different antigens or peptides i.p. in IFA normally induced T_H2-type responses, but the immunization with an ovalbumin-derived peptide resulted in a T_H0-type response (25).

BALB/c mice recognized one immunodominant epitope in Bos d 2, which raises the question of the role of this epitope in

determining the quality of immune response against the allergen, especially because the peptide (ELEKYQQLNSERGVN) containing the epitope was exactly the same as that recognized by all the cow-dust-asthmatic patients (10). As the immunology of lipocalins is poorly characterized, it is not known whether the corresponding regions in lipocalin allergens in general are important in immune responses against them. However, the studies conducted in the BALB/c mouse with β -lactoglobulin indicated that the region in β -lactoglobulin corresponding to the immunodominant epitope found in Bos d 2 contains a T cell epitope (36–38). In the same way as observed with the antigens (Tables 1 and 2), our results show that the quality of immune response against the peptide with the immunodominant epitope was strongly influenced by the immunization route and the responding cell type in that the lymph node cell response against the peptide was T_H1 -biased after s.c. immunization in CFA (Tables 3 and 4). This may account for the finding that the immunodominant peptide failed to induce IL-4 production in lymph node cells (Table 4). Another possibility is that other epitopes in Bos d 2 are able to modify the response as the lymph node cell response after immunization with Bos d 2 in CFA induced IL-4 production (Table 2). In addition, the outcome of immune response may be regulated by a delicate balance between the priming and stimulation doses; the molar concentrations of the peptide used both in immunization and stimulation were higher than those for Bos d 2.

Indeed, a factor influencing the outcome of immune response can be the dose of antigen in priming. It has been reported that the low doses of antigen favor T_H2 -type responses (39,40). The effect may be attributed to the low ligand density on antigen-presenting cells (APC), which has been shown to favor T_H2 -type responses (41–43). As another point of view, studies with altered peptide ligands (APL) suggest that signaling through TCR can be qualitatively different due to the flexible recognition of antigen by TCR (44,45). Therefore, T cell repertoire and the selection of cells becoming recruited is probably critical for the outcome of the immune response. For example, when mice were first primed with a superagonist APL having an I-A^b-binding capacity similar to the wild-type peptide, stimulation with the wild-type peptide resulted in the production of IFN- γ mRNA, not IL-4 mRNA as was the case if mice were primed with the wild-type peptide (41). Furthermore, the findings of Murray *et al.* suggest that if T cells capable of recognizing the ligand efficiently are missing, even the high densities of the ligand on APC are not able to deviate the response to T_H1 dominance (43,46). As to Bos d 2, the weak immunogenicity of Bos d 2 suggests that it is recognized inefficiently, although the contribution of other mechanisms, including peripheral tolerance (47,48), cannot be excluded. Bos d 2 may be presented at a low density on APC and/or the T cell repertoire recognizing Bos d 2 is deficient. Both humans and mice contain numerous endogenous lipocalins (<http://www.expasy.ch/cgi-bin/nicesite.pl?PS00213>, 1 September 2001) and it can be expected that this has resulted in an extensive thymic deletion of T cells capable of recognizing endogenous lipocalins. It can be hypothesized that the surviving self-specific T cells, present in low numbers in the periphery,

recognize self-mimicking lipocalin allergens with their low-affinity TCR (49) in a suboptimal way and this favors the T_H2 -deviated immune response. Taken together, this study was performed as an approach to the question of allergenicity of Bos d 2, a lipocalin allergen. To address the significance of immune recognition for the allergenicity of lipocalin family of proteins, further studies are required on various factors involved, including the priming and stimulation doses of the immunodominant and other epitopes in Bos d 2, as well as on immunologic properties shared between different lipocalin allergens.

Acknowledgements

The skilful technical assistance of Virpi Fisk and Riitta Korhonen is gratefully acknowledged. Grants from Kuopio University Hospital (projects 5021601 and 5021605) and from the Academy of Finland (contract 2939) supported this work.

Abbreviations

APL	altered peptide ligand
APC	antigen-presenting cell
CFA	complete Freund's adjuvant
Con A	concanavalin A
GST	glutathione S-transferase
HEL	hen egg lysozyme
IFA	incomplete Freund's adjuvant
n	natural
OD	optical density
pp	<i>Pichia pastoris</i> -produced
r	recombinant
SI	stimulation index
TT	tetanus toxoid

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