

Probing the mechanisms of low immunogenicity of a lipocalin allergen, Bos d 2, in a mouse model

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Summary

Background Bos d 2, a major bovine allergen of the lipocalin family, stimulates very weakly cow dust-asthmatic subjects' peripheral blood mononuclear cells and the spleen cells of several inbred mouse strains immunized with the allergen.

Objective To identify the immune mechanisms accounting for the weak stimulatory capacity of Bos d 2.

Methods The spleen cell responses of BALB/c mice immunized with the allergen and with hen egg lysozyme and tetanus toxoid as control antigens were examined using several *in vitro* methods.

Results Analysis of the numbers of spleen cells in the antigen-stimulated *in vitro* cultures with the vital dye 7-amino-actinomycin D showed that Bos d 2 induced a smaller expansion of cells in comparison with the control antigens. Increased cell death *in vitro* did not account for the weak response against Bos d 2. The number of spleen cells reacting against Bos d 2 also proved to be the lowest when they were analysed by labelling the stimulated cells with 5-6-carboxyfluorescein diacetate succinimidyl ester or by enumerating cytokine-secreting cells by ELISPOT. Eliminating CD8⁺ cells in the *in vitro* culture did not enhance the response against Bos d 2. Bos d 2 was also the weakest of the antigens to stimulate the production of soluble cytokines. Adding IL-2, IL-4 or antibody against TGF- β in the antigen-stimulated spleen cell cultures enhanced the proliferative responses against all the antigens, whereas adding IL-12 or antibody against IL-4 or IL-10 did not enhance the responses.

Conclusion The results exclude several mechanisms of peripheral tolerance as an explanation for the poor immune response against Bos d 2, and suggest that the allergen is recognized by a low number of specific T cells. The weak immunogenicity of Bos d 2 may be related to its allergenicity.

Keywords allergenicity, animal allergen, immunogenicity, immune response, lipocalin, T cell

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Introduction

The allergenicity of a protein is most probably determined by a variety of factors, including physical and chemical properties, and it may be promoted by the biological function of the molecule [1]. Taking this into account, a crucial element for the sensitization to occur is the recognition of the allergen by the immune system. We have previously shown that the proliferative responses of Bos d 2-allergic asthmatic patients' peripheral blood mononuclear cells (PBMC) against natural Bos d 2 are very weak [2], despite the facts that the allergen is the major IgE-binding component in cow dander [3] and that it is able to induce positive reactions in both skin prick tests and bronchial provocation tests among Bos d 2-allergic subjects [4]. Recently, we reported that natural Bos d 2 is also a weak immunogen for several inbred mouse strains [5]. In further experiments with recombinant Bos d 2 and the control antigens hen egg lysozyme and tetanus toxoid, the IgG1 response of the responder mouse

strain BALB/c against Bos d 2 was weaker than against the control antigens, even after repeated immunizations. Importantly, the IgG2a response against Bos d 2 was virtually non-existent, which was not the case with control antigens. In all, the immune response against Bos d 2 was observed to be T helper cell type 2 (Th2) deviated both in mouse and in man [2, 5].

A number of regulatory mechanisms of the immune system might be responsible for the distinctive reaction against Bos d 2. We have suggested that the presence of endogenous lipocalins in humans could contribute to the Th2 deviation of the immune response against lipocalin allergens [6]. As lipocalins can exhibit considerable amino acid identity between species (e.g. 57% identity between the major dog allergen Can f 1 and human tear lipocalin, 61% between the major horse allergen Equ c 1 and pig salivary lipocalin, and 38% between Bos d 2 and mouse odourant-binding protein 1a) (the SIB BLAST network service at the Swiss Institute of Bioinformatics, 13 May 2002) [7], lipocalin allergen-reactive T cells of high avidity may have been deleted during thymic maturation [8, 9]. The remaining lipocalin-reactive T cells would recognize lipocalin allergen epitopes weakly or in a deviant way. For example, altered peptide ligands have been observed to induce changes in the cytokine secretion patterns of T cells [10], and a weak

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stimulation through T cell receptor has been shown to favour Th2-type responses [11–13].

In addition to the possible deletion of Bos d 2-reactive T cells during thymic development, the mechanisms of peripheral tolerance could be operative in regulating the immune response against Bos d 2. The purpose of this study was to elucidate the immune mechanisms responsible for the decreased proliferative responsiveness of spleen cells against Bos d 2 in the BALB/c mouse, the only strain out of six examined able to respond to Bos d 2 by a distinct production of antibody [5]. It was confirmed that the proliferative response of spleen cells *in vitro* against Bos d 2 was weak in comparison with the responses against the control antigens. None of the peripheral mechanisms of immune regulation examined could be shown to account for the weak reactivity against the allergen. The results suggest that the principal factor contributing to the poor reactivity against Bos d 2 is a weak recognition of the allergen by a low number of specific T cells.

Materials and methods

Antigens

The antigens used in the study were recombinant (r) Bos d 2 produced in *Pichia pastoris* [14, 15], hen egg lysozyme (HEL; L-6876, Sigma, St Louis, MO, USA) and tetanus toxoid (TT; National Public Health Institute, Helsinki, Finland). Recombinant Bos d 2 has been shown to have the same capacity to induce skin prick test reactions, to bind IgE in ELISA, and to induce the proliferation of human T cell clones as the natural allergen [15].

Cell samples

Female BALB/c mice aged 8 to 9 weeks were obtained from M & B (Ry, Denmark) and maintained under pathogen-free conditions throughout the study. The mice were immunized intraperitoneally (i.p.) with 50 µg of rBos d 2, HEL or TT in Inject Alum (Pierce, Rockford, IL, USA). Control mice were injected with phosphate-buffered saline (PBS). Spleens were removed 10 days after immunization and the spleen cells of each group (spleens from three to five mice) were isolated by Lympholyte M (Cedarlane, Hornby, Ontario, Canada) density gradient centrifugation and resuspended in complete DMEM culture medium as described previously [5]. For enzyme-linked immunospot (ELISPOT) assays, single-cell suspensions were prepared by pressing minced spleens through a 70-µm nylon mesh (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) into HL-1 medium (HL-1 Complete Serum-free Medium; BioWhittaker, Walkersville, MD, USA) containing 2 mM L-glutamine and the antibiotics. Experiments were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 18 March 1986; adopted in Finland on 31 May 1990). The study was approved by the Animal Care and Use Committee of the University of Kuopio.

Labelling of cells with CFSE and 7-AAD and flow-cytometric analyses

All flow-cytometric analyses were performed with a FACScan cytometer (Becton Dickinson, Mountain View, CA, USA) and the CellQuest™ 3.1 software (Becton Dickinson).

The divisions of spleen cells upon antigen stimulation were analysed quantitatively by monitoring the attenuation of fluorescence of 5-6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) dye. Isolated mononuclear spleen cells in PBS/5% fetal calf serum (FCS) were incubated for 5 min at room temperature with CFSE at a final concentration of 5 µM. After four washings with PBS/5% FCS, the cells were suspended at 2×10^6 cells/mL in complete medium. The cells were then stimulated for 4 days in round-bottomed 96-well microtitre plates (2×10^5 cells/well) with rBos d 2, HEL or TT at 100 µg/mL in a humidified 5% CO₂ incubator at 37 °C. Cultures without a stimulant served as a control. After incubation, the CFSE-labelled cells of three parallel wells were combined in a tube, washed with PBS and resuspended in 0.5 mM EDTA/PBS for 5 min at 37 °C. After centrifugation, the cells were resuspended in 0.5 mL PBS/5% FCS. Altogether 20 000 events were collected with a flow cytometer, and mononuclear cells were gated based upon the forward and side scatter view. CFSE fluorescence profiles (the numbers of events) between immunization groups were normalized with the CellQuest software.

To determine the numbers of live and dead cells after antigen stimulation, 5×10^5 mononuclear spleen cells per well were stimulated for 3 days as described above. Cells from three parallel wells were combined and washed once in PBS containing 2% FCS and 0.1% NaN₃. The supernatant was removed carefully and the cells were incubated with the vital dye 7-amino-actinomycin D (7-AAD; BD PharMingen; 20 µL/million cells) for 10 min at room temperature. To allow direct comparison between the numbers of spleen cells after stimulation with different antigens, 0.5 mL of PBS/2% FCS/0.1% NaN₃ was added to each tube, and events were collected with a flow cytometer for 90 s [16].

Proliferation assays

The proliferation assays of spleen cells (2×10^5 /well) were performed in triplicate or in quadruplicate as described previously [5]. Antigen concentrations (rBos d 2, HEL and TT) ranged from 0.1 µg/mL to 100 µg/mL.

In some experiments, spleen cells depleted *in vitro* of CD8⁺ cells were used. CD8⁺ cells were removed from mononuclear cell fractions by magnetic separation (Dynabeads® Mouse CD8 (Ly-2); Dynal, Oslo, Norway) following the instructions of the manufacturer. High purity of cell preparations (less than 2% CD8⁺ cells, ungated view) was verified by flow cytometry using CD4FITC (Sigma, H129.19), CD8PE (Sigma, 53-6.7) and CD3Cy-chrome (PharMingen, San Diego, CA, USA, 145-2C11) antibodies.

Where indicated, human recombinant interleukin (IL)-2 at 10, 20 and 50 U/mL, mouse rIL-4 at 10, 50 and 200 U/mL, mouse rIL-12 at 10, 100 and 200 U/mL (all purchased from PharMingen) or monoclonal antibodies against murine IL-4 (clone 30340), IL-10 (clone JES052A5) and TGF-β (clone 1D11; all from R & D Systems Europe Ltd, Abingdon, UK) at 1 µg/mL or 10 µg/mL were added to *in vitro* cultures. In experiments with monoclonal antibodies (mAb) against the cytokines, purified rat IgG (Sigma, cat.nro I-4131) at the same concentration as mAbs served as an additional control.

ELISPOT assays

ELISPOT analyses were performed as described elsewhere [17] with minor modifications. Ninety-six-well ImmunoSpot M200

plates (Cellular Technology Ltd, Cleveland, OH, USA) were pre-coated overnight at +4 °C with capture antibodies in PBS (0.1 mL/well). After washing with sterile PBS, the plates were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Washing was repeated and 0.1 mL of antigens (rBos d 2, HEL or TT) in HL-1 medium was added in duplicate to the wells at concentrations of 25 and 100 µg/mL. The spleen cells were added at a density of 10^6 cells/well. Cultures with an anti-CD3 antibody (ATCC, CRL-1975) and those without a stimulant served as positive and negative controls, respectively. After incubation in a humidified 5% CO₂ incubator at 37 °C for 24 h (IL-2 and interferon (IFN)-γ) or 48 h (IL-4 and IL-5), the plates were washed with PBS and PBS/0.05% Tween. Biotinylated secondary antibodies were added to PBS/1% BSA/Tween and the plates were incubated overnight at +4 °C. After washing with PBS/Tween, streptavidin-horseradish peroxidase (Dako A/S, Glostrup, Denmark) at a dilution of 1/2000 in PBS/BSA/Tween was added. Incubation was at room temperature for 2 h. The plates were then washed with PBS/Tween and PBS. Colour reaction was developed for 1 h using a solution containing 800 µL of 3-amino-9-ethyl-carbazole (Sigma, 100 mg dissolved in 10 mL N-N-dimethylformamide (Merck, Darmstadt, Germany)), 24 mL of 0.1 M sodium acetate, pH 5.0, and 12 µL of H₂O₂. The reaction was stopped by washing the plates with sterile water, and the numbers of spots were counted under a stereomicroscope. The capture antibodies used were 11B11 at 4 µg/mL for IL-4, TRFK5 at 5 µg/mL for IL-5, JES6-1A12 at 2 µg/mL for IL-2 and R4-6A2 at 4 µg/mL for IFN-γ. Biotinylated secondary antibodies were BVD6-24G2 for IL-4, TRFK4 for IL-5, both at 4 µg/mL, JES6-5H4 for IL-2 and XMG1.2 for IFN-γ, both at 2 µg/mL. All antibodies were from PharMingen.

Induction and measurement of cytokine production

Cytokine stimulation and the measurement of soluble cytokines were performed as described previously [5]. In brief, the spleen cells were stimulated at a density of 3×10^6 cells/mL in a volume of 1.5 mL with rBos d 2, HEL or TT at a concentration of 100 µg/mL. Cultures without a stimulant served as a negative control. After incubation in a humidified 5% CO₂ incubator at 37 °C for 48 h (IFN-γ and IL-2) or 96 h (IL-4, IL-5 and IL-10), culture supernatants were collected and the concentrations of cytokines were measured in duplicate using ELISA with commercial reagents (PharMingen) according to the manufacturer's instructions. The measurement of IL-2 using the CTLL-2 cell line with rIL-2 (Strathmann, Biotech GmbH, Hamburg,

Germany) as a standard has been described previously [5]. The limits of detection were 5 pg/mL for IL-4, 30 pg/mL for IL-5, 150 pg/mL for IL-10, 100 pg/mL for IFN-γ and 0.1 U/mL for IL-2.

Results

Proliferative spleen cell response against Bos d 2 is weak

Groups of mice were immunized i.p. with rBos d 2 and the control antigens HEL and TT in Imject Alum. CFSE labelling was applied to determine the proliferation of mononuclear spleen cells upon *in vitro* stimulation with the antigens. The analyses revealed that Bos d 2 induced a smaller fraction of mouse spleen cells to proliferate than the control antigens HEL and TT (Fig. 1). This observation was in line with the finding that the expansion of cells after stimulation with the antigens was the smallest with Bos d 2, as indicated by the number of live cells in the culture (Table 1). This was probably not due to the increased cell death *in vitro* as the number of dead spleen cells (7-AAD⁺) of rBos d 2-immunized mice was at the same level after culturing with or without the allergen (Table 1). Comparison of the ratios of dead vs. live cells in the cultures showed that

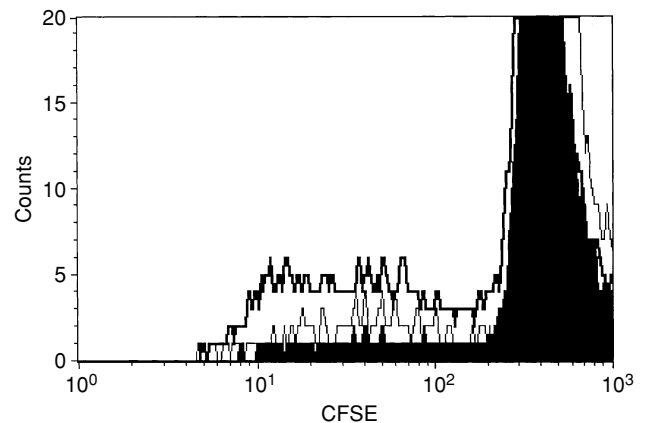


Fig. 1. *In vitro* proliferation of mononuclear spleen cells monitored by decrease of CFSE fluorescence. Mononuclear spleen cells from BALB/c mice immunized 10 days earlier with rBos d 2 (shaded area), HEL (thin line), or TT (thick line) were labelled with CFSE and then stimulated with the antigens at 100 µg/mL. After a 4-day culture, flow-cytometric analyses were performed. The data are representative of three independent experiments.

Table 1. Flow-cytometric quantification of live and dead mononuclear spleen cells from rBos d 2, HEL or TT-immunized BALB/c mice after a 3-day culture with the antigens at 100 µg/mL and without a stimulant

Antigen	Number of cells (events)*			
	Live		Dead	
	0 µg/mL	100 µg/mL	0 µg/mL	100 µg/mL
rBos d 2	14461 ± 3974	21885 ± 4209	39416 ± 8495	39642 ± 6745
HEL	15738 ± 2917	31191 ± 3086	46341 ± 9865	28548 ± 6073
TT	15499 ± 3547	35381 ± 5211	44131 ± 11927	30287 ± 7316

*Live cells were determined as the mononuclear cell fraction gated in the forward and side scatter view and the dead cells as the 7-AAD⁺ cell fraction in the ungated forward and side scatter view (cell debris excluded), as described in Materials and methods. The results are presented as mean event counts ± SEM of three independent experiments.

immunization alone without *in vitro* antigen stimulation yielded similar results between immunization groups (Fig. 2). In the antigen-stimulated cultures the ratio was the highest with Bos d 2. However, as it remained below the ratio of spontaneous cell death, it can be inferred that Bos d 2 did not induce increased death of spleen cells. The removal of CD8⁺ spleen cells *in vitro* before stimulation with Bos d 2 did not enhance the proliferative response (Fig. 3).

Bos d 2 induces low-level cytokine production from a low number of spleen cells

The number of cytokine-secreting spleen cells was quantified by the ELISPOT technique. Upon *in vitro* stimulation, rBos d 2 induced the lowest number of spleen cells to secrete IL-4, IL-5 and IL-2, whereas the number of IFN- γ -secreting cells remained at the level of PBS-treated mice for all antigens (Fig. 4). Interestingly, IL-4-producing cells could be easily detected with ELISPOT although IL-4 was hardly found in the culture supernatants (Fig. 5). This could be due to the fact that the secreted IL-4 remains locally, concentrated between a T cell and a target cell [18]. HEL was observed to induce the highest number of IL-4-secreting cells (Fig. 4). Secretion of the other cytokines was related to the stimulatory capacity of the antigens (Figs 4 and 5), except for IFN- γ , which remained at (Fig. 4) or below (Fig. 5) the detection level.

IL-2 and IL-4 promote antigen-specific spleen cell responses

The weak responsiveness of spleen cells against rBos d 2 upon *in vitro* stimulation could be due to a delicate regulative balance between appropriate cytokines and/or anergy. Adding IL-2 or IL-4 to the antigen-stimulated spleen cell cultures promoted specific proliferation with all antigens (Fig. 6). IL-12 did not enhance the responses (Fig. 6). It is to be noticed, however, that the spleen cell response of rBos d 2-immunized mice stimulated with rBos d 2 plus IL-2 or IL-4 did not reach the levels of the corresponding HEL or TT controls. Interestingly, the proportional increase of spleen cell responses with IL-4, i.e., in comparison with the response without the cytokine, was the greatest for rBos d 2-immunized mice (Fig. 6). The situation was more

complex with IL-2. When IL-2 was added, the proportional increase of the response was the greatest for HEL-immunized mice at a lower concentration of antigen (12.5 $\mu\text{g/mL}$), whereas at a higher concentration of antigen (100 $\mu\text{g/mL}$) the increase of the response was proportionally the greatest for rBos d 2-immunized mice (Fig. 6).

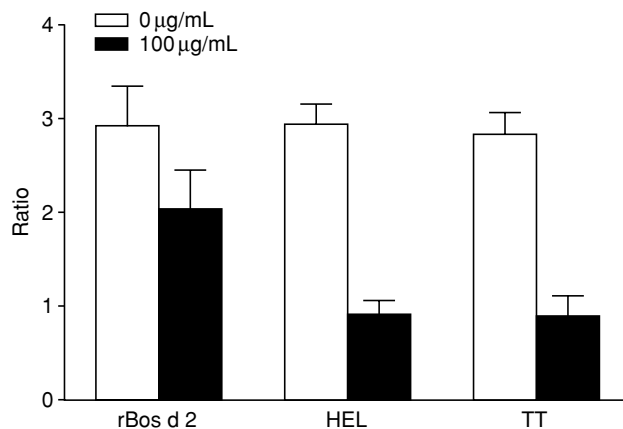


Fig. 2. Ratios of dead vs. live mononuclear spleen cells from rBos d 2, HEL or TT-immunized BALB/c mice after a 3-day culture with the antigens at 100 $\mu\text{g/mL}$ and without a stimulant. The data originate from Table 1 and are presented as mean ratio $\pm$ SEM of three independent experiments.

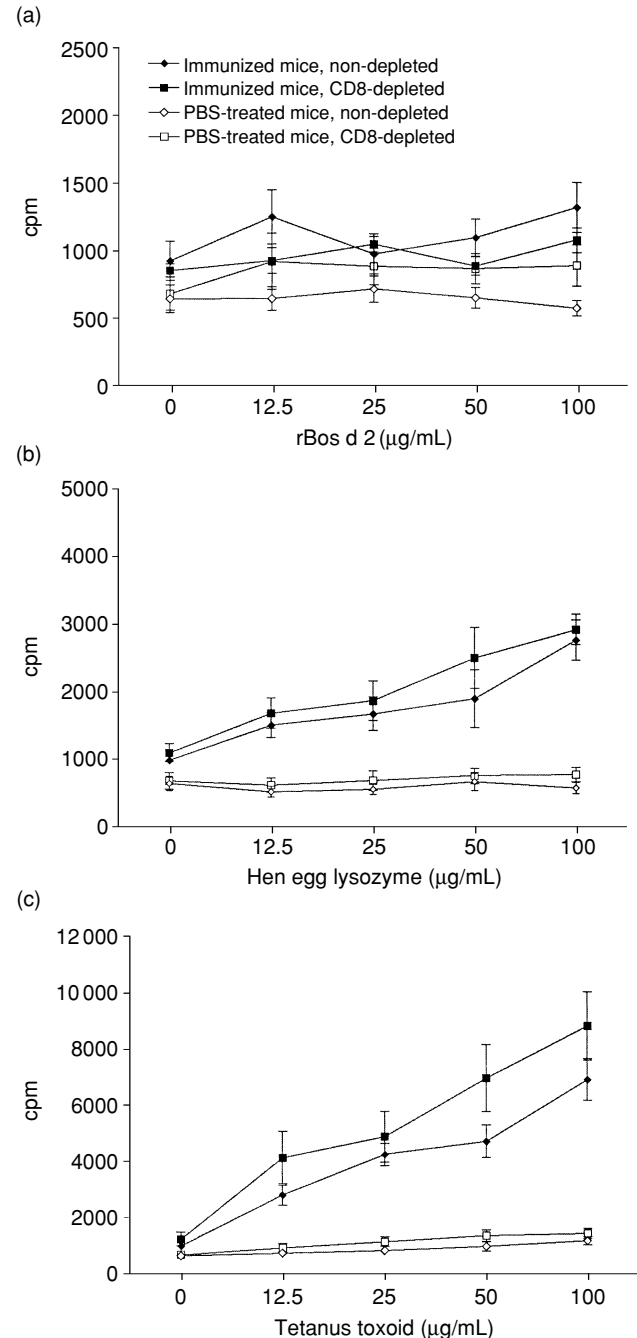


Fig. 3. Proliferative response of mononuclear spleen cells *in vitro* depleted of CD8⁺ cells upon stimulation with rBos d 2 (a), HEL (b), or TT (c). The mice were immunized with the antigens or treated with PBS 10 days before the *in vitro* stimulation. The results are presented as mean cpm $\pm$ SEM of three independent experiments.

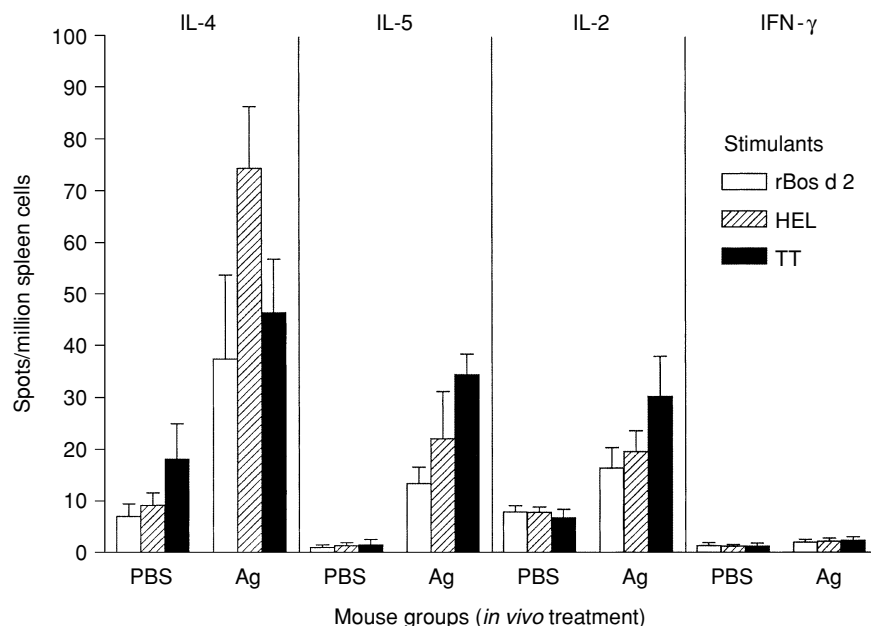


Fig. 4. ELISPOT analysis of cytokine production by splenocytes from BALB/c mice immunized with the antigens (Ag group: rBos d 2, HEL or TT) or mice treated with PBS upon stimulation with the antigens at 100 µg/mL. The mice were injected with the antigens or PBS 10 days before the *in vitro* stimulation. The results are presented as mean spot numbers $\pm$ SEM of three independent experiments.

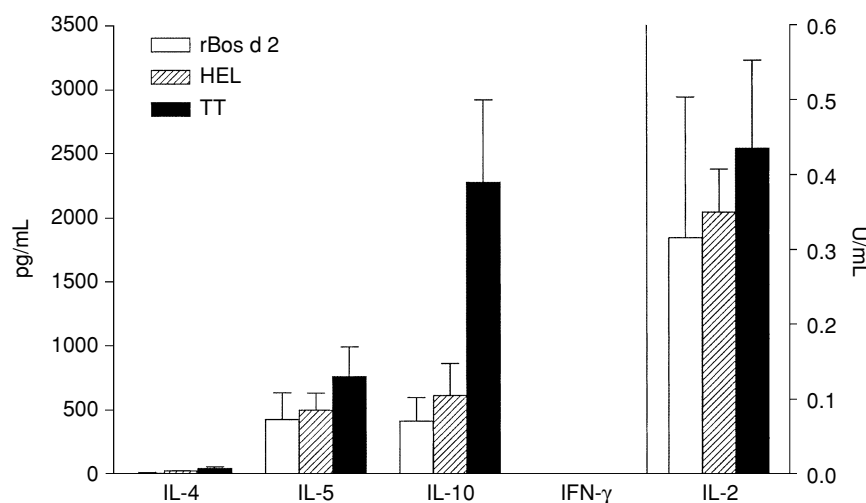


Fig. 5. Production of soluble cytokines by splenocytes from rBos d 2, HEL or TT-immunized BALB/c mice upon stimulation with the antigens at 100 µg/mL. The results are presented as mean concentrations $\pm$ SEM of three independent experiments. The level of cytokine production by unstimulated cells remained below the level of detection (see Materials and methods), except for IL-2 of rBos d 2-immunized mice ($0.21 \text{ U} \pm 0.13$). The cytokine levels produced by stimulated and unstimulated cells from PBS-treated mice were below the level of detection (data not shown).

Spleen cell response against rBos d 2 is not suppressed by IL-4, IL-10 or TGF-β

The effect of cytokines was further examined by neutralizing them *in vitro*. In repeated experiments, the neutralization of IL-4 or IL-10 in the antigen-stimulated cultures did not enhance the proliferation of spleen cells of any of the immunization groups, whereas anti-TGF-β promoted the proliferation for all groups (Fig. 7). The enhancement by anti-TGF-β was in part independent of antigen stimulation. The spleen cell response of rBos d 2-immunized mice upon stimulation with the allergen plus anti-TGF-β did not reach that of control mice with control antigens plus the anticytokine.

Discussion

A distinctive feature of human PBMC [2] and murine spleen cell [5] responses against Bos d 2 is that cellular (proliferative) responses *in vitro* upon stimulation with the allergen are very weak. As the reasons for this are unclear and may be related to the allergenicity of Bos d 2, the role of several immunoregulatory mechanisms possibly operative in the periphery was examined in a mouse model. HEL and TT were chosen again as control antigens as HEL is not a strong immunogen in the BALB/c mouse [19], whereas TT induces strong immune responses in several mouse strains [5] and in humans [2]. The

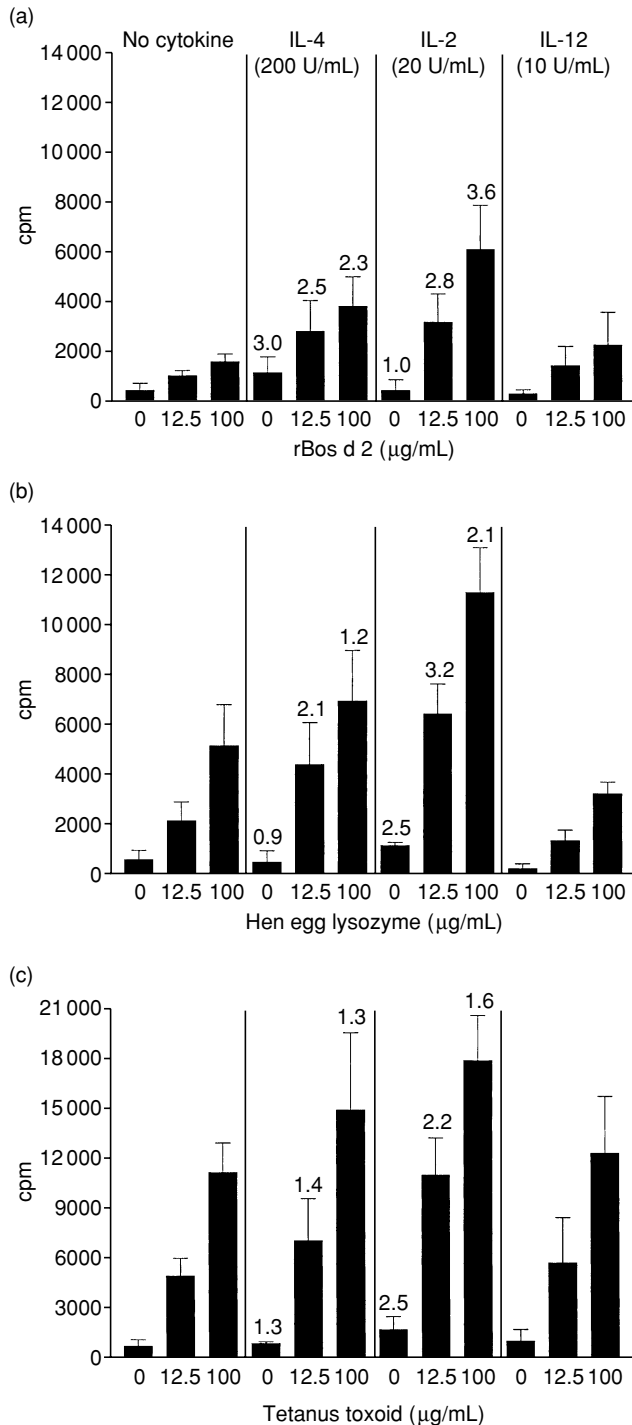


Fig. 6. Effect of exogenous IL-2, IL-4 or IL-12 on the proliferative response of mononuclear spleen cells from BALB/c mice immunized with rBos d 2 (a), HEL (b), or TT (c) upon stimulation with the antigens. As IL-2 and IL-4 induced the proliferation of spleen cells independently of antigen stimulation (medium only plus the cytokine: in all experiments < 6150 cpm for IL-4, < 5480 cpm for IL-2 and < 1710 cpm for IL-12 with PBS-treated mice), the cpm values obtained with PBS-treated mice were subtracted from those of immunized mice. The figures above the bars indicate how much stronger the response was in comparison with the response of the culture without the cytokine. The results are presented as mean cpm $\pm$ SEM of three independent experiments.

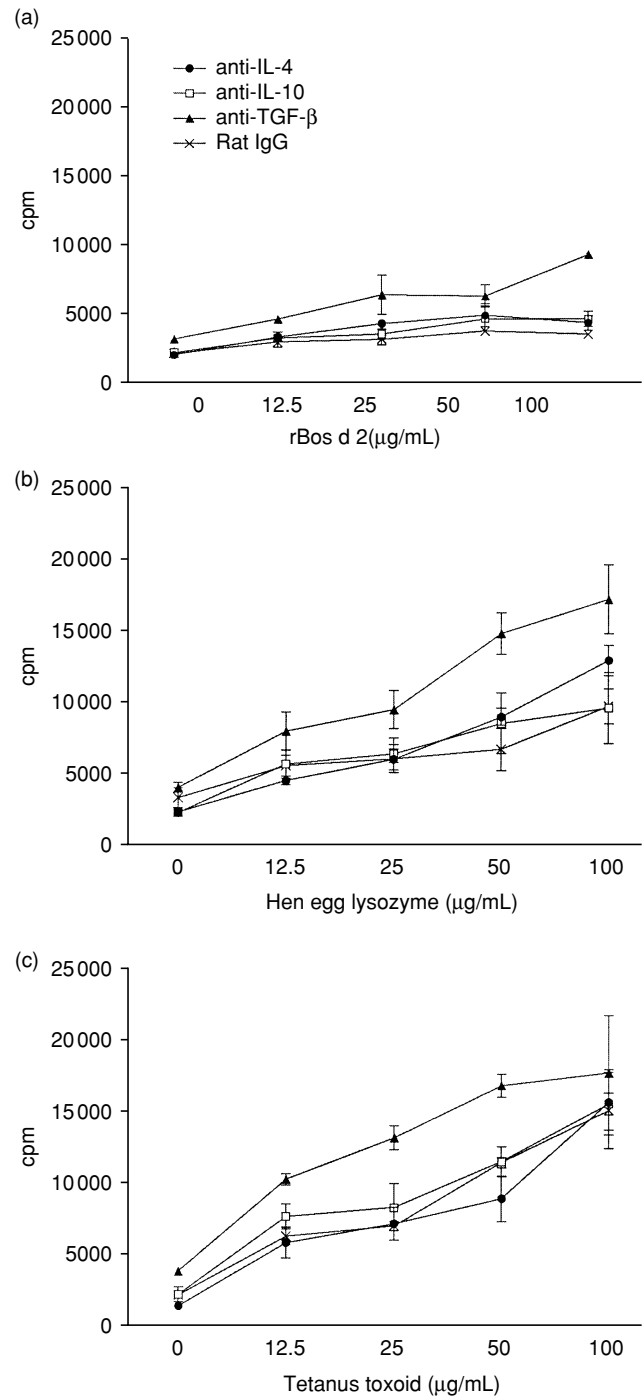


Fig. 7. Effect of neutralizing antibodies against IL-4, IL-10 or TGF-β on the proliferative response of mononuclear spleen cells from BALB/c mice immunized with rBos d 2 (a), HEL (b), or TT (c) upon stimulation with the antigens. The antibodies and rat IgG as a control were used at 1 µg/mL. The use of the antibodies at 10 µg/mL gave similar results (data not shown). The medium background of PBS-treated mice with anti-TGF-β was < 2540, and it was < 1380 cpm with other antibodies. The spleen cells of PBS-treated mice did not exhibit dose-response effect upon stimulation with the antigens. The data are representative of three independent experiments.

selection of alum as an adjuvant ensured the Th2 dominance of immune responses with all antigens [20].

Analyses performed using several *in vitro* methods confirmed that rBos d 2 was less efficient in stimulating spleen cells than the control antigens HEL and TT. This was shown as the shallow dose–response curves in proliferation tests (Figs 3, 6, and 7), as a lower number of live cells in the culture after stimulation with the allergen (Table 1), and as a lower number of cells responding to rBos d 2 than to control antigens (Figs 1 and 4). The number of cell divisions induced by the three antigens was largely the same (Fig. 1). The low number of spleen cells responding to rBos d 2 most probably did not result from the deletion of Bos d 2-specific T cells in the *in vitro* culture, as the numbers of dead spleen cells of rBos d 2-immunized mice did not differ between the cultures with and without rBos d 2 (Table 1) and the ratio of dead vs. live cells in the Bos d 2-stimulated culture did not exceed that of unstimulated cells (Fig. 2). CD8⁺ T cells, considered to be important regulators of Th2-deviated immune responses in some rodent models [21], did not seem to play a role in the poor response against Bos d 2 (Fig. 3).

A characteristic feature of anergy is the absent or strongly decreased IL-2 production in association with a block in proliferation [22]. Our results show that anergy does not appear a principal mechanism accounting for the weak proliferation of Bos d 2-immunized mice' spleen cells, as the difference in IL-2 production induced by Bos d 2 and the control antigens was not great. Although exceptions have been reported [23], anergy is characteristically reversed by exogenous IL-2 [24]. In this study, IL-2 enhanced proliferative responses in all groups (Fig. 6). However, the response with Bos d 2 plus IL-2 did not reach the responses obtained with control antigens plus IL-2. As to the role of CD4⁺CD25⁺ regulatory T cells, their inhibitory effect on the proliferative response against Bos d 2 was not addressed specifically in this study. However, it has been reported that adding IL-2 also overcomes this form of anergy [25].

Recently, a form of T cell anergy was discovered, defined as IFN- γ -mediated anergy [26]. As shown by our results (Figs 4 and 5), the lack of IFN- γ production was not antigen-specific but most probably dependent on the characteristics of the BALB/c mouse [27], on alum adjuvant [20] and/or on the immunization route used [20], all of which favour the Th2 deviation of the immune response. Moreover, adding IL-12 to the antigen-stimulated spleen cell cultures did not enhance the responses (Fig. 6), suggesting that IFN- γ -mediated anergy [26] does not account for the weak proliferative response against Bos d 2.

It is well established that Th1 and Th2 cells exert potent cross-regulation on each other [27]. As the immune responses in our experimental setting are clearly Th2-biased (Figs 4 and 5), it is unlikely that Th1 cells would be responsible for the reduced expansion of Bos d 2-specific T cells. On the other hand, IL-4, the key cytokine of the Th2-type response, involved for example in transplantation tolerance [28] and in down-regulating autoimmune pathology [29], might suppress the proliferation of spleen cells of Bos d 2-immunized mice upon stimulation with the allergen. However, this was not the case, as anti-IL-4 mAb did not enhance proliferation (Fig. 7). On the contrary, adding IL-4 in antigen-stimulated cultures augmented the proliferation of spleen cells in all groups (Fig. 6). Interestingly, when the proliferation in the antigen-stimulated cultures with extra IL-4

was compared with that of cultures without extra IL-4, the increase in proliferation was proportionally the greatest in the culture with Bos d 2. This finding is compatible with the idea that the immune response against Bos d 2 is strongly Th2-deviated, as the proliferation of Th2 cells is sustained by IL-4, the growth factor for Th2 cells [30]. Moreover, as anergic T cells fail to respond to IL-4 [31], our observation suggests that anergy, if present (see above), affects only a proportion of Bos d 2-specific T cells.

T cell populations secreting the immunosuppressive cytokines IL-10 and/or TGF- β have been observed to play an important role in regulating the immune responsiveness of pathogenic T cells, for example in the murine models of colitis [32, 33] and autoimmune diseases [29, 34]. In allergen immunotherapy, IL-10 is considered to be the major factor in creating the specific T cell anergy [35]. Our results do not support the idea that IL-10 or TGF- β is involved in inhibiting the proliferative response against Bos d 2 (Figs 5 and 7). The Bos d 2-stimulated spleen cell cultures produced only low levels of IL-10 in comparison with control antigens (Fig. 5), and anti-IL-10 mAb had no effect on the proliferation of these cells (Fig. 7). The effect of anti-TGF- β mAb on the proliferation of spleen cells was independent of the stimulating antigen (Fig. 7).

Our results indicate that neither anergy, suppressive cytokines nor increased cell death plays a decisive role in inhibiting the cellular response against Bos d 2 in the periphery. Theoretically, the possibility cannot be excluded that endogenous lipocalin-derived peptides could be involved in down-regulating the response against Bos d 2 by mechanisms such as partial T cell stimulation [36] or antagonism [37]. However, the results of ELISPOT analyses, performed in a serum-free medium, and the experiments on anergy with extra IL-2 do not support this. Therefore, it seems conceivable that the low number of Bos d 2-specific T cells could account for the poor stimulatory capacity of Bos d 2. Bos d 2 may also be recognized by T cells more weakly than the control antigens. If the weak recognition of the allergen is the principal factor responsible for the weak response, its significance for Th2 deviation should be addressed. Our results are compatible with our hypothesis that the presence of endogenous lipocalins would have resulted in the thymic deletion of lipocalin-specific high-avidity T cells and this is the reason why the cellular response against exogenous lipocalin allergens is weak [6].

Acknowledgements

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