

Mucosal tolerance as therapy of type I allergy: intranasal application of recombinant Bet v 1, the major birch pollen allergen, leads to the suppression of allergic immune responses and airway inflammation in sensitized mice

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Summary

Background Several studies have demonstrated that mucosal administration of soluble antigens can prevent the onset or reduce the severity of certain autoimmune diseases or allergies. Few studies exist showing the efficacy of mucosal tolerance for therapy of such diseases.

Objective The aim of the present study was to modulate an allergic immune response by intranasal antigen administration in an already sensitized organism.

Methods A murine model of allergic asthma to birch pollen (BP) and its major allergen Bet v 1 was utilized. Sensitized mice were intranasally treated with recombinant (r)Bet v 1 in different concentrations and at different intervals. On the day the mice were killed, blood and bronchoalveolar lavage fluids were taken and immediate type I skin tests were performed. T cell proliferation and cytokine production (interleukin (IL)-5, interferon (IFN)- γ) were measured in spleen and lung cell cultures.

Results Mucosal treatment with rBet v 1 ($3 \times 50 \mu\text{g}$ in 4 day intervals) led to a reduction of type I skin reactions, suppressed immunoglobulin (Ig)G1/IgE antibody levels and markedly decreased IL-5 and IFN- γ production *in vitro* in spleen and lung cell cultures. Moreover, lung inflammation (i.e. eosinophilia and IL-5 levels in bronchoalveolar lavage fluids) was significantly suppressed by the treatment.

Conclusion Our results demonstrate that intranasal treatment with rBet v 1 reduced systemic allergic immune responses as well as airway inflammation in BP-sensitized mice. We therefore suggest that mucosal tolerance induction with recombinant allergens could be a promising concept for the therapy of allergic diseases.

Keywords type I allergy, recombinant allergens, immunotherapy, intranasal treatment, suppression/ tolerance, *in vivo* animal model

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Introduction

Type I allergy represents a major health problem in industrialized countries. 15–20% of the population in middle and northern Europe suffer from allergic symptoms such as allergic rhinoconjunctivitis, allergic asthma or anaphylactic shock due to the hyper-reactivity of the immune system towards environmental, mainly airborne antigens. Among the inhalant allergens, pollen of the white birch (*Betula verrucosa*) represents an important source. The major allergen of birch pollen (BP) is the 17 kDa molecule Bet v 1 [1], which has been cloned, sequenced [2], and expressed in recombinant form in *Escherichia coli* [3]. Several experimental studies have been undertaken to characterize the immune responses to Bet v 1 *in vivo* and *in vitro* [4–7].

About 95% of BP-allergic patients display IgE binding to Bet v 1, and more than 60% react exclusively to it [8,9].

The only curative treatment for patients suffering from pollinosis is specific immunotherapy (SIT). SIT is performed by injecting increasing doses of allergens to induce immunological hyporesponsiveness [10–12]. Even though this treatment is effective in the majority of patients, it has several disadvantages, such as allergic side-reactions in some patients, long duration of the treatment or frequent injections, leading to low compliance of the patients. Thus, there is strong interest in the development of novel strategies, which are at least as effective as SIT, but of shorter duration and less invasive.

It is generally recognized that mucosal administration of soluble antigens leads to a state of antigen-specific immunological unresponsiveness, termed oral/mucosal tolerance. The fact that the immune system normally is unresponsive to environmental antigens, such as food and inhalant antigens, indicates the important role of mucosal tolerance to prevent hypersensitivity reactions towards these antigens. Based on this physiological situation, oral tolerance has been suggested

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as a treatment strategy against inflammatory diseases in humans. Several experimental studies demonstrated that mucosal administration of antigen can prevent the onset or reduce the severity of certain Th1-based autoimmune diseases [13–18]. It was further described that inhalative or intranasal antigen administration can also prevent Th2-based immune responses [19–24]. Despite this substantial number of experimental models few studies have investigated the effects of mucosal tolerance in already primed animals, which is the situation most relevant to clinical disease.

We have established a murine model of inhalative type I allergy to BP and its major allergen Bet v 1 [25]. Using this model we recently reported that nasal as well as oral application of recombinant (r)Bet v 1 prior to sensitization prevented the development of allergic immune responses *in vivo* and *in vitro* [23], suggesting mucosal tolerance induction as a prophylactic measure against type I allergy.

In the present study we investigated whether mucosal/intranasal tolerance could also be successfully induced in already sensitized animals. Our results demonstrate that mucosal tolerance induction with recombinant allergens may represent a promising alternative treatment to conventional immunotherapy.

Materials and methods

Animals

Female, 6-week old BALB/c mice were obtained from Charles River (Sulzfeld, Germany). All experiments were approved by the Animal Experimentation Ethics Committee of the University of Vienna and the Ministry of Education, Science and Culture.

Antigens

Recombinant Bet v 1 was obtained from Biomay GmbH (Linz, Austria) [2,3].

Birch pollen (BP) (Allergon, Ängelholm, Sweden) was used for the preparation of a BP extract. One hundred gram BP were dissolved in 1 L phosphate buffered saline (PBS) and extracted by stirring at 4 °C for 5 h. After centrifugation at 4000 *g* for 60 min at 4 °C the supernatant was filtered and dialysed (Spectra/Por 1; molecular weight 6–8000 Dalton; Spectrum, Houston, TX, USA) against PBS for 12 h. The protein concentration of the BP extract was determined according to the method of Bradford et al. [26] and analysed by SDS PAGE [27] and immunoblot as described [28]. The BP extract was lyophilized and stored at –20 °C.

Sensitization and tolerization (Experimental design, Fig. 1)

For sensitization 1 µg rBet v 1 adsorbed to Al(OH)₃ (Serva, Heidelberg, Germany; 2 mg/animal) was injected intraperitoneally (i.p.) three times in 14 day intervals (Fig. 1).

Sensitization was followed by intranasal treatment with rBet v 1 or NaCl (sham-treatment) applied in a volume of 30 µL into the nostrils of the mice. In pilot experiments multiple intranasal treatment protocols were performed to find the optimal dose and frequency of antigen administration: 15 µg daily for 10 days, 25 µg every second day (days 0, 2 and 4), 50 µg every third day (days 0, 3 and 6) and 100 µg once per week (days 0, 7 and 14). Immunomodulation was achieved by intranasal application of 50 µg rBet v 1, applied on days 0, 3 and 6, and was therefore chosen as the optimal treatment schedule in the present study (Fig. 1).

Seven days after the last intranasal treatment an aerosol challenge with 1% w/v BP extract was performed on two consecutive days (51 and 52). The allergen suspension was aerosolized through a nebulizer (De Vilbiss nebulizer 646; Somerset, PA, USA) into a chamber, in which the mice were exposed for 20 min/day as previously described [25].

Blood sampling

Blood samples were taken before and after sensitization, 7 days after intranasal treatment and after aerosol-challenge by tail bleeding. Sera were prepared and stored at –20 °C until analysed.

Cutaneous type I hypersensitivity reaction

After aerosol challenge (day 55) intradermal skin tests were performed [25]. 100 µL of 0.5% Evans blue (Sigma, St Louis, MO, USA) were intravenously injected into the tail vein of the mice. Subsequently, 30 µL of BP (2.5 µg/mL) and rBet v 1 (2.5 µg/mL) were injected intradermally into the shaved abdominal skin. The mast cell degranulation compound 48/80 (20 µg/mL; Sigma) was used as a positive and PBS as a negative control. After 20 min the animals were killed, skinned and the colour intensity (score 1–3) of the reaction was compared with the individual positive control.

Bronchoalveolar lavage for the determination of cellular infiltration within the lung

Bronchoalveolar lavage fluids (BALF) were collected as previously described [25]. Lungs were washed twice with 0.8 mL ice cold PBS and approximately 1.4 mL erythrocytes free BALF was recovered. BAL samples were spun onto microscope slides,

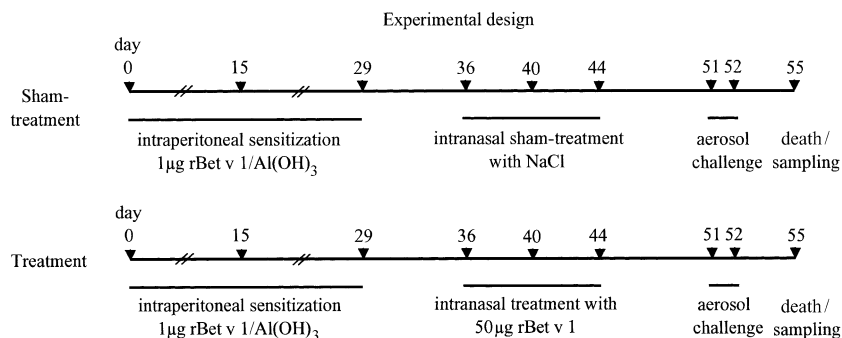


Fig. 1. Experimental design. Two groups of mice ($n = 8/\text{group}$) were used in the experiment. All mice were sensitized three times intraperitoneally (i.p.) with rBet v 1 adsorbed to Al(OH)₃. Subsequently, mice were intranasally (i.n.) treated with rBet v 1 ('treatment' group – treat), or i.n. sham-treated with NaCl ('sham-treatment' group – sham). Thereafter all mice were aerosol-challenged with a 1% BP solution on 2 consecutive days.

air dried and stained with haemacolor staining solution (Hemacolor Rapid Blood Smear Staining, Merck, Darmstadt, Germany) [29]. At least 200 cells were counted and differentiated by light microscopy according to standard morphologic criteria. The total number of counted cells was set to 100%, and the percentages of eosinophils, macrophages, lymphocytes and neutrophils were expressed as relative values.

Determination of allergen-specific antibodies in serum

Antigen-specific antibodies were measured by ELISA as previously described [25]. Microtitre plates (Nunc, Maxisorp, Roskilde, Denmark) were coated with rBet v 1 (2 µg/mL carbonate buffer pH 9.6) overnight at 4 °C. Serum samples were diluted 1 : 1000 for IgG1, 1 : 500 for IgG2a and 1 : 10 for IgE. Rat anti-mouse IgG1, IgG2a and IgE antibodies were used at a concentration of 1 µg/mL (Pharmingen, San Diego, CA, USA) and thereafter peroxidase-conjugated mouse anti-rat IgG antibodies (1 : 2000 with 0.5% BSA/P/T; Jackson Immuno Laboratories Inc., West Grove, PA, USA) were added. Antibody levels were expressed in arbitrary ELISA units which were calculated from a standard curve obtained with a pool of hyperimmune-standard sera. (Hyperimmune sera were prepared from mice, 10 times i.p. immunized in 14 days intervals with 1 µg rBet v 1 adsorbed to Al(OH)₃).

Lympho-proliferation in lung and spleen cell cultures

When the mice were killed, spleens and lungs were taken aseptically, minced and filtered through sterile filters and cell suspension prepared as previously described [24]. Proliferation assays were performed by plating cell suspensions at a concentration of 2×10^5 /well into 96-well round-bottomed plates (Nunc) and stimulating the cells with BP (5 µg/well) and rBet v 1 (2 µg/well) for 4 days. The ratio of the mean proliferation with antigen (cpm) and with medium control values (cpm) (i.e. stimulation index (SI)) was calculated.

Cytokine production in lung and spleen cell cultures

For determination of IL-5, IFN-γ and IL-10 production, the spleen and lung cell suspensions were cultured in 48-well plates (Costar, Cambridge, MA, USA) with and without BP (25 µg/well) at a concentration of 5×10^6 /well. Supernatants were taken 24 h for IL-5 and 48 h for IFN-γ and IL-10 after antigen stimulation and stored at -20 °C until analysis [24]. Levels of IL-5 and IL-10 were measured with mouse ELISA kits

(Endogen, Cambridge, MA, USA). The sensitivity of the IL-5 ELISA was < 5 pg/mL, that of the IL-10 ELISA < 12 pg/mL.

IFN-γ levels were measured in 96-well plates (Nunc) coated with rat anti-mouse IFN-γ (Endogen) at a concentration of 0.5 µg/mL carbonate buffer pH 9.6 for 6 h at room temperature (20 °C). Thereafter biotin-labelled rat anti-mouse IFN-γ antibodies (Endogen) were applied at a concentration of 0.1 µg/mL, followed by peroxidase-conjugated streptavidin (1 : 10 000 in PBS/4% bovine serum albumin (BSA); Endogen) for 30 min. For colour development tetramethylbenzidine (TMB) substrate (Chemicon, Temecula, CA, USA) was used and the absorbance measured at 450 nm with a Dynatech microplate reader. The sensitivity of the assay was < 15 pg/mL. Results reflect the measured cytokine levels in pg/mL after subtraction of baseline levels of non-stimulated cultures.

Statistics

For statistical analysis the Mann-Whitney *U*-test was used.

Results

Allergen-specific antibody responses in tolerized vs. sensitized mice

Allergen-specific antibody levels in serum were measured before and after sensitization, after intranasal treatment and at the day the mice were killed. The intranasal treatment with rBet v 1 reduced the allergen-specific IgG1 antibody levels in sensitized mice ($P < 0.05$). Consequently, at the day the mice were killed, the rBet v 1-treated animals displayed markedly reduced IgG1 antibody levels compared to the sham-treated sensitized controls ($P < 0.01$, Fig. 2). Also, a gradual decrease of specific IgE antibody levels was observed within the rBet v 1-treated group ($P < 0.05$, Table 1), the difference between the treated and the sham-treated animals was however, not statistically significant at the day the mice were killed. After the intranasal treatment with rBet v 1 there was an increase in IgG2a levels (not statistically significant) in the treatment group compared to the sham-treatment group and to the pre-treatment values (Table 1).

In accordance with the reduction of IgE antibody levels, the immediate type skin reactivity to Bet v 1 and BP was slightly reduced in the rBet v 1-treated compared to the sham-treated control mice (data not shown).

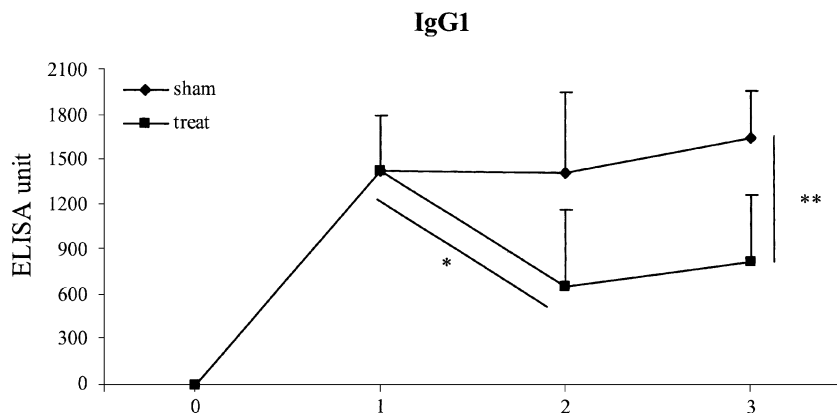


Fig. 2. Allergen-specific IgG1 antibody levels. Bet v 1-specific IgG1 antibody levels were measured in sera of mice i.n. treated with rBet v 1 (square dots – treat) or sham-treated with NaCl (triangular dots – sham) after sensitization. (0) Antibody levels of preimmune sera, (1) after sensitization, (2) after intranasal treatment, and (3) after aerosol-challenge, at the day the mice were killed. Antibody levels were measured by ELISA and reported as mean arbitrary ELISA units according to a standard curve. Error bars indicate standard deviation. ** $P < 0.01$, * $P < 0.05$ as determined by Mann-Whitney *U*-test.

Table 1. Allergen-specific antibody responses (IgE and IgG2a) in sera of intranasally sham-treated (sham) and rBet v 1-treated (treat) mice

Treatment	Antibody isotype	Post-sensitization	Post-treatment	Killed
sham	IgE	264.77 ± 115.15	188.90 ± 124.71	170.69 ± 99.94
treat	IgE	264.77 ± 115.15	158.01 ± 86.55*	142.59 ± 90.47*
sham	IgG2a	79.32 ± 84.36	83.82 ± 94.95	75.05 ± 90.76
treat	IgG2a	79.32 ± 84.36	148.03 ± 124.63	140.12 ± 120.86

Antibody levels were measured by ELISA in sera after sensitization, after intranasal treatment and after aerosol-challenge, at the day the mice were killed. Antibody levels are reported as mean arbitrary ELISA units ($\pm$ SD). * $P < 0.05$ as determined by Mann-Whitney *U*-test.

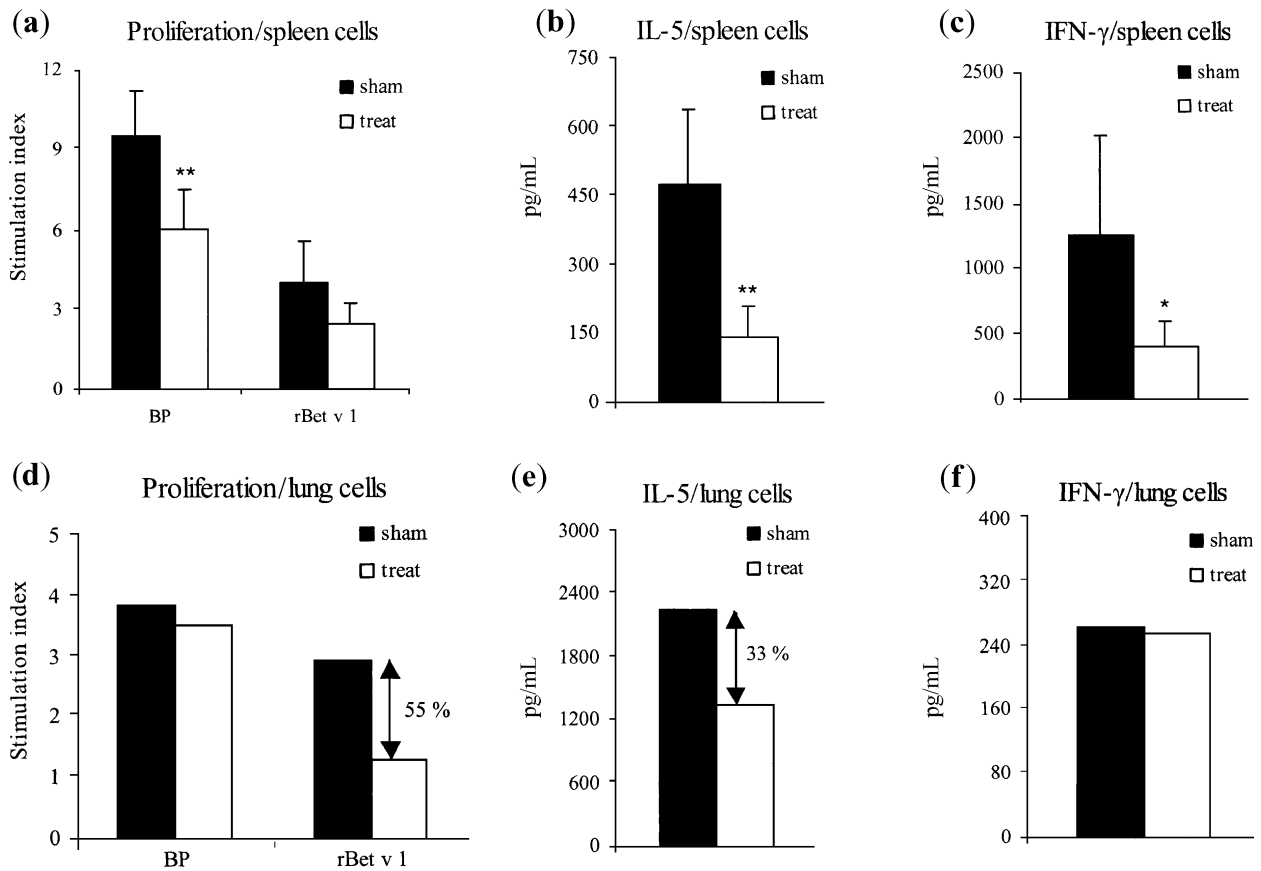


Fig. 3. Lympho-proliferation and cytokine production in spleen and lung cell cultures. Lympho-proliferation and cytokine production were measured in spleen cell cultures and pooled lung cell cultures of sham-treated controls (black bars – sham) and of rBet v 1-treated mice (open bars – treat). Antigen-specific (BP, rBet v 1) proliferative responses were measured by ^3H -incorporation in spleen cells (a) or lung cells (d) and indicated as stimulation indices (i. e. ratio of mean proliferation with antigen and medium control values). IL-5 levels (pg/mL) were measured in supernatants of spleen cell (b) and lung cell cultures (e) 24 h after stimulation with BP. IFN- γ levels (pg/mL) were measured in supernatants of spleen (c) and lung cells (f) 48 h after stimulation with BP. The values represent cytokine concentrations after subtraction of the baseline values. Error bars indicate standard deviation. ** $P \leq 0.01$, * $P \leq 0.05$ as determined by Mann-Whitney *U*-test. In pooled lung cell samples only percentages of changes were given.

Proliferative responses in spleen and lung cell cultures

The lympho-proliferative responses to BP and rBet v 1 were measured in spleen and pooled lung cell cultures. Spleen cells of rBet v 1-treated mice proliferated markedly less upon stimulation with BP ($P < 0.01$) and with rBet v 1 (Fig. 3a). Also, proliferative responses in pooled lung cell cultures of rBet v 1-treated mice were reduced upon stimulation with antigen, particularly with rBet v 1 (55% reduction) compared with those of sham-treated controls (Fig. 3d).

Cytokine production in spleen and lung cell cultures

To characterize the *in vitro* cytokine production profile, IL-5 and IFN- γ levels were measured in spleen and pooled lung cell cultures.

IL-5 production was significantly reduced in spleen cell cultures of rBet v 1-treated mice compared to sham-treated controls ($P < 0.01$, Fig. 3b). IL-5 levels, measured in lung cell cultures of rBet v 1-treated mice, were reduced by 33% in comparison to the sham-treated controls (Fig. 3e).

Also, IFN- γ production of spleen cells was significantly suppressed after the intranasal allergen treatment ($P < 0.05$, Fig. 3c). IFN- γ levels in lung cell cultures did not, however, differ between the two groups (Fig. 3f).

No significant differences of IL-10 levels were detected in spleen cell cultures of sham-treated ($76.43 \text{ pg/mL} \pm 22.32$) vs. rBet v 1-treated ($71.53 \text{ pg/mL} \pm 27.21$) mice.

Airway inflammation

To assess the effects of the intranasal treatment in sensitized mice on airway inflammation, IL-5 levels and cellular infiltration, in particular eosinophils, were analysed in BALFs.

IL-5 levels were significantly reduced in mice intranasally treated with rBet v 1 compared to the sham-treated control animals ($P < 0.01$, Fig. 4a). In accordance with the reduced IL-5 levels, the percentage of eosinophils was also significantly lower in the Bet v 1-treated than in the sham-treated mice ($P < 0.05$, Fig. 4b). In addition, the relative number of lymphocytes ($P < 0.05$) and neutrophils was reduced after the intranasal treatment with rBet v 1. Consequently, the relative percentage of macrophages was significantly increased ($P < 0.05$) after intranasal allergen treatment.

For control purposes, differential cell counting of BAL cells from naïve mice was performed, showing that 99–100% of the cells were macrophages and that there were no eosinophils or lymphocytes.

Discussion

Based on the fact that long duration and frequent allergen injections are major reasons for patients objecting to undergoing specific immunotherapy, an obvious advantage of mucosal treatment would be the ease and non-invasiveness of this strategy. In the present study we give evidence that mucosal tolerance induction with recombinant allergens could be a promising therapeutic concept for treatment of immediate type hypersensitivity reactions. Intranasal application of rBet v 1 led to the reduction of allergen-specific IgG1/IgE antibody levels as well as Bet v 1-specific lymphocyte proliferation and cytokine production in BP-sensitized mice. Moreover, airway inflammation (IL-5 and eosinophilia) was significantly suppressed by the intranasal allergen treatment.

Fifty μg of rBet v 1 applied three times in 4 day intervals appeared to be the optimal dose and application frequency for

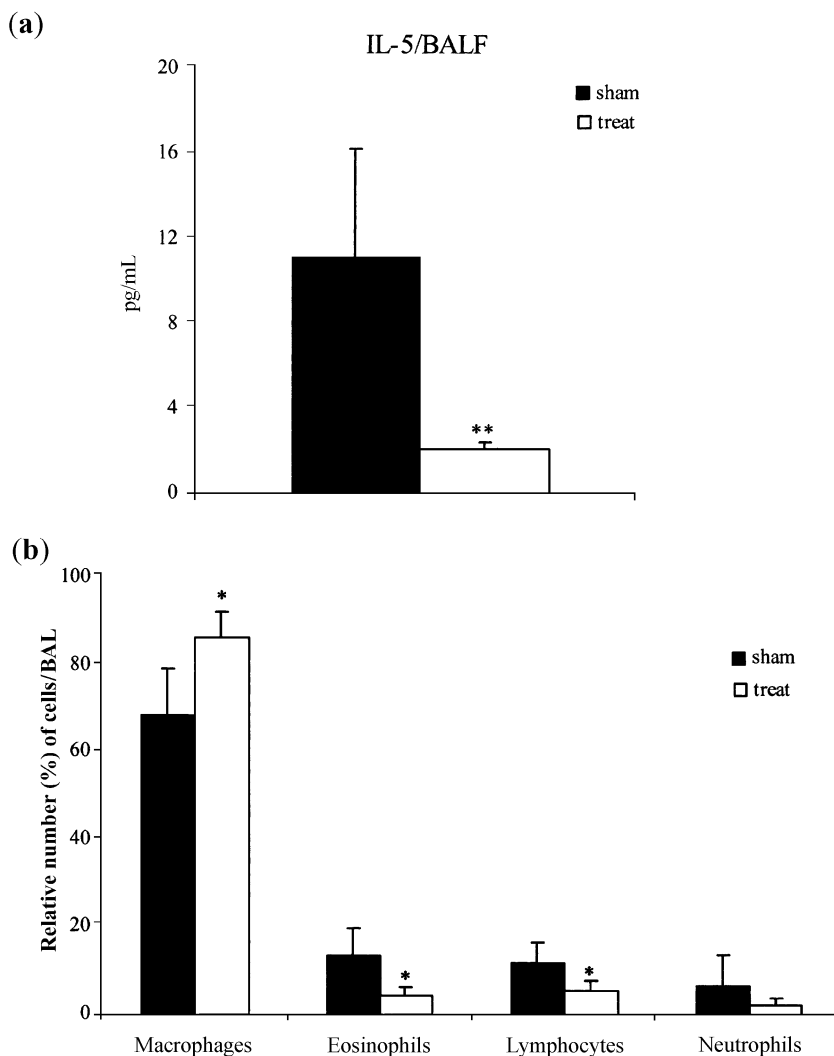


Fig. 4. Airway inflammation. IL-5 levels and cellular infiltration in bronchoalveolar lavage fluids (BALF) were measured as parameters of airway inflammation. (a) IL-5 levels (pg/mL) were measured in supernatants of BALFs. (b) The numbers of macrophages, eosinophils, lymphocytes and neutrophils were expressed as a percentage of the total number of counted cells. Black bars (sham) represent values of sham-treated mice, open bars (treat) indicate that of rBet v 1-treated mice. Error bars indicate standard deviation. ** $P < 0.01$, * $P < 0.05$ as determined by Mann–Whitney U -test.

inducing immunosuppression in Bet v 1-sensitized mice. Lower (i.e. <25 µg) or higher (i.e. >100 µg) doses could not influence allergen-specific immune responses in the sensitized animals (data not shown). Similar to our observations, it was demonstrated in a model of oral tolerance induction in ovalbumin-sensitized mice that an increase in antigen dosage *per se* did not improve tolerance induction [30], but that also the time of antigen application and the frequency of antigen application were important factors for successful tolerance induction in already primed animals [30–33].

It was postulated that the induction of mucosal tolerance more easily affects ongoing T cell responses (delayed type hypersensitivity (DTH) reactions) than established antibody responses [30,31,33,34]. In that respect Leishman et al. demonstrated that DTH reactions, T cell proliferation as well as Th1 and Th2 cytokine production *in vitro*, but not antibody production, were suppressed after oral treatment [30,31]. The authors suggested that at the time of tolerance induction, B cells might have been already primed by antigen and T cell-derived cytokines, and would therefore undergo further expansion when the antigen is fed after sensitization [30,31]. However, our results showed that both T cell responses (lymphocyte proliferation and cytokine production *in vitro*) as well as allergen-specific antibody responses, in particular IgG1 antibodies, were markedly reduced after the intranasal treatment, indicating that both T and B cell responses were susceptible to intranasal tolerance induction. As we already noted in a previous study in naive mice, the intranasal route seems to be more effective than the oral route in tolerance induction [23], which might also be the case in primed animals – probably related to the lack of acidic/enzymatic antigen degradation processes. Similar to our findings, a recent study demonstrated a reduction of IgG1 and IgE antibody levels following intranasal tolerance induction with a bee venom allergen in primed mice [35]. This reduction was associated with an increase in IgG2a antibody levels and IFN-γ production. In our model we observed a moderate increase in IgG2a as well, however, IFN-γ production – along with IL-5 – was significantly reduced after the treatment, indicating that the observed immunomodulation cannot be solely explained by a shift from Th2 to Th1 like responses.

Airway inflammation, characterized by the presence of IL-5 and eosinophils within the airways, is a typical feature of allergic asthma [36,37]. Here we give strong evidence for the therapeutic application of mucosal tolerance also in allergic asthma, since both IL-5 levels in the bronchoalveolar lavage, as well as IL-5-dependent eosinophil recruitment and lymphocyte infiltration were suppressed by the intranasal treatment. It has previously been demonstrated in naive animals that high-dose antigen instillation into the trachea can prevent eosinophilic lung inflammation [38]. The authors demonstrated that the suppressive effect was reversible by application of neutralizing anti-TGF-β antibodies, suggesting TGF-β as a major factor responsible for the down-modulation of Th2-like immune responses. Similarly, we have previously shown that TGF-β might be involved in Bet v 1-induced tolerance in naive mice [23]. However, there is evidence from literature that the mechanisms of tolerance induction differ in the primed organism. It has been reported that TGF-β production was not enhanced in mice tolerized by feeding antigen after priming [39]. Similar results were obtained from a model in which the inhibition of eosinophilic recruitment into mouse airways induced by high-dose

oral tolerance was not effected by anti-TGF-β treatment [40]. Along with these lines our pilot studies indicate that, in contrast to mice tolerized prior to sensitization [23], TGF-β mRNA levels were not enhanced in intranasally treated mice compared to the sensitized controls (data not shown). Another cytokine produced by regulatory T cells, which has been shown to play a key role in several models of tolerance induction, is IL-10 [41,42]. However our results did not give any evidence that IL-10 might be involved in tolerance induction, since no differences in IL-10 levels were seen between the groups. As none of the measured cytokines were increased after the intranasal treatment, mechanisms of active suppression or immunodeviation, as discussed above, seem unlikely in our model. Studies by Leishman et al. [30], Lamont et al. [34] and Astori et al. [35] suggested anergy as a critical mechanism for tolerance induction in primed mice. Our results, showing a suppression of allergen-specific cellular immune responses accompanied by reduced antibody responses (IgG1/IgE), may support their finding. The moderately increased IgG2a levels may be explained by the possibility that the primed IgG2a-producing B cells were exceptionally sensitive to even reduced T cell help upon repeated antigen challenge. However, it will be necessary to dissect the role of the different mechanisms in detail in our model.

In conclusion, our study demonstrated that mucosal treatment with rBet v 1 was effective in suppressing systemic allergic immune responses and airway inflammation in sensitized mice. Despite the fact that the mechanisms are still unclear and need further investigation, our results give rise to optimism that intranasal tolerance induction with recombinant allergens may be a useful alternative treatment for conventional immunotherapy against type I allergic diseases in the close future.

Acknowledgements

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